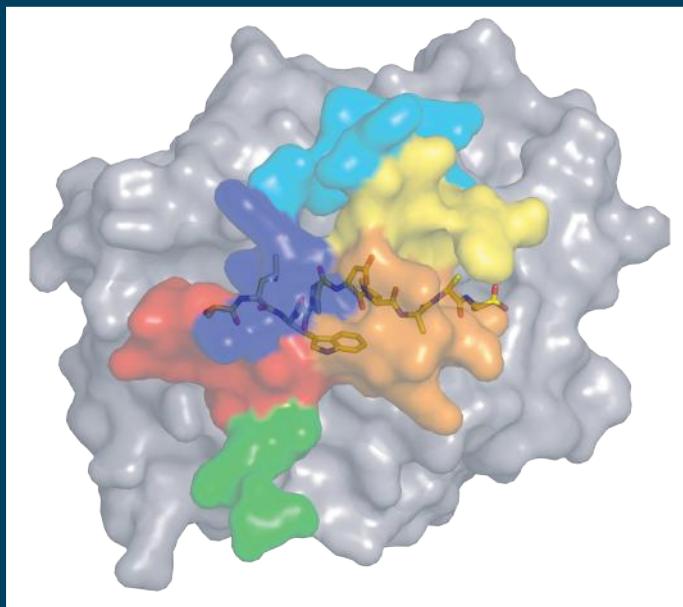
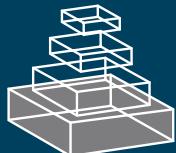


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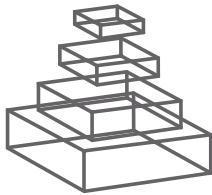


INVESTIGATING AND HARNESSING T-CELL FUNCTIONS WITH ENGINEERED IMMUNE RECEPTORS AND THEIR LIGANDS

Topic Editor
Bruno Laugel



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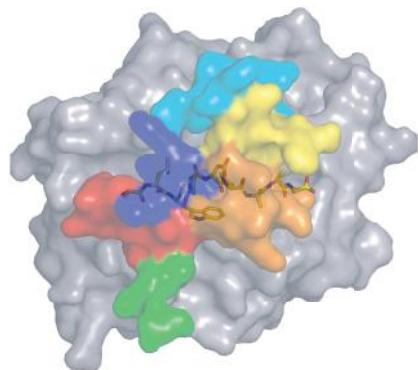
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INVESTIGATING AND HARNESSING T-CELL FUNCTIONS WITH ENGINEERED IMMUNE RECEPTORS AND THEIR LIGANDS

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The image shows the docking of a T-cell receptor (TCR) on a peptide-major histocompatibility (MHC) complex. The colours indicate the docking footprints of the TCR's six complementary-determining region loops on the MHC molecule (in grey) and the peptide (yellow). The image is courtesy of Dr David Cole, Cardiff University School of Medicine.

in the biology of CD8⁺ T-cells that translate into promising therapeutic prospects, as illustrated by recent breakthroughs in cancer therapy, they have proven more challenging to apply to CD4⁺ T-cells.

This Research Topic aims to provide a comprehensive view of the recent insights provided by the use of engineered antigen receptors and their ligands on T-cell activation and how they have been or could be harnessed to design efficient immunotherapies.

T-cells are an essential component of the immune system that provide protection against pathogen infections and cancer and are involved in the aetiology of numerous autoimmune and autoinflammatory pathologies. Their importance in disease, the relative ease to isolate, expand and manipulate them *ex vivo* have put T-cells at the forefront of basic and translational research in immunology. Decades of study have shed some light on the unique way T-cells integrate extrinsic environmental cues influencing an activation program triggered by interactions between peptide-MHC complexes and the antigen-recognition machinery constituted of clonally distributed T-cell receptors and their co-receptor CD4 or CD8. The manipulation of these molecular determinants in cellular systems or as recombinant proteins has considerably enhanced our ability to understand antigen-specific T-cell activation, to monitor ongoing T-cell responses and to exploit T-cells for therapy. Even though these principles have given numerous insights

Table of Contents

- 05 Bench, bedside, toolbox: T-cells deliver on every level**
Bruno Laugel
- 08 An altered gp100 peptide ligand with decreased binding by TCR and CD8 α dissects T cell cytotoxicity from production of cytokines and activation of NFAT**
Niels Schaft, Miriam Coccoris, Joost Drexhage, Christiaan Knoop, I. Jolanda M. de Vries, Gosse J. Adema and Reno Debets
- 18 Cellular-level versus receptor-level response threshold hierarchies in T-cell activation**
Hugo A. van den Berg, Kristin Ladell, Kelly Miners, Bruno Laugel, Sian Llewellyn-Lacey, Mathew Clement, David K. Cole, Emma Gostick, Linda Wooldridge, Andrew K. Sewell, John S. Bridgeman and David A. Price
- 31 Structural and biophysical insights into the role of CD4 and CD8 in T cell activation**
Yili Li, Yiyuan Yin and Roy A. Mariuzza
- 42 Co-receptor CD8-mediated modulation of T-cell receptor functional sensitivity and epitope recognition degeneracy**
Barbara Szomolay, Tamsin Williams, Linda Wooldridge and Hugo Antonius van den Berg
- 53 Corrigendum: Co-receptor CD8-mediated modulation of T-cell receptor functional sensitivity and epitope recognition degeneracy**
Barbara Szomolay, Tamsin Williams, Linda Wooldridge and Hugo Antonius van den Berg
- 56 Individual MHC-restricted T-cell receptors are characterized by a unique peptide recognition signature**
Linda Wooldridge
- 62 Immune parameters to consider when choosing T-cell receptors for therapy**
Scott R. Burrows and John J. Miles
- 68 Advances in T-cell epitope engineering**
Johanne M. Pentier, Andrew K. Sewell and John J. Miles
- 72 Re-directing CD4 $^{+}$ T cell responses with the flanking residues of MHC class II-bound peptides: the core is not enough**
Christopher J. Holland, David K. Cole and Andrew Godkin
- 81 Analysis, isolation, and activation of antigen-specific CD4 $^{+}$ and CD8 $^{+}$ T cells by soluble MHC-peptide complexes**
Julien Schmidt, Danijel Dojcinovic, Philippe Guillaume and Immanuel Luescher
- 95 Monitoring the dynamics of T cell clonal diversity using recombinant peptide:MHC technology**
J. Lori Blanchfield, Shayla K. Shorter and Brian D. Evavold
- 104 "Model T" cells: a time-tested vehicle for gene therapy**
Sid P. Kerkar

- 111 *TCR-engineered T cells meet new challenges to treat solid tumors: choice of antigen, T cell fitness, and sensitization of tumor milieu***
Andre Kunert, Trudy Straetemans, Coen Govers, Cor Lamers, Ron Mathijssen, Stefan Sleijfer and Reno Debets
- 127 *Role of T cell receptor affinity in the efficacy and specificity of adoptive T cell therapies***
Jennifer D. Stone and David M. Kranz
- 143 *Structure-based, rational design of T cell receptors***
V. Zoete, M. Irving, M. Ferber, M. A. Cuendet and O. Michielin
- 162 *Increased peptide contacts govern high affinity binding of a modified TCR whilst maintaining a native pMHC docking mode***
David K. Cole, Malkit Sami, Daniel R. Scott, Pierre J. Rizkallah, Oleg Y. Borbulevych, Penio T. Todorov, Ruth K. Moysey, Bent K. Jakobsen, Jonathan M. Boulter, Brian M. Baker and Yi Li
- 170 *Young T cells age during a redirected anti-tumor attack: chimeric antigen receptor-provided dual costimulation is half the battle***
Andreas A. Hombach and Hinrich Abken
- 174 *Molecular insights for optimizing T cell receptor specificity against cancer***
Michael Hebeisen, Susanne G. Oberle, Danilo Presotto, Daniel E. Speiser, Dietmar Zehn and Nathalie Rufer
- 184 *Beyond the antigen receptor: editing the genome of T-cells for cancer adoptive cellular therapies***
Angharad Lloyd, Owen N. Vickery and Bruno Laugel
- 191 *Expression of concern: Co-receptor CD8-mediated modulation of T-cell receptor functional sensitivity and epitope recognition degeneracy***
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Bench, bedside, toolbox: T-cells deliver on every level

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Decades of research have shed light on many aspects of T-cells, spawning a myriad of diagnostic and therapeutic applications in the process. Chief among those properties is the unique ability of T-cells to scan the intra-cellular protein content to detect anomalies in tissues, be it the presence of pathogens or cellular transformation. The tri-partite interaction between the T-cell receptor (TCR), its co-receptor CD4 or CD8, and peptide-major histocompatibility (pMHC) ligands determines the outcome of an encounter between a T-cell and an antigen presenting cell and can result in ignorance or trigger a cellular activation program central to adaptive immunity. Over the years, tremendous insights into the rules that govern this interaction have been gained at the molecular and cellular levels, resulting in the development of technologies and tools that improve our understanding of the dynamics of antigen-specific T-cell responses *in vivo* as well as therapeutic modalities aimed at harnessing the power of T-cells through vaccines, cellular therapies, and biologics. The selection of 18 articles that constitute this Research Topic reflects these advances in many ways and provides a snapshot of the current focus in the field, with an emphasis on the efforts made in order to translate our knowledge of T-cell biology into tools for therapy, diagnosis, and immune-monitoring.

From a fundamental point of view two primary research articles examine how T-cells discriminate between pMHC antigens and integrate signals that result in different cellular outcomes. Schaft et al. tested a panel of altered peptide ligands of human glycoprotein (gp)100 and identified a partial agonist that dissociates signaling networks downstream of TCR triggering (1). The altered peptide ligand they identified elicits cytotoxicity but negligible or no cytokine secretion nor NFAT-mediated transcription, an intriguing observation that appears related to the extent of binding by TCR and CD8 α and reveals the intricacies of signal transduction downstream of the TCR. In an extensive study of T-cell activation, van den Berg et al. examined the response of a human CD8 $+$ T-cell clone against several agonists of different affinities for the TCR (2). Their results support a model of epitope discrimination at the cellular level based on the integration of TCR signals, whereby the sum of signals read by a T-cell determines the functional response, rather than by the individual properties of receptor-ligand interactions. These two reports further highlight the analog nature of signal processing in T-cells, which enables diverse functional outcomes based on the concatenation of input signals rather than a binary response mediated via a simple on/off switch mechanism.

Also in the domain of basic research the articles by Li et al. and Szomolay et al. offer comprehensive insights into the roles of the

co-receptors CD4 and CD8. In the former article, the authors summarize the literature on the structural and biophysical properties of the pMHC/co-receptor interaction and discuss the implications on the topological organization of the entire antigen receptor machinery on the T-cell membrane, a parameter that likely influences the initiation and transduction of TCR signals (3). Szomolay et al. focus on the modulation of antigen recognition and ligand specificity by the co-receptor CD8 (4). Based on existing experimental data they formulate mathematical models that predict dynamic variations of T-cell response specificity and magnitude as a function of pMHC/CD8 binding kinetics and of CD8 expression levels on the cell surface, the latter phenomenon likely constituting an adaptive mechanism tuning responsiveness at different developmental stages. On the subject of antigen specificity, Wooldridge describes in details the extent of the cross-reactivity inherent to the TCR and the consequent degeneracy of T-cell antigen recognition (5). These parameters have clear implications when it comes to the pre-clinical development of T-cell based therapies, especially with respect to safety issues that relate to potential off-target effects.

Moving closer to translational research Burrows and Miles discuss the different parameters to consider when selecting TCRs for use in cellular therapy or as biologics (6). Again this article emphasizes the importance of assessing the antigen specificity and degeneracy profiles of therapeutic TCR candidates both in syngeneic and allogeneic systems. On the flip side of the TCR/pMHC interaction, Pentier et al. propose strategies to optimize T-cell epitopes in the context of therapeutic vaccination, including the design of synthetic antigen mimics that could circumvent the labile nature of native L-amino-acid peptides (7). Also relevant to the optimization of peptide ligands, Holland et al. provide fascinating insights into peculiar- and little-appreciated aspects of MHC class II epitope presentation, namely the influence of flanking residues that extend outside the MHC groove, on the interaction between the TCR and its antigen as well as T-cell activation (8).

A remarkable technological advance of molecular immunology has been the use of recombinant pMHC molecules to monitor T-cell responses by flow cytometry. Schmidt et al. review the development of these tools in detail from their initial description as monomeric reagents used to probe T-cell clones by photo-affinity labeling to their popularization as tetramers and higher order multimers for accurate and detailed *ex vivo* analysis of polyclonal T-cell responses (9). The authors also give an extensive account of recent technical improvements made in the manufacture of “switchable” class I pMHC multimers for the isolation of “untouched” antigen-specific T-cells and class II pMHC molecules and the challenges inherent to antigen-specific analysis of CD4 $+$ T-cell responses

by flow cytometry. As further illustration of the great strides made in pMHC technology Evavold and colleagues summarize the groundbreaking 2-dimension adhesion frequency assay they have developed and that allows monitoring TCR/pMHC interactions in their natural membrane environment (10). They also define new ways this technology can be used to advance our understanding of T-cell biology, for instance the detection and characterization of elusive CD4+ T-cells.

A large part of the Research Topic focuses on T-cell based cellular cancer therapies, perhaps the most promising domain of therapeutic application of T-cell biology at the moment. This approach has seen recent remarkable clinical success and is currently actively pursued around the globe. Kerkar starts by giving a general overview of T-cell based therapies for cancer and other disease indications (11). In addition to classical T-cell re-direction using viral vectors expressing TCRs or chimeric antigen receptors (CARs) the author discusses different therapeutic strategies using T-cells as vehicles such as the delivery of cytokines to diseased tissues. Kunert et al. remind us of the recent clinical successes of T-cell adoptive therapies by offering a comparative overview of clinical trials evaluating different experimental therapies in development, including immune checkpoint blocking antibodies and small molecule inhibitors (12). The authors proceed to define what parameters likely determine the success rates of TCR gene therapy, from the choice of target antigens to the cues that influence T-cell fitness or pre-conditioning patient treatment, and suggest strategies to overcome current challenges in the field.

Since the vast majority of tumor-associated antigens are directly derived from self proteins most naturally occurring peripheral TCRs bind to tumor pMHC with low affinity compared to microbial epitopes. Consequently, antigen receptor engineering that seeks to optimize and improve the recognition of tumor epitopes by increasing the affinity of the TCR is an important focus in the field of cancer cellular therapies. Stone and Kranz review in detail the TCR affinity-optimization efforts to date, mostly based on *in vitro* protein evolution platforms such as yeast and phage display, highlighting the benefits of the approach in terms of enhanced anti-tumor reactivity but also its pitfalls, in particular risks of autoimmune adverse effects in the case of high-affinity TCRs cross-reacting with non-tumor self epitopes (13). The authors further suggest strategies to identify potential off-target cross-reactive epitopes during the pre-clinical development of affinity-optimized TCRs. On the same topic Zoete et al. argue in favor of a rational, structure-guided approach to TCR/pMHC affinity-optimization (14). The authors describe their *modus operandi* to this endeavor, which is based on the *in silico* modeling of mutations within the complementary determining region loops of the TCR based on solved and modeled structures of TCR/pMHC complexes. An important take home message of these articles is that affinity enhancement should be within the physiological range of affinities observed for natural TCRs as supra-normal affinities seem to both result in inefficient activation as well as enhanced cross-reactivity. However, with respect to cross-reactivity, this view is somewhat counterbalanced by the article of Cole et al. who report the first structure of a high-affinity TCR generated by random mutagenesis and isolated by phage display (15). This TCR only bears mutations within

the hypervariable CDR3 β loop and owes its enhanced binding properties to additional contacts with the peptide rather than the MHC molecule, explaining the relative lack of increase in affinity for known cross-reactive ligands compared to the index epitope. Directed mutations that seek to mimic this design may be the way forward for TCR affinity-optimization.

Even though the articles of this topic focus heavily on the use of TCRs for cancer cellular therapy this shouldn't play down the promises of CARs, which have also shown spectacular clinical results. This small injustice is repaired thanks to the article of Hombach and Abken, who review recent CAR engineering principles intended to promote long-term persistence and functionality of re-directed T-cells *in vivo* by triggering co-stimulatory signaling pathways subsequent to antigen engagement (16).

In addition to receptor engineering, a complementary and promising avenue to improve the efficacy of T-cell based cancer cellular therapies lies in the inactivation of immune-suppressive mediators of the tumor milieu. Recent clinical successes obtained with blocking antibodies targeting CTLA-4 or PD-1 as monotherapies raise the question of whether combining such approaches with T-cell adoptive transfer would provide additional clinical benefit, as it is hoped it will with vaccines. In accordance, Rufer and colleagues discuss TCR affinity-optimization along with other potential therapeutic strategies that include targeting co-inhibitory receptors with blocking monoclonal antibodies, impairing downstream inhibitory signaling and second messenger pathways with small molecule inhibitors or activating co-stimulatory receptors with agonistic antibodies (17). Generally speaking the combination of T-cell therapy with the inactivation of co-inhibitory receptors expressed by T-cells is a recurrent theme in the articles of the research topic and in the broader literature. The implementation of such therapeutic interventions is also a matter of discussion. Co-administration of blocking monoclonal antibodies or recombinant proteins with cellular therapies is usually the most popular option. However, recent progress in genome engineering technologies offers new angles for co-inhibitory receptor inactivation in the context of cellular therapies. Lloyd et al. briefly review the literature on protein-guided and RNA-guided endonucleases as a means to inactivate specific genes in human cells (18). They hypothesize that the co-delivery of anti-tumor antigen receptors with genome editing agents targeting immune checkpoint receptor genes may represent a cost-efficient and safe way of improving cancer ACTs without the need for combining different therapeutic modalities such as the adoptive transfer of cells as well as the infusion of biologics.

In summary, these 18 articles give an overview of several themes currently under investigation, and of their challenges, in the field of human T-cell biology. It is noteworthy that a large part of the Research Topic addresses applied aspects of T-cell immunology; this might be an indication that decades of intense fundamental research might be about to pay off and translate into effective treatments as well as viable commercial products.

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An altered gp100 peptide ligand with decreased binding by TCR and CD8 α dissects T cell cytotoxicity from production of cytokines and activation of NFAT

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Altered peptide ligands (APLs) provide useful tools to study T cell activation and potentially direct immune responses to improve treatment of cancer patients. To better understand and exploit APLs, we studied the relationship between APLs and T cell function in more detail. Here, we tested a broad panel of gp100_{280–288} APLs with respect to T cell cytotoxicity, production of cytokines, and activation of Nuclear Factor of Activated T cells (NFAT) by human T cells gene-engineered with a gp100-HLA-A2-specific TCR $\alpha\beta$. We demonstrated that gp100-specific cytotoxicity, production of cytokines, and activation of NFAT were not affected by APLs with single amino acid substitutions, except for an APL with an amino acid substitution at position 3 (APL A3), which did not elicit any T cell response. A gp100 peptide with a double amino acid mutation (APL S4S6) elicited T cell cytotoxicity and production of IFN γ , and to a lesser extent TNF α , IL-4, and IL-5, but not production of IL-2 and IL-10, or activation of NFAT. Notably, T cell receptor (TCR)-mediated functions showed decreases in sensitivities for S4S6 versus gp100 wild-type (wt) peptide, which were minor for cytotoxicity but at least a 1000-fold more prominent for the production of cytokines. TCR-engineered T cells did not bind A3-HLA-A2, but did bind S4S6-HLA-A2 although to a lowered extent compared to wt peptide-HLA-A2. Moreover, S4S6-induced T cell function demonstrated an enhanced dependency on CD8 α . Taken together, most gp100 APLs functioned as agonists, but A3 and S4S6 peptides acted as a null ligand and partial agonist, respectively. Our results further suggest that TCR-mediated cytotoxicity can be dissected from production of cytokines and activation of NFAT, and that the agonist potential of peptide mutants relates to the extent of binding by TCR and CD8 α . These findings may facilitate the design of APLs to advance the study of T cell activation and their use for therapeutic applications.

Keywords: activation of nuclear factor of activated T cells, altered peptide ligands, cytokine production, cytotoxicity, human T lymphocytes, T cell receptor

INTRODUCTION

T lymphocytes are potent mediators of anti-tumor immune responses. In fact, T cell receptor (TCR) genes derived from anti-tumor T lymphocytes have been successfully used to redirect other, non-tumor-specific T lymphocytes to tumor cells, and have shown promising clinical activities in the treatment of tumor-bearing patients (1, 2). Adoptive T cell therapy to tumors is based on the ability of TCRs to selectively recognize antigens, i.e., peptides that are presented by Major Histocompatibility Complex (MHC) molecules. The clinical use of TCR-engineered T lymphocytes directed against the human leukocyte antigen (HLA)-A2-restricted antigens MART-1, gp100, or NY-ESO-1 resulted in objective responses in patients with metastatic melanoma up to 45% (3, 4). Importantly, the avidity and antigen reactivity of parental T cell clones, used as a source for TCR genes, are preserved by TCR gene transfer (5–7). Moreover, cytotoxic responses of TCR-engineered T cells toward a panel of gp100 peptide mutants are identical to those of parental CTL clones (7).

Studies with mutated peptides, so called altered peptide ligands (APLs), have eloquently demonstrated that T cell recognition of antigen is flexible and that binding of different APLs can result in distinct and selective T cell signaling and functions (8, 9). APLs can be classified depending on the T cell responses they elicit; e.g., agonists induce the full range of T cell activation such as proliferation, cytokine secretion, and cytotoxic killing; partial agonists sub-optimally activate T cells and cause a selective pattern of effector functions; null agonists do not activate T cells; whereas antagonists specifically inhibit T cell activation induced by the wild-type (wt) peptide [reviewed in (10, 11)]. Interestingly, melanoma cells can process and present antagonistic APLs themselves, thereby potentially providing cues that prevent maximal intra-tumoral T cell activation and facilitate immune evasion (12). Immune suppression mediated by antagonistic peptide variants can be reversed by APLs with highly agonist properties that are able to sensitize T cells and yield resistance against effects of inhibitory APLs (12, 13). Importantly, APLs have already been

used in immunotherapeutic strategies with the intent to more effectively skew immune responses against autoimmune diseases, infectious diseases, and cancer [reviewed in (10)].

Numerous APLs have been designed for cancer epitopes and include, amongst others, MUC1-HLA-A2 (14), HER1-HLA-A2 (15), HER2-HLA-A2 (16), HER2-HLA-A24 (17), MelanA-HLA-A2 (18), gp100-HLA-A2 [epitopes 154, 209, and 280 (19)], TRP2-HLA-A2 (20), PSA-HLA-A2 (21), and NY-ESO1-HLA-A2 (22). Such APLs have principally been designed to improve the binding affinity of peptide to the MHC molecule, allowing induction of improved T cell responses against wt epitope. For example, MelanA-HLA-A2-specific T cell responses have rapidly and reproducibly been induced with the highly immunogenic APL with a Leucine at anchor position 2 (L2) (18, 23). However, enhanced immunogenicity of APLs may not necessarily be accompanied by the induction of a curative T cell response specific for the native epitope in patients with cancer. In fact, the modified MelanA epitope may alter TCR binding and prime T cells with different TCRs compared to the wt peptide (24). Indeed in patients with melanoma, T cells elicited by APL L2 demonstrated higher frequencies but weaker functional T cell avidity toward the native epitope (25). This is not necessarily a general finding as gp100 APLs (gp100_{154–162} A8 and gp100_{280–288} V9) were clinically equally effective when compared to wt peptides when used in combination with a DC vaccine (26). Collectively, however, these studies challenge the value and clinical applicability of APLs. Further and detailed studies into APLs and their effects on various T cell parameters are needed to gain a better understanding of the perimeters of T cell specificity and sensitivity. In addition, a correct definition of agonist and potential antagonist properties of APLs will allow successful translation of selected APLs to clinical settings. It is noteworthy that besides the setting of vaccination, where the frequency of the relevant TCR may be insufficient, the clinical potential of APLs may be extended to the setting of adoptive T cell therapy, which ensures a high frequency of the expected TCR in patients.

Here, we have used a panel of gp100_{280–288} APLs and explored APL characteristics in relation to T cell recognition and different T cell responses. To this end, we have transferred a defined TCR, i.e., a gp100-HLA-A2-specific TCR, into human T cells, and tested the effect of individual and double amino acid substitutions of the wt gp100 peptide on T cell responses. Analyses of gp100 APLs revealed that all single amino acid mutants retain their agonistic properties, except for the A3 mutant and double amino acid S4S6 mutant that acted as a null ligand and partial agonist, respectively. Findings showed that TCR-mediated cytotoxicity can be dissected from production of cytokines and activation of nuclear factor of activated T cells (NFAT), and suggest that the agonist potential of APLs relates to the extent a peptide mutant is bound by TCR and CD8 α .

MATERIALS AND METHODS

CELLS AND REAGENTS

Peripheral blood lymphocytes (PBL) from healthy donors were isolated by centrifugation through Ficoll-Isopaque (density = 1.077 g/cm³; Pharmacia Biotech, Uppsala, Sweden). Obtaining and handling of human samples, such as PBL, were

according to national and institutional guidelines and approved by the Erasmus MC Cancer Institute's ethical committee. Primary human T lymphocytes were cultured as described elsewhere (7). The TAP-deficient TxB cell hybrid and HLA-A2-positive T2, and the gp100-positive, HLA-A2-positive melanoma cell line FM3 were maintained in DMEM (Gibco BRL, Paisley, Scotland, UK) supplemented with 10% Bovine Calf Serum (BCS: Hyclone, Logan, UT, USA) and the antibiotics streptomycin (100 μ g/ml) and penicillin (100 U/ml). The HLA-A2-positive melanoma cell lines BLM and BLMgp100 (the latter transfected with human gp100-encoding cDNA) were cultured as described previously (27, 28). The Jurkat T cell clone E6.1 was expanded in RPMI 1640 medium supplemented with L-glutamine, 10% BCS, and antibiotics.

PEPTIDES AND PEPTIDE-MHC MULTIMERS

Peptides used in this study were: the gp100_{280–288} wt peptide YLEPGPVTA, the gp100 APLs A1–A8, G9, and S4S6, indicating an Alanine, Glycine, or Serine substitution at the indicated amino acid position of the wt peptide, and an irrelevant HLA-A2-binding EBV BMLF-1 wt peptide (GLCTLVAML). Peptide preparations were synthesized as described earlier (7) and found to be >90% pure as analyzed by analytical HPLC. MHC class I binding of peptides was analyzed via stabilization of HLA-A2 on T2 cells, as described previously (29, 30). The gp100 wt peptide, the gp100 APLs A3 and S4S6, and the BMLF-1 wt peptide were used to generate peptide-HLA-A2 monomers (Sanquin Blood Supply Foundation, Amsterdam, Netherlands). Multimers of peptide and HLA-A2 were freshly prepared by incubating streptavidin^{PE} and the corresponding soluble monomers at a 1:4 molar ratio for 1 h at 4°C as described elsewhere (31).

CLONING AND TRANSFER OF TCR GENES

Genes encoding gp100_{280–288}-HLA-A2-specific TCR $\alpha\beta$ were PCR-amplified from CTL clone 296 (CTL-296) and cloned into the retroviral vector bullet, as described previously (7). Primary human T lymphocytes of healthy donors, pre-activated with anti-CD3 mAbs were transduced with TCR-positive retroviruses produced by the packaging cell line Phoenix-Amp (32, 33). A retroviral vector encoding human CD8 α (34) was used to transduce Jurkat T cells prior to transfer of TCR genes (35). Transduction with Mock genes served as a negative control.

FLOW CYTOMETRY AND CELL-SORTING

T cells were analyzed for TCR transgene expression by flow cytometry using either PE-conjugated anti-TCRV β 27 mAb (Beckman-Coulter, Marseille, France) (nomenclature of TCRV regions according to <http://imgt.org>) or PE-conjugated gp100 wt peptide-HLA-A2 complexes (ProImmune Ltd., Oxford, UK), as described previously (7, 35). In short, 0.1–0.5 \times 10⁶ T cells were incubated with mAb on ice for 30 min or peptide-MHC complexes (see Peptides and Peptide-MHC Multimers) at room temperature for 1 h, washed, fixed (1% PFA). T cells were gated according to forward and sideward scatter properties using a FACSCalibur (Becton Dickinson, Alphen a/d Rijn, Netherlands) equipped with Cell-Quest software (BD Biosciences). Binding of peptide-MHC was

analyzed within viable gate of TCR-engineered T cells with marker set at <1% positive binding for non-stained TCR-engineered T cells. TCR-engineered T cells were MACS-enriched using gp100 wt peptide-HLA-A2 multimers and anti-PE MACS MicroBeads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

CYTOTOXICITY ASSAY

Cytotoxic activity of T cells was assayed in standard 6 h ^{51}Cr -release assays using the following target cells: T2 cells pulsed with 1 μM or titrated amounts of either gp100 wt peptide, gp100 APLs, BMLF-1 wt peptide, or the melanoma cell lines BLM, BLMgp100, or FM3. Antigen-specificity was confirmed by addition of anti-TCRV β 27 mAb or mouse immunoglobulin (mIg; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) (both at 1 $\mu\text{g}/\text{ml}$ final), and the contribution of CD8 interactions was studied by addition of anti-CD8 α mAb (clone 4H8, 10 $\mu\text{g}/\text{ml}$ final, Sanquin Blood Supply Foundation) to T cells 30 min before cultivation with target cells. To inhibit NK cell activity during the cytotoxicity assay, an excess of non-labeled K562 cells was added to target cells prior to assay. The percentage of specific ^{51}Cr -release was determined as follows: [(test counts – spontaneous counts)/(maximum counts – spontaneous counts)] \times 100% (36).

CYTOKINE MEASUREMENTS

To quantify the production of cytokines, 6×10^4 T cells were cultured in the presence of 2×10^4 T2 cells pulsed with titrated amounts of peptide for 18 h. Supernatants were harvested, and levels of IL-2, IL-4, IL-5, IL-10, IFN γ , and TNF α were determined via Cytokine Bead Array (CBA kit; BD Biosciences), according to the manufacturer's instructions. In some experiments, CBA data were supplemented with ELISAs (Sanquin Blood Supply Foundation), which were performed according to the manufacturer's instructions. As a positive control, T cells were stimulated with PMA and PHA.

NFAT REPORTER GENE ASSAY

Reporter gene assays for NFAT were performed as described in detail elsewhere (35). In short, exponentially growing TCR/CD8 α -co-transduced Jurkat T cells (5×10^6) were electroporated with a construct containing Firefly Luciferase coupled to six response elements of NFAT [FLuc-NFAT(REF6)]. Twenty hours post-transfection, Jurkat T cells were transferred to round-bottom 96-well plates at 2×10^5 cells/well and co-incubated with target cells at 10^5 cells per well for 6 h in RPMI 1640 medium supplemented with 1% BCS at 37°C and 5% CO₂. FM3 cells were pre-incubated O/N with IFN γ (PeproTech, NJ, USA, 10 ng/ml) and IL-1 β (PeproTech, 30 ng/ml), and co-cultivation of these melanoma cells with Jurkat T cells was performed in the presence of anti-CD28 mAb (clone 15E8, 2 $\mu\text{g}/\text{ml}$, Sanquin Blood Supply Foundation). As a positive control for activation of NFAT, T cells were stimulated with PMA and ionomycin. Following stimulation, cells were lysed and luciferase activities were determined. Luciferase activities were expressed relative to a non-stimulated condition (medium only, which was set to 1.0; Figure 1) or in absolute light units corrected for a non-stimulated condition (LU corrected for medium only; Figure 5).

PRESENTATION OF THE DATA

All assays, i.e., cytotoxicity, cytokine production, and NFAT activation assays have at least three data points of which mean values (with all values within 15% of mean) were used for graphical presentation. For each graph the experiment as a whole was repeated several times (as indicated) and data of a representative experiment was shown (the latter based on the mean value of triplicate data points per experiment).

RESULTS

SINGLE AMINO ACID SUBSTITUTIONS OF THE gp100 wt PEPTIDE DO NOT AFFECT T CELL FUNCTIONS EXCEPT FOR E3 TO A SUBSTITUTION, WHICH RESULTS IN A NULL LIGAND

In order to study gp100 peptide requirements of various TCR-mediated responses, both primary human T cells and Jurkat T cells were retrovirally transduced with TCR α and β genes that originated from the gp100/HLA-A2-specific CTL clone 296, and MACSsorted for high and equal levels of TCR expression. Flow cytometry with TCRV β mAb showed that gp100 TCR expression levels were about 90% [mean fluorescence intensity (MFI): 103] and 93% (MFI: 214) for primary human T lymphocytes and Jurkat T cells, respectively (data not shown). CD8 expression on primary human T cells was >50% and Jurkat T cells were co-transduced with the human CD8 α gene (expression level: 100%; MFI: 590) (data not shown). Antigen-specific responses of TCR-engineered T cells were validated at the level of T cell cytotoxicity, production of TNF α , and activation of NFAT. Figure 1A demonstrates that TCR-transduced primary human T lymphocytes were able to lyse gp100-positive, HLA-A2-positive but not gp100-negative, HLA-A2-positive melanoma cell lines. The antigen-specificity of this response was further confirmed by the use of anti-TCRV β mAb (Figure 1A). In addition, Figures 1B,C demonstrate that TCR-transduced human T lymphocytes produced TNF α and activated the transcription factor NFAT in an antigen-specific manner. Mock-transduced T cells did not show cytotoxic reactivities, TNF α production, and NFAT activation in response to any of the tumor cell lines tested. Data shown in Figure 1 confirm previous data from our group (7, 35) and further validate the antigen-specificity and use of the CTL296-derived TCR $\alpha\beta$ for our *in vitro* analyses of APLs.

Using APLs with single amino acid substitutions, we studied the same three T cell responses with the following observations. *First*, all APLs ($n = 9$) sensitized T2 target cells for TCR-mediated lysis, except for APL with an E to A substitution at position 3 (i.e., APL A3) (Figure 2A). *Second*, although quantities of TNF α produced varied depending on the peptide used, all APLs, except for APL A3, induced production of this cytokine (Figure 2B). *Third*, again all APLs induced a clear activation of NFAT, except for APL A3 (Figure 2C). Mock-transduced T cells did not respond upon stimulation with any of the APLs tested, neither did TCR-transduced T cells respond to stimulation with BMLF-1 wt peptide (data not shown and Figure 2). Observations presented in Figure 2 extend earlier data showing that the cytotoxic responses of the parental CTL-296 versus gp100 APL with single amino acid mutations are preserved following TCR gene transfer into human T cells (7).

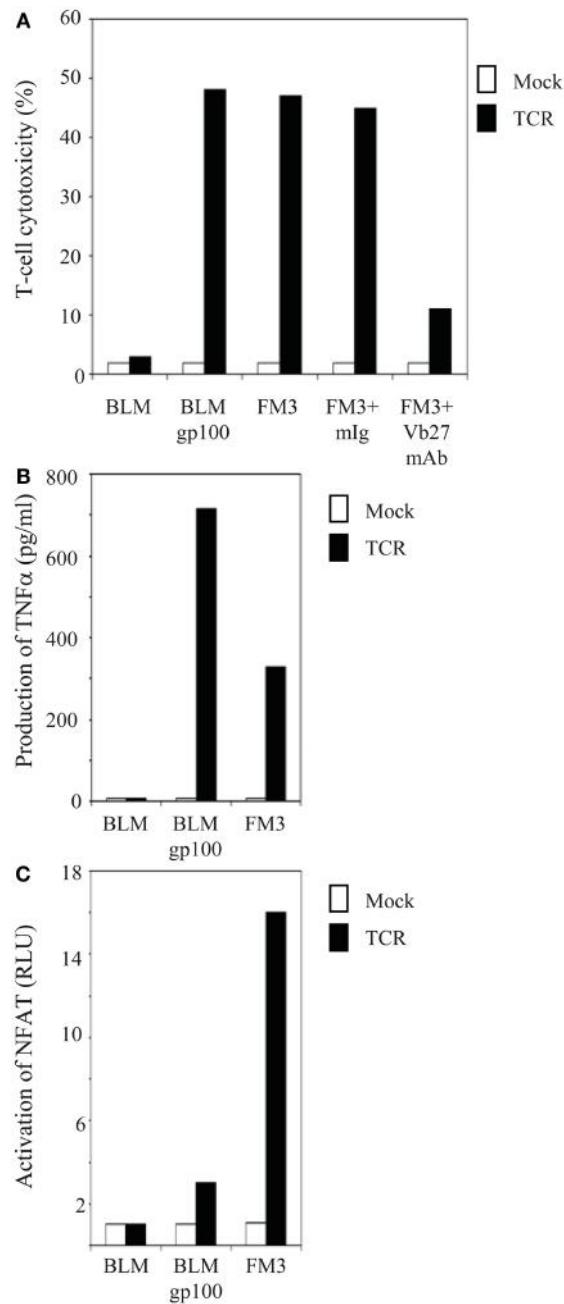


FIGURE 1 | T cells gene-engineered with a gp100/HLA-A2-specific TCR demonstrate antigen-specific reactivity against melanoma cells.

(A) TCR-transduced human T cells lyse gp100-positive, HLA-A2-positive melanoma cells. Primary human T cells transduced with TCR (black bars) or Mock genes (white bars) were tested in a 6 h ^{51}Cr -release assay with the following target cells: the gp100-negative, HLA-A2-positive melanoma cell line BLM and the gp100-positive, HLA-A2-positive melanoma cell lines BLMgp100 and FM3. The effector to target cell ratio was 30:1. Inhibition studies were performed with T cells and FM3 target cells using anti-TCR β 27 mAb (Vb27) or mIg. Specific lysis was calculated, and results of one (out of three) representative experiment are shown.

(B) TCR-transduced human T cells produce TNF α in response to gp100-positive, HLA-A2-positive melanoma cells.

(Continued)

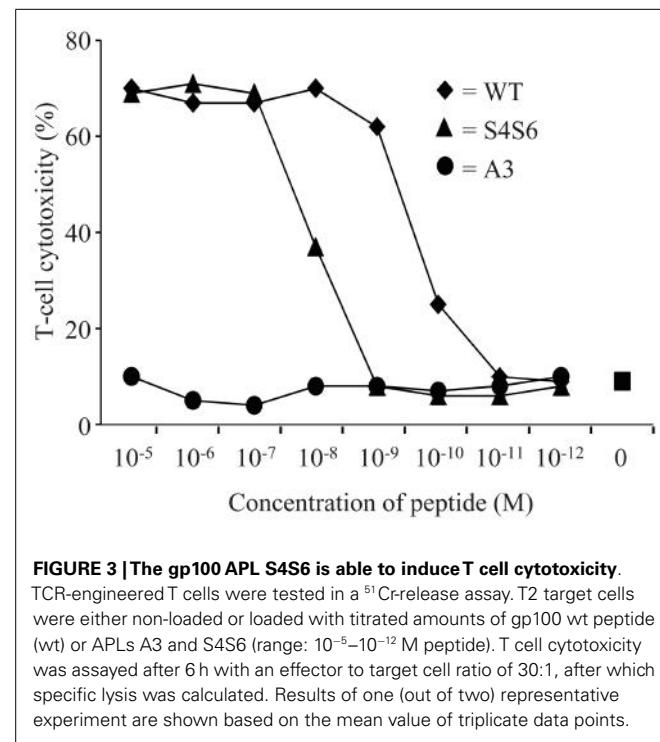
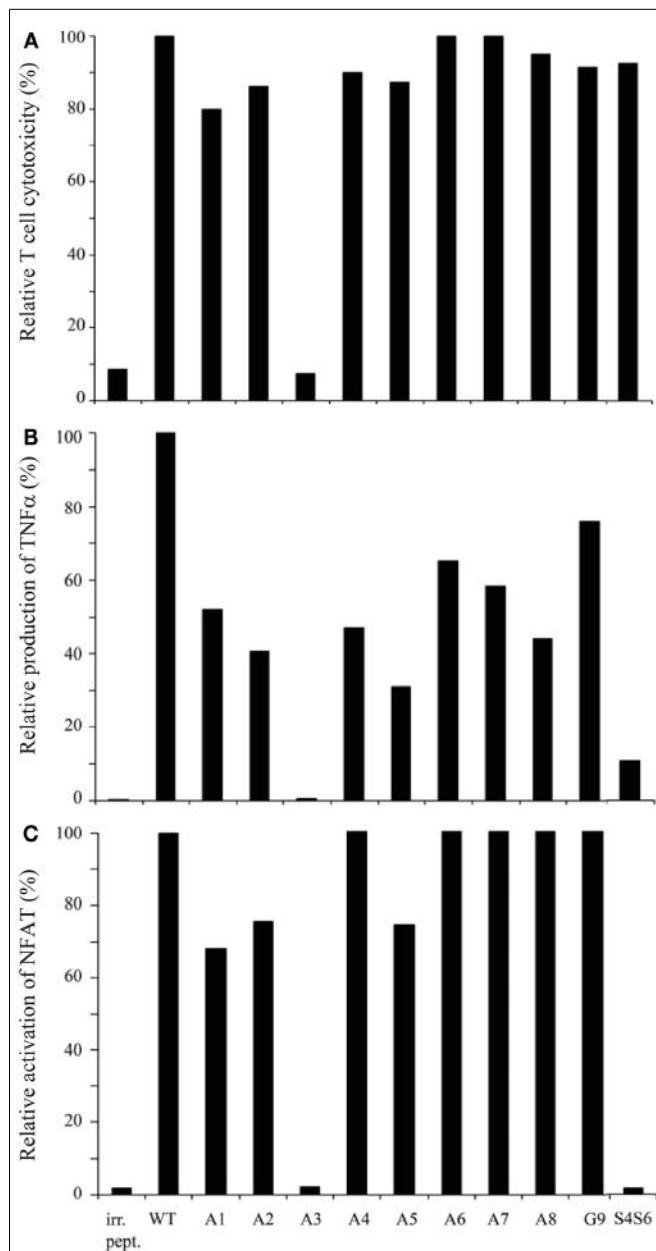
FIGURE 1 | Continued

T cells and melanoma cells as described in **(A)** were co-cultivated for 18 h at an effector to target cell ratio of 3:1, after which TNF α levels (in pg/ml) were determined in supernatants by ELISA. Results of one (out of three) representative experiment are shown. **(C)** TCR-transduced human T cells activate NFAT in response to gp100-positive, HLA-A2-positive melanoma cells. Jurkat T cells transduced with TCR (black bars) or Mock genes (white bars) were transfected with an NFAT reporter construct and subsequently co-cultivated for 6 h with the same target cells as described in **(A)** at an effector to target cell ratio of 2:1. Luciferase activities were determined in cell lysates and expressed relative to medium only [in Relative Light Units (RLU)]. Medium stimulations of TCR- and Mock-transduced Jurkat T cells were 0.024 and 0.018, respectively, and were both set to 1.0. Results of one (out of two) representative experiment are shown.

A P4 AND P6 TO S SUBSTITUTION VARIANT OF THE gp100 wt PEPTIDE FUNCTIONS AS A PARTIAL AGONIST AND DISSECTS T CELL CYTOTOXICITY FROM PRODUCTION OF CYTOKINES AND ACTIVATION OF NFAT

Next to the gp100 APLs with single amino acid substitutions, we generated an APL with a double amino acid mutation. The rationale behind designing this APL was to experimentally address whether replacement of both Proline amino acids, which are characterized by a rigid backbone, would affect and perhaps enhance T cell functions. Since Prolines at positions 4 and 6 in the wt gp100 peptide (YLEPGPVTA), when individually replaced by Alanine, did not alter function of T cells, we next replaced both Prolines by Serines, which are characterized by a more flexible backbone. This S4S6 peptide, when tested in the same set of assays as described above, was able to induce a cytotoxic T cell response and production of low levels of TNF α , but was not able to induce activation of NFAT in human T cells (**Figures 2A–C**).

Findings with gp100 APLs suggest that APLs A1, A2, A4, A5, A6, A7, A8, G9 act as full agonists; APL A3 acts as a null ligand; and APL S4S6 acts as a partial agonist (**Figure 2**). To verify whether APLs A3 and S4S6 present a true null ligand and partial agonist, respectively, we performed extensive peptide dose-response studies. Results of repeated experiments were highly consistent and showed that APL A3 did not elicit T cell cytotoxicity (**Figure 3**), production of cytokines (**Figure 4**), and activation of NFAT (**Figure 5**) when tested over a 7-log range of peptide concentrations (10^{-5} – 10^{-12} M peptide). APL S4S6, however, induced decreased T cell cytotoxicity (**Figure 3**), an extremely lowered production of cytokines (**Figure 4**), but no activation of NFAT (**Figure 5**). When looking at wt peptide, we noted that over a large range of concentrations, T cell responses were comparable in all three assays, arguing that results with APLs are not due to differences in assay sensitivities. In example, wt peptide induced maximal T cell cytotoxicity, IFN γ production as well as NFAT activation over a 4-log range (10^{-5} – 10^{-8} M peptide), whereas these T cell responses became suboptimal from 10^{-9} to 10^{-10} M peptide onward, and were negligible from 10^{-10} to 10^{-11} M or lower. Instead, quantities of S4S6 peptide that were able to induce maximal T cell cytotoxicity were 10–100-fold higher when compared to wt peptide (**Figure 3**). With respect to cytokines, wt peptide selectively induced levels of IFN γ , and to a lesser extent TNF α , IL-2, IL-4, and IL-5, but



not IL-10 (Figure 4). S4S6 peptide, however, triggered maximal production of IFN γ at concentrations that were 10^5 – 10^6 -fold higher when compared to wt peptide (Figure 4). Production of IL-4 and IL-5 upon stimulation with S4S6 peptide was only detectable at the highest peptide concentrations used (at most 750 pg/ml when stimulating with 10^{-5} M peptide) (Figure 4). Using TNF α ELISAs, in our experience more sensitive than CBA, we again only observed detectable production of TNF α at the highest peptide concentrations used (at most 500 pg/ml when stimulating with 10^{-5} M peptide) (data not shown). Lastly, activation of NFAT was not observed for S4S6 peptide independent of the peptide concentration tested (Figure 5).

APL S4S6-INDUCED T CELL FUNCTIONS SHOW ENHANCED DEPENDENCY ON CD8 α

In an effort to explain our results with APLs A3 and S4S6, we examined their ability to bind to HLA-A2, their ability to bind (when complexed to HLA-A2) to the gp100 TCR, and to what extent T cell function, in case of S4S6, relies on the co-receptor CD8 α .

To study HLA-A2 binding of APLs, we performed an HLA-A2 stabilization assay using T2 cells. This assay showed that APLs A3 and S4S6 bind as efficient to HLA-A2 as does wt peptide (i.e., stabilization factors at 50 μM were 1.29, 1.14, and 1.25 for A3, S4S6, and wt peptide, respectively). The only APLs within our panel that bound significantly less to HLA-A2 are those that harbored a non-conservative mutation in one of the anchoring residues (i.e., APLs A2 and G9, but not V9, having stabilization factors at 50 μM of 0.00, 0.13, and 1.27, respectively). The abilities of APLs A3 and S4S6 to be stably bound by HLA-A2 allowed the synthesis of peptide-MHC multimers and detection of their

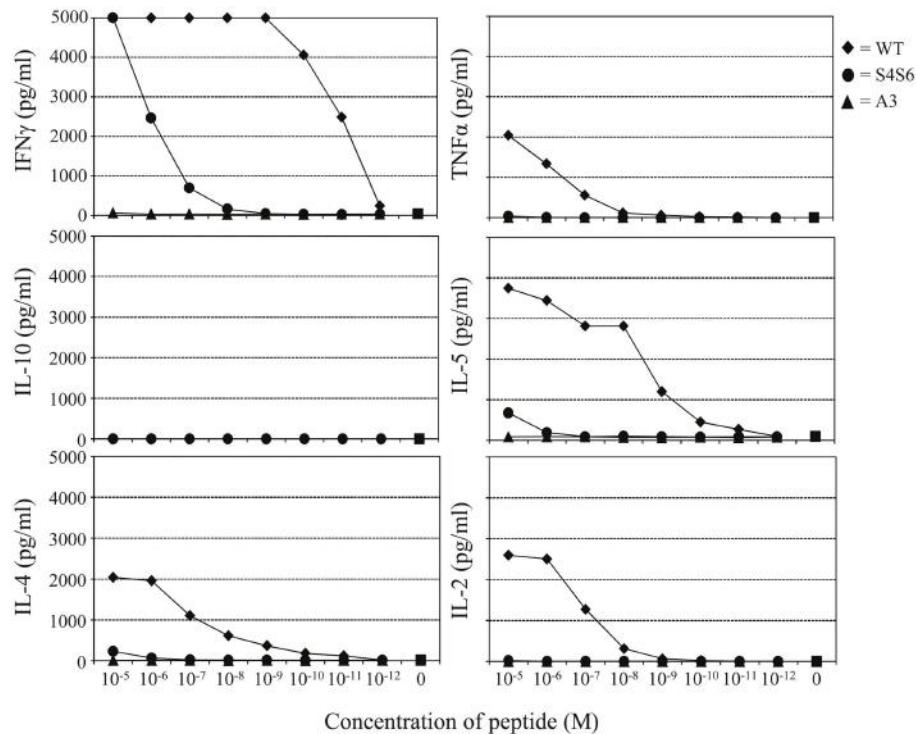


FIGURE 4 | The gp100 APL S4S6 induces a changed cytokine production profile. TCR-engineered T cells were tested for the production of IFN γ , TNF α , IL-2, IL-4, IL-5, and IL-10 using a Cytokine Bead Array (CBA). T2 target cells were either non-loaded or loaded with titrated amounts of gp100 wt peptide (wt) or APLs A3 and S4S6 (range:

10⁻⁵–10⁻¹² M peptide). Following an 18 h co-cultivation at an effector to target cell ratio of 3:1, supernatants were collected and measured for cytokine content. Results of one (out of three) representative experiment are shown based on the mean value of triplicate data points.

binding by TCR-engineered T cells by flow cytometry. When analyzing the binding of titrated amounts of peptide-HLA-A2 multimers, we observed that neither A3-HLA-A2, nor (the negative control) BMLF-1-HLA-A2 were bound by T cells (Figure 6). In contrast, S4S6-HLA-A2 was bound by T cells, although to a lower extent than wt peptide-HLA-A2 (Figure 6). Finally, we established the contribution of CD8 α to S4S6-induced T cell functions, to which end we have tested T cell cytotoxicity in the presence of a CD8 α blocking antibody (Figure 7). Anti-CD8 α antibody fully neutralized gp100 TCR-mediated cytotoxicity upon stimulation with S4S6 peptide, whereas decrease in cytolytic activity was negligible upon stimulation with wt peptide (Figure 7; right versus left panels).

DISCUSSION

In this study, we have characterized peptide requirements for T cell cytotoxicity, production of cytokines, and activation of NFAT. To this end, TCR $\alpha\beta$ genes originating from the gp100_{280–288}-HLA-A2-specific 296 CTL clone were transferred in human T cells, and receptor-mediated responses were tested versus gp100-positive and -negative melanoma cells (Figure 1) and a broad panel of gp100 APLs (Figure 2). We observed that APLs with single amino acid substitutions functioned as agonists. The E to A peptide mutant (i.e., APL A3) is an exception to this observation since it functions as a null ligand. The P to S double amino acid

substitution variant (i.e., APL S4S6) functions as a partial agonist and is able to dissect cytotoxicity from cytokine production and NFAT activation.

Our observation that the performance of APLs with single amino acid substitutions was identical among the different T cell parameters tested, i.e., T cell cytotoxicity, production of cytokines, and activation of NFAT (Figure 2), was confirmed for a second gp100 TCR. Primary human T cells transduced with TCR $\alpha\beta$ genes derived from the gp100_{280–288}-HLA-A2-specific MPD CTL clone (37) showed cytolytic responses which were in complete accordance with TNF α production upon stimulation with APLs with single amino acid mutations [(7), and data not shown]. In case of the CTL-296-derived TCR, APL A3 behaved as a null agonist with respect to T cell cytotoxicity, production of cytokines, and activation of NFAT (Figure 2). This non-responsiveness is not caused by less efficient presentation of A3 peptide since the binding capacity of this mutant to HLA-A2 molecules is in the same range as that of the wt peptide. Rather, the non-responsiveness is caused by the TCR's inability to bind A3-HLA-A2 complexes (Figure 6). The classification of APL A3 as a null agonist, and not as a potentially weak or partial agonist, is justified by the observation that peptide concentrations over a 7-log range did not affect T cell activities (Figures 3–5). In addition, we were unable to show an inhibitory effect of excess A3 peptide on wt peptide-induced responses in TCR-transduced human T cells, suggesting that this

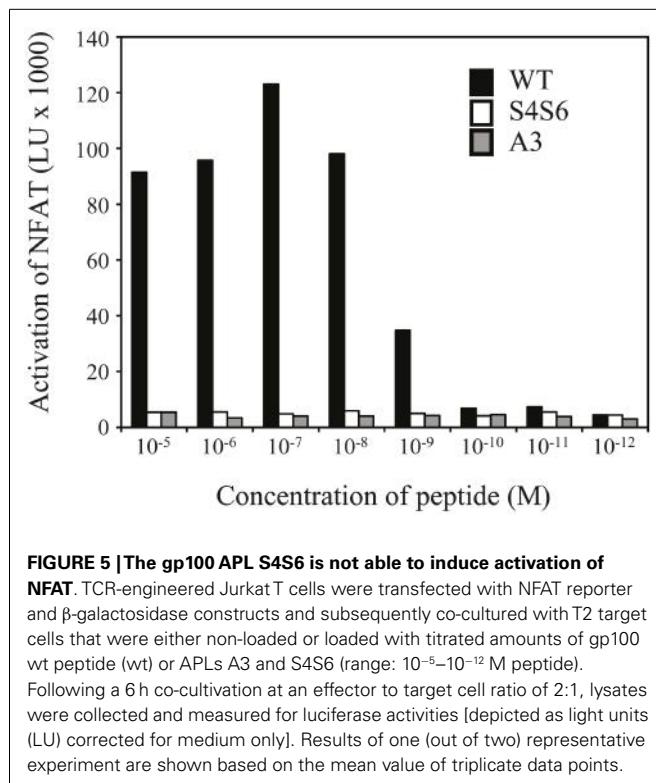


FIGURE 5 | The gp100 APL S4S6 is not able to induce activation of NFAT. TCR-engineered Jurkat T cells were transfected with NFAT reporter and β -galactosidase constructs and subsequently co-cultured with T2 target cells that were either non-loaded or loaded with titrated amounts of gp100 wt peptide (wt) or APLs A3 and S4S6 (range: 10^{-5} – 10^{-12} M peptide). Following a 6 h co-cultivation at an effector to target cell ratio of 2:1, lysates were collected and measured for luciferase activities [depicted as light units (LU) corrected for medium only]. Results of one (out of two) representative experiment are shown based on the mean value of triplicate data points.

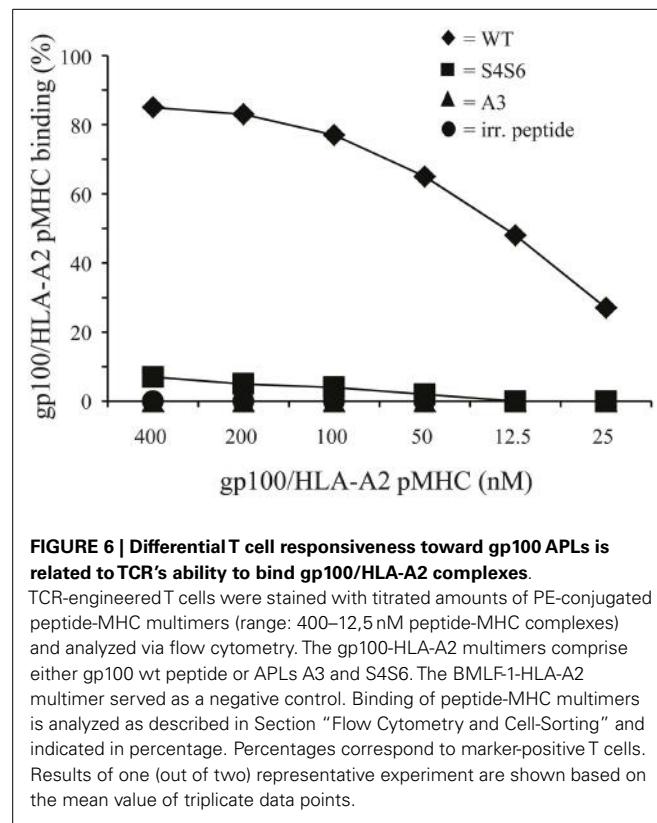


FIGURE 6 | Differential T cell responsiveness toward gp100 APLs is related to TCR's ability to bind gp100/HLa-A2 complexes.

TCR-engineered T cells were stained with titrated amounts of PE-conjugated peptide-MHC multimers (range: 400–12.5 nM peptide-MHC complexes) and analyzed via flow cytometry. The gp100-HLa-A2 multimers comprise either gp100 wt peptide or APLs A3 and S4S6. The BMLF1-HLa-A2 multimer served as a negative control. Binding of peptide-MHC multimers is analyzed as described in Section “Flow Cytometry and Cell-Sorting” and indicated in percentage. Percentages correspond to marker-positive T cells. Results of one (out of two) representative experiment are shown based on the mean value of triplicate data points.

peptide variant is not acting as an antagonist (data not shown). The possibility that APL A3 acts as a supra-agonist, i.e., a null ligand that enhances the reactivation of memory CTL responses (38), is currently under investigation.

Altered peptide ligand S4S6 was classified as a partial agonist since it was able to induce cytotoxicity, production of only selected cytokines, but not activation of NFAT when compared to wt peptide (Figures 3–5). When using titrated amounts of peptide we demonstrated that 10–100-fold more S4S6 peptide was needed to induce cytotoxicity when compared to wt peptide (Figure 3). The decreased sensitivity of TCR-mediated cytotoxicity was relatively minor and at least a 1000-fold less when compared to the decreased sensitivities of TCR-mediated cytokine production and NFAT activation for S4S6 versus wt peptide (see below). Induction of cytotoxic activity is considered an early T cell response and generally requires small amounts of antigenic peptide and hardly any TCR down-regulation. Cytolytic proteins, e.g., perforin and granzymes, are pre-synthesized and thereby lower the threshold to trigger their release, which may explain the success of partial agonists to induce cytotoxicity (39–43). S4S6 peptide induced the production of IFN γ and to a lesser extent of IL-4, IL-5, and TNF α , but not IL-2 and IL-10 (Figure 4) or the activation of NFAT (Figure 5). In fact, TCR-mediated production of IFN γ required 10^5 – 10^6 -fold higher concentrations of S4S6 when compared to wt peptide, and production of IL-4, IL-5, and TNF α required $>10^6$ -fold higher concentrations of S4S6, whereas production of IL-2, IL-10, and activation of NFAT could not even be measured at the highest concentration of S4S6 tested. These findings are in line with the notion that *de novo* synthesis of cytokines, which in

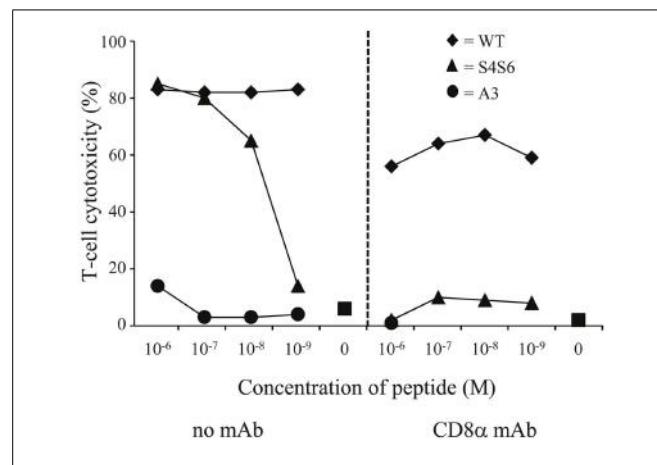


FIGURE 7 | The gp100 APL S4S6 renders the gp100/HLa-A2-specific TCR CD8 dependent. TCR-engineered T cells were tested in a 51 Cr-release assay as described in legend to Figure 3 either without or with blocking anti-CD8 mAb (left and right panels, respectively). T2 cells were either non-loaded or loaded with titrated amounts of gp100 wt peptide (wt) or APLs A3 and S4S6 (range: 10^{-6} – 10^{-9} M peptide). Results of one (out of two) representative experiment are shown based on the mean value of triplicate data points.

many cases depends on NFAT activation, generally requires higher amounts of antigenic peptide and a much stronger TCR signal, i.e., high levels of TCR occupancy and TCR down-regulation (43). Production of cytokines and activation of NFAT, considered late T cell responses, may therefore not be triggered by a partial agonist.

Partially agonistic peptides can selectively stimulate some T cell effector functions by inducing a pattern of signal transduction that is qualitatively different from the pattern induced by any concentration of the native peptide (9, 44–46). Partial agonistic signaling patterns are characterized by differential phosphorylation of TCR subunits, recruitment but no activation of ZAP-70, activation of MAP kinases (although for a shortened time period) and/or phenotypically distinct Ca^{2+} fluxes (11, 47, 48). A shortened period of MAP kinase activation and/or weakened Ca^{2+} flux could explain the observed lack of NFAT activation in T cells following stimulation with S4S6 peptide. Translocation of NFAT is reported to take place during “the TCR and co-receptor microclustering stage” in the formation of an immunological synapse (49). It would be interesting to find out whether S4S6 peptide would allow for TCR engagement but not proceeding to TCR microclustering and/or its coalescence into a central synapse. Another property of APL S4S6 may include its potential ability to induce T cell anergy. This state of T cell hyporesponsiveness is generally induced by triggering the TCR in the absence of sufficient T cell co-stimulation or in the presence of proficient T cell co-inhibition, and accompanied by the expression of anergy-associated genes, which subsequently contributes to impaired TCR signaling (50). Although there exist multiple forms of anergy, and it is mainly studied in CD4 T cells, the induction of anergy-associated genes appears to depend on the activation of NFAT. Hence, we argue that APL S4S6, because of its inability to activate NFAT, does not contribute to an anergic state of T cells.

The partial T cell responsiveness induced by APL S4S6 is not related to an altered ability of S4S6 peptide to bind to HLA-A2, but rather to a substantially decreased ability of the gp100 TCR to bind S4S6-HLA-A2 complexes (Figure 6). The new Serine residues, although not affecting binding to HLA-A2, apparently changed peptide-MHC conformation such that TCR chains showed a decreased fit for APL S4S6. Interestingly, APLs A2 and G9 with non-conservative amino acid substitutions at one of the peptide anchor positions to bind to HLA-A2 showed a significantly lowered stabilization of HLA-A2, yet were clearly able to induce cytotoxicity, TNF α production, and activation of NFAT (Figure 2).

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Cellular-level versus receptor-level response threshold hierarchies in T-cell activation

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Peptide-MHC (pMHC) ligand engagement by T-cell receptors (TCRs) elicits a variety of cellular responses, some of which require substantially more TCR-mediated stimulation than others. This threshold hierarchy could reside at the receptor level, where different response pathways branch off at different stages of the TCR/CD3 triggering cascade, or at the cellular level, where the cumulative TCR signal registered by the T-cell is compared to different threshold values. Alternatively, dual-level thresholds could exist. In this study, we show that the cellular hypothesis provides the most parsimonious explanation consistent with data obtained from an in-depth analysis of distinct functional responses elicited in a clonal T-cell system by a spectrum of biophysically defined altered peptide ligands across a range of concentrations. Further, we derive a mathematical model that describes how ligand density, affinity, and off-rate all affect signaling in distinct ways. However, under the kinetic regime prevailing in the experiments reported here, the TCR/pMHC class I (pMHCI) dissociation rate was found to be the main governing factor. The CD8 coreceptor modulated the TCR/pMHCI interaction and altered peptide ligand potency. Collectively, these findings elucidate the relationship between TCR/pMHCI kinetics and cellular function, thereby providing an integrated mechanistic understanding of T-cell response profiles.

Keywords: T-cell activation, T-cell cross-reactivity, T-cell receptor

INTRODUCTION

T lymphocyte antigen receptors mediate adaptive immune responses via interactions with disease-associated peptide ligands presented on the surface of target cells by major histocompatibility complex (MHC) molecules. In the case of CD8⁺ T-lymphocytes (CTLs), which constitute the classical T-cell effector subset, the clonotypically expressed T-cell receptors (TCRs) engage specific peptide-MHC class I (pMHCI) molecules to elicit several functions that are instrumental in eliminating the pathogenic threat (1).

The six hyper-variable complementarity-determining regions (CDRs) of the TCR govern molecular interactions with pMHCI (2). These CDRs confer the specificity of molecular recognition, allowing CTLs to attack diseased cells without causing undue harm to healthy cells (1). Nonetheless, a certain degree of degeneracy within the antigen recognition system is unavoidable (3). Indeed, ample experimental evidence supports the notion that a single TCR clonotype can interact productively with numerous peptide ligands, which typically vary in their ability to elicit different types of cellular response (4–14). Furthermore, some cellular responses are more readily evoked than others (15–18). It seems reasonable to explain this phenomenon by postulating a hierarchy of thresholds. Such a hierarchy may reside either at the level of individual TCR/CD3 complexes or at the cellular level. In the former scenario, the threshold parameters relate to distinct components of

the TCR triggering process, with different responses being elicited at different stages in the development of a mature signalosome. Essentially, this is the kinetic discrimination model proposed by Rabinowitz et al. (19), whereby early responses require a shorter TCR/pMHCI dwell-time than late responses. This model is logically distinct from the kinetic proofreading model that accounts for the existence of dwell-time thresholds *per se* (20).

In the cellular-level scenario, TCR triggering delivers a stereotypical signal that elicits distinct responses across a single quantitative gradient (e.g., concentration of the relevant signaling factor). The contrast between these two scenarios is illustrated diagrammatically in Figure 1. A third possibility is that the hierarchy comprises a combination of both receptor-level and cellular-level modes. It has hitherto been unclear which of these three alternatives prevails in T-cell signaling.

In this study, we used a mathematical model to investigate the extent to which each of the three possibilities (receptor-level hierarchy, cellular-level hierarchy, or a combination of both) agree with experimental data. Our model accounts for the functional sensitivity of TCR-mediated responses to a given pMHCI ligand on the basis of TCR/pMHCI interaction kinetics. The concept that TCR/pMHCI kinetics governs functional sensitivity was pioneered by Lanzavecchia et al. (21) and subsequently formulated as a mathematical model (22, 23). Bridgeman et al. (24) recently summarized the available evidence across reported systems.

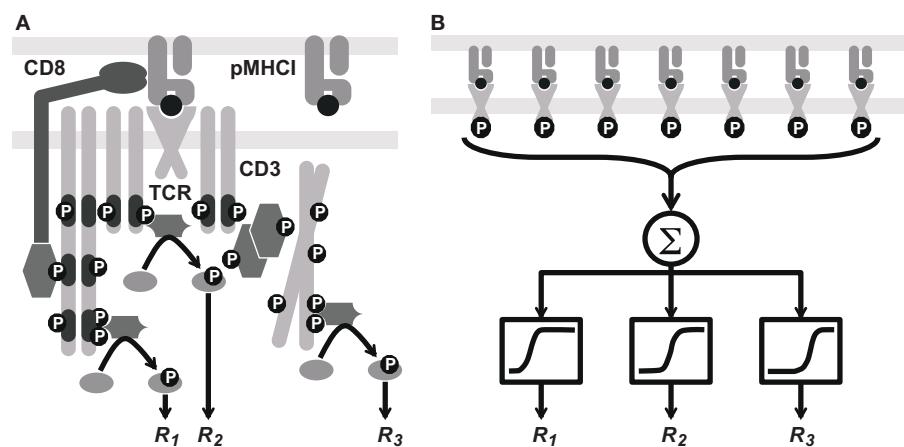


FIGURE 1 | Two hypotheses on the level of the response hierarchy. (A)

Signaling cascades are initiated at different points along the TCR/CD3 reaction pathway, which includes phosphorylation events and the docking of kinases and linker proteins. Thus, responses R_1 , R_2 , and R_3 would correspond to distinct values of the TCR triggering threshold, i.e., $T_R^{[1]} < T_R^{[2]} < T_R^{[3]}$. **(B)** Cellular integration of signals from the triggered TCRs is shown as a summation (Σ) box. This signal passes through a non-linear threshold that

determines whether the cell will respond. Here, R_1 , R_2 , and R_3 represent various cellular responses, such as the expression of different cytokine species. Each receives the same integrated signal W as an input, but different values of W are required to initiate a response. This is depicted schematically by sigmoid curves whose midpoint lies to the left, in the middle, or to the right. These differences correspond to distinct values of the cellular activation threshold, i.e., $W_{act}^{[1]} < W_{act}^{[2]} < W_{act}^{[3]}$. These two hypotheses are not mutually exclusive.

Analysis of TCR/pMHCI interaction kinetics demonstrates that functional sensitivity is not dependent on a single biophysical parameter, but rather on the interplay between association rate, dissociation rate, and ligand densities (23, 25, 26). Under the experimental conditions prevailing in the present study, however, the dissociation rate (the reciprocal of the mean dwell-time of the TCR/pMHCI interaction) emerged as the dominant biophysical parameter. Moreover, we estimated the extent to which the CD8 coreceptor modulates this parameter using data from parallel experiments conducted in the absence of an extracellular MHCI/CD8 interaction.

Five distinct cellular responses were investigated: (i) mobilization of the degranulation marker CD107a; (ii) secretion of macrophage inflammatory protein 1- β (MIP-1 β); (iii) secretion of tumor necrosis factor- α (TNF- α); (iv) secretion of interleukin-2 (IL-2); and (v) secretion of interferon- γ (IFN- γ). Measured simultaneously and independently by flow cytometry, the magnitude of each response was quantified as a function of fluorescence and plotted against peptide concentration. The resulting dose-response curves indicated that the five cellular responses were elicited by pMHCI stimulation according to a pronounced hierarchy. Analysis of these curves by means of the mathematical model, in conjunction with biophysical data, indicated that the cellular-level response threshold hierarchy hypothesis provides the most parsimonious explanation.

MATERIALS AND METHODS

EXPERIMENTAL PROCEDURES

Cells and peptide ligands

The CTL clone ILA1 recognizes residues 540–548 (ILAKFLHWL in single-letter amino acid code, abbreviated as ILA) of the human telomerase reverse transcriptase protein presented in the context of the human MHCI allotype HLA-A*0201 (HLA-A2). Cell culture

was performed as described previously (27). The altered peptide ligands used in this study are referred to here as 3G, 5Y, 8T, 8E, and 3G8T; these ligands were largely characterized previously (27, 28), and display equivalent binding to HLA-A2 (29).

Bioassay

Stable C1R transfectants expressing wild-type HLA-A2 (C1R-A2) or CD8-null HLA-A2 (C1R-A2null), the $\alpha 3$ domain of which contains the double mutation D227K/T228A that abrogates CD8 binding (30), were pulsed with peptide as indicated for 1 h at 37°C. For each condition, assays were set up simultaneously in 96-well plates using 4.5×10^5 C1R cells per well, thereby ensuring that all cellular parameters were consistent across ligand stimulations. Subsequently, brefeldin A (10 μ g/ml; BD Biosciences) and monensin (0.7 μ l/ml; BD Biosciences) were added together with a pre-titrated concentration of the directly conjugated monoclonal antibody (mAb) α CD107a-FITC [BD Pharmingen; Ref. (31)]. Serum-starved CTLs, incubated in Roswell Park Memorial Institute (RPMI) medium (Life Technologies) containing 2% fetal calf serum (FCS) for 16 h prior to assay, were subsequently added at 9×10^5 cells per well. After incubation for 6 h, which generally allows sufficient time for even the most sluggish response to appear, cells were washed in phosphate-buffered saline (PBS) containing 1% FCS and incubated for 10 min at room temperature with Aqua fixable live/dead cell stain (Life Technologies) to enable the exclusion of dead cells from the analysis. Pre-titrated concentrations of α CD3-H7allophycocyanin (BD Biosciences), α CD8-QD705 (Life Technologies), and α CD19-V500 (BD Horizon) were then added for 20 min at 4°C. After two further washes in PBS/1% FCS, cells were fixed/permeabilized using a Cytofix/Cytoperm™ kit (BD Biosciences) according to the manufacturer's instructions, then stained intracellularly with pre-titrated concentrations of

α MIP-1 β -PE (BD Pharmingen), α IFN γ -V450 (BD Horizon), α IL-2-allophycocyanin (BD Pharmingen), and α TNF α -PECy7 (BD Pharmingen) for 20 min at 4°C.

Flow cytometry

Stained cell samples were acquired and recorded using a customized FACS Aria II flow cytometer (BD Biosciences) equipped for the simultaneous detection of 18 fluorescent parameters. Polychromatic analyses were conducted using FlowJo software version 9.5.2 (TreeStar Inc.). The following gating tree was applied: (i) single cells were identified based on their light scatter properties; (ii) Boolean gating was carried out to exclude artifacts and fluorochrome aggregates; (iii) viable CD3 $^+$ CD19 $^-$ events were selected; (iv) outliers were eliminated in a side-scatter versus CD3 display; and (v) gates were set on cells positive for individual functional read-outs. The frequencies and median fluorescence intensity (MFI) values of each functional response were exported for data analysis to *Excel* and onward for simultaneous curve fitting in *Mathematica*.

Surface plasmon resonance

Soluble TCR, derived from the ILA1 CTL clone, was manufactured as described previously (32, 33). Binding analysis by surface plasmon resonance (SPR) was performed using a BIAcore T100™ equipped with a CM5 sensor chip (34). Biotinylated pMHCI (200–400 response units) was immobilized to streptavidin, which was chemically linked to the chip surface. The pMHCI was injected at a slow flow rate (10 μ l/min) to ensure uniform distribution on the chip surface. Combined with the small amount of pMHCI bound to the chip surface, this reduced the likelihood of dissociation rate limiting mass transfer effects. The ILA1 TCR was purified and concentrated to \sim 100 μ M on the day of SPR analysis to reduce the likelihood of TCR aggregation affecting the results. At least 5 serial dilutions were prepared in duplicate and injected over the relevant sensor chips at a flow rate of 45 μ l/min. All experiments were conducted at 25°C. Results were analyzed using *BIAevaluation*, *Excel*, and *Origin*.

THEORY AND DATA ANALYSIS

Assumptions

The assumptions of the TCR triggering model are as follows: (i) TCRs on the T-cell surface become “triggered” (i.e., are induced to become signalosomes) during an interaction with a pMHCI ligand; and (ii) the T-cell accumulates the signals emanating from triggered TCRs over space (cell:cell interface) and time (duration of cell:cell interaction), proceeding with a response when this signal exceeds a cellular activation threshold. The mathematical formulation of these assumptions is based on the kinetics of the TCR/pMHCI interaction.

The TCR triggering rate equation

The model describes the kinetics of interactions between TCR and pMHCI molecules in the interface area between a T-cell and an antigen-presenting cell (APC). This area is occupied by TCRs on the T-cell side of the interaction and by pMHCI complexes on the APC side. Let R_T be the total number of TCRs and Z_T

be the total number of pMHCI molecules. Both are subject to a conservation law:

$$R_T = C + R \quad (1)$$

$$Z_T = C + Z \quad (2)$$

where C is the number of TCR/pMHCI complexes, R is the number of TCRs not engaged in a complex (i.e., “free” TCR molecules) and Z is the number of free pMHCI molecules. Kinetic equilibrium is expressed by the law of mass action equation:

$$k_{\text{on}} RZ = k_{\text{off}} C. \quad (3)$$

This equation, together with equations (1) and (2), leads to a quadratic in C with solution

$$C = \frac{R_T + Z_T + k_{\text{off}}/k_{\text{on}}}{2} \left(1 - \sqrt{1 - \frac{4R_T Z_T}{R_T + Z_T + k_{\text{off}}/k_{\text{on}}}} \right) \quad (4)$$

(the other root is irrelevant since it exceeds both R_T and Z_T). The rate at which TCRs are being triggered can be expressed as the rate at which TCR/pMHCI complexes dissociate times the probability that any given TCR/pMHCI association event results in a triggering of the associated TCR/CD3 complex:

$$W = k_{\text{off}} C P_{\text{trig}} \quad (5)$$

where W denotes the TCR triggering rate and P_{trig} the triggering probability for an individual interaction event. Since triggering of a TCR/CD3 complex requires completion of a series of phosphorylation and docking events, it is reasonable to assume that triggering can only happen if the peptide ligand remains engaged for at least a certain amount of time. This minimum duration is the *TCR triggering threshold* T_R . If the lifetime of the TCR/pMHCI complex is exponentially distributed, the triggering probability is given by:

$$P_{\text{trig}} = \exp\{-k_{\text{off}} T_R\}. \quad (6)$$

Combined, equations (4–6) yield the general triggering rate equation:

$$W = \frac{k_{\text{off}} \exp\{-k_{\text{off}} T_R\}}{2} (R_T + Z_T + k_{\text{off}}/k_{\text{on}}) \times \left(1 - \sqrt{1 - \frac{4R_T Z_T}{R_T + Z_T + k_{\text{off}}/k_{\text{on}}}} \right). \quad (7)$$

Simple forms in special cases [equations (13–15)] are obtained by using the first-order approximation to the square root in equation (7).

Connecting the theoretical model to experimental observations

The available data comprise cellular response measurements (read-outs) of T-cell activation following exposure to APCs incubated with various peptide ligands across a range of concentrations, as well as TCR/pMHCI association and dissociation

rates for each peptide ligand, measured via SPR. To relate these measurements to the TCR triggering theory, a number of auxiliary assumptions are required. In particular, if Y is the peptide incubation concentration, the following proportionality is assumed:

$$Z_T = \alpha Y. \quad (8)$$

If K_D is the dissociation constant determined via SPR, the following proportionality is assumed:

$$\frac{k_{\text{off}}}{k_{\text{on}}} = \kappa K_D. \quad (9)$$

The proportionality constant κ is required to convert rates as measured via SPR to the 2-dimensional environment of the cell:cell interaction area. To connect the model to data, two compound parameters are introduced: $\zeta = \alpha/\kappa$ (dimensionless), and $\rho = R_T/\kappa$ (M). With these parameters, the TCR triggering rate $w = W/\kappa$ (in M·s⁻¹) assumes a simpler form:

$$w = \frac{k_{\text{off}} \exp\{-k_{\text{off}} T_R\}}{2} (\rho + \zeta Y + K_D) \times \left(1 - \sqrt{1 - \frac{4\rho\zeta Y}{\rho + \zeta Y + K_D}}\right). \quad (10)$$

The read-out in the present study is median fluorescence intensity (MFI), which is a valid measure of activation in view of its good correlation with the fraction of responding cells. Accordingly, the read-out U is assumed to be proportional to the fraction of responding cells. This assumption is validated by response data at the level of individual cells (see Results below). The equation for the read-out is as follows:

$$U = U_{\text{min}} + (U_{\text{max}} - U_{\text{min}}) \mathbb{P}(\text{respond}) \quad (11)$$

where U_{min} and U_{max} are nuisance parameters associated with the read-out procedure; these parameters take specific values for each type of cellular response and are assumed not to vary across peptide ligands. The probability that a T-cell responds is modeled as follows:

$$\mathbb{P}(\text{respond}) = \mathbb{P}(w_{\text{act}} \leq w) \quad (12)$$

where w is the scaled TCR triggering rate given by equation (10) and w_{act} is the *cellular activation threshold*. This latter quantity is assumed to have a log-normal distribution over the population of T-cells in the experiment. Stochastic variation between the T-cells within the responding population is thus taken into account. The parameters are estimated by simultaneous least-squares fitting over the set of available ligands and read-outs. The median of the log-normal distribution provides the estimate of w_{act} .

Implementation of response hierarchy hypotheses

We examined five different cellular responses and compared three hypotheses: (i) for each type of cellular response, both T_R and w_{act} have distinct values; (ii) there is a common value of T_R across responses, whereas w_{act} has a different value for each response; and

(iii) there is a common value of w_{act} across responses, whereas T_R has a different value for each response. As the least-squares fit is carried out simultaneously, these hypotheses can be implemented by specifying either common or particular values for these parameters.

RESULTS

KINETIC PARAMETERS AND LIGAND NUMBERS INTERACT TO DETERMINE THE RATE OF TCR TRIGGERING

The triggering equation (7) reduces to various simplified forms, depending on which receptors are kinetically limiting. These forms are of immunological interest since they illuminate the controversy over which kinetic parameter primarily governs T-cell activation. First, if the term $k_{\text{off}}/k_{\text{on}}$ is much larger than both R_T and Z_T , which applies when both cells have introduced comparatively low numbers of ligands into the interaction area, the following approximation is accurate:

$$W = R_T Z_T k_{\text{on}} \exp\{-k_{\text{off}} T_R\} \quad (k_{\text{off}}/k_{\text{on}} \gg \max\{R_T, Z_T\}), \quad (13)$$

that is, the triggering rate will be proportional to both TCR and pMHC numbers: signaling is *affinity-limited*.

On the other hand, if the cells express numbers of ligands in the interaction area that are much larger than the 2-dimensional dissociation constant $k_{\text{off}}/k_{\text{on}}$, there are two further good approximations:

$$W = R_T k_{\text{off}} \exp\{-k_{\text{off}} T_R\} \quad (k_{\text{off}}/k_{\text{on}} \ll Z_T) \quad (14)$$

$$W = Z_T k_{\text{off}} \exp\{-k_{\text{off}} T_R\} \quad (k_{\text{off}}/k_{\text{on}} \ll R_T); \quad (15)$$

in the first case, signaling is *TCR-limited* and in the second, it is *MHC-limited*. These equations imply that under the receptor-limited regimes, the TCR triggering rate displays an optimum relative to k_{off} at the point $k_{\text{off}} = T_R^{-1}$, whereas under the affinity-limited regime, W increases monotonically in k_{on} and decreases monotonically in k_{off} .

When both R_T and Z_T are much larger than $k_{\text{off}}/k_{\text{on}}$, the TCR triggering rate is proportional to $\min\{R_T, Z_T\}$ as shown in Figure 2A, whereas at lower receptor densities, $W \propto R_T Z_T$ as per equation (13). The transition between the two regimes happens where the receptor densities traverse the 2-dimensional dissociation constant $k_{\text{off}}/k_{\text{on}}$. Thus, depending on receptor densities, the triggering rate may be affected solely by changes in R_T (TCR copy numbers), Z_T (pMHC copy numbers), or both. The MHC-limited case corresponds to the serial triggering (serial engagement) hypothesis proposed by Valitutti et al. (35–37); however, the present theory is more general in the sense that the serial triggering mechanism arises as a special case.

Figure 2B shows that the TCR triggering rate increases with both k_{on} and mean dwell-time k_{off}^{-1} when k_{on} is low (relative to receptor densities; scaling is explained in the legend to Figure 2). However, when k_{on} increases, the triggering rate exhibits a weak dependence on k_{on} and a non-monotone dependence on k_{off} , attaining a maximum at $k_{\text{off}} = 1/T_R$.

The *avidity effect* is routinely exploited to determine the functional sensitivity of the TCR to a given ligand. This effect hinges on mutual compensation by pMHC ligand numbers and the TCR

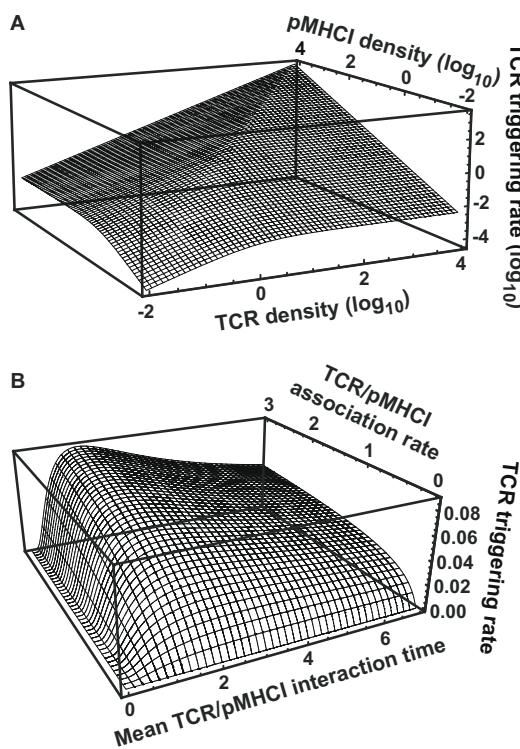


FIGURE 2 | TCR triggering rate depends on TCR/pMHC1 association and dissociation rates as well as the densities of TCR and pMHC1 molecules.

(A) Dependence of the rate of TCR triggering on receptor densities. Quantities are dimensionless: TCR triggering rate is scaled as $WT_R^2 k_{on}$, TCR density is scaled as $R_T k_{on}/k_{off}$, pMHC1 density is scaled as $Z_T k_{on}/k_{off}$, and $k_{off} = 1/T_R$ is assumed (this means that the ligand is optimal under MHC-limited conditions). **(B)** Dependence of the rate of TCR triggering on kinetic parameters. The dependence on the mean TCR/pMHC1 interaction time ($1/k_{off}$) is non-monotone, indicating that a maximally strong agonist must satisfy $k_{off} = 1/T_R$. In contrast, the dependence on k_{on} is monotone. Affinity is customarily expressed by the dissociation constant $K_D = k_{off}/k_{on}$. Accordingly, whether or not an improvement of affinity correlates with an enhanced TCR triggering rate depends on where the system is initially located on the surface of the graph, as well as on the relative contributions that changes in association and dissociation rates make to the overall change in affinity. Quantities are dimensionless: TCR triggering rate is scaled as $WT_R/(R_T + Z_T)$, association rate is scaled as $k_{on}T_R(R_T + Z_T)$, and the mean lifetime of the interaction is scaled as $1/(k_{off}T_R)$. Here, $R_T = Z_T$. Similar qualitative behavior results when the two ligand densities are unequal.

triggering rate per ligand; i.e., a T-cell can be activated by a strong agonist even at low copy numbers whereas a poor agonist may be effective but only at sufficiently high copy numbers. In equation (15), copy numbers are expressed by Z_T and the intrinsic quality of the ligand is expressed by the quantity $k_{off}\exp\{-k_{off}T_R\}$. The latter attains a maximum at $k_{off} = 1/T_R$. If the triggering rate W needs to exceed a certain threshold value to activate the T-cell, sufficient pMHC1 copy numbers can compensate for a suboptimal intrinsic triggering rate. However, increasing Z_T may transfer the system into the TCR-limited regime (Figure 2B), under which conditions pMHC1 density-based compensation cannot be achieved, cf. equation (14). Moreover, physiological bounds must exist on how many copies can be present in the interaction area and poor

agonists will be unable to activate the T-cell unless unusually high numbers of the ligand are presented (e.g., the cell transcribes very large quantities of the protein).

INTERCELLULAR VARIATION IN RESPONSE HIERARCHY THRESHOLDS

An overview of the experimental data is provided in Figure 3. In keeping with the avidity effect, the fraction of cells that exhibited at least n distinct responses (where $1 \leq n \leq 5$) increased with peptide concentration; individual ligands differed with respect to the minimal concentration required to elicit n cellular responses. There was appreciable variability with regard to which functions were elicited, as depicted in Figure 3B, which displays the data obtained at peptide concentrations of 10^{-6} M. The responses generally conformed to the following series:

$$\text{MIP-1}\beta < \text{CD107a} < \text{TNF}\alpha < \text{IL-2} < \text{IFN-}\gamma$$

(with the lowest threshold on the left). However, a small proportion of cells exhibited a slightly different ordering of threshold values, as would be expected given the natural variability between cells with respect to the threshold value of any one given response.

These results indicate that, for each response, there is variation between cells with respect to the cellular threshold value. This supports the assumption made in the section *Connecting the theoretical model to experimental observations* above, namely that the sigmoid dose-response shape of the population-level read-out can be accounted for by postulating a statistical distribution at the cell population level with respect to the cellular threshold for any given response.

THE RESPONSE HIERARCHY RESIDES AT THE LEVEL OF CELLULAR SIGNAL INTEGRATION

The parameters of the mathematical model were estimated by means of least-squares fitting of model predictions to the data; the biophysical data are summarized in Table 1 and Figure A1 in the Appendix. Figure 4 shows the results of least-squares curve fitting for the dual-hierarchy hypothesis, the receptor-level response hierarchy hypothesis, and the cellular-level response hierarchy hypothesis. The goodness-of-fit should be assessed in the light of the parsimoniousness of the model. There is less than one parameter per curve. This compares favorably to the standard practice of fitting a separate sigmoid model to each curve, which requires three or more parameters per curve. The fit is very good for such extreme parameter-count efficiency.

There is no substantial difference in the quality of fit between the dual-threshold model and the cellular-threshold model, whereas the fit to the receptor-threshold model is consistently less good. Thus, the data are best explained by postulating a hierarchy only at the level of the cellular activation threshold.

The MIP-1 β response was found to have the lowest activation threshold. Relative to this value, the ratios of median threshold values were found to be as follows: CD107a/MIP-1 β = 1.44; TNF α /MIP-1 β = 11.7; IL-2/MIP-1 β = 225; and IFN- γ /MIP-1 β = 231. These findings broadly agree with the consensus series derived from the data reported in Figure 3. The estimated median activation threshold values are markedly higher for IL-2 and IFN- γ , which required a stimulus over two orders of magnitude larger compared to the more readily elicited responses.

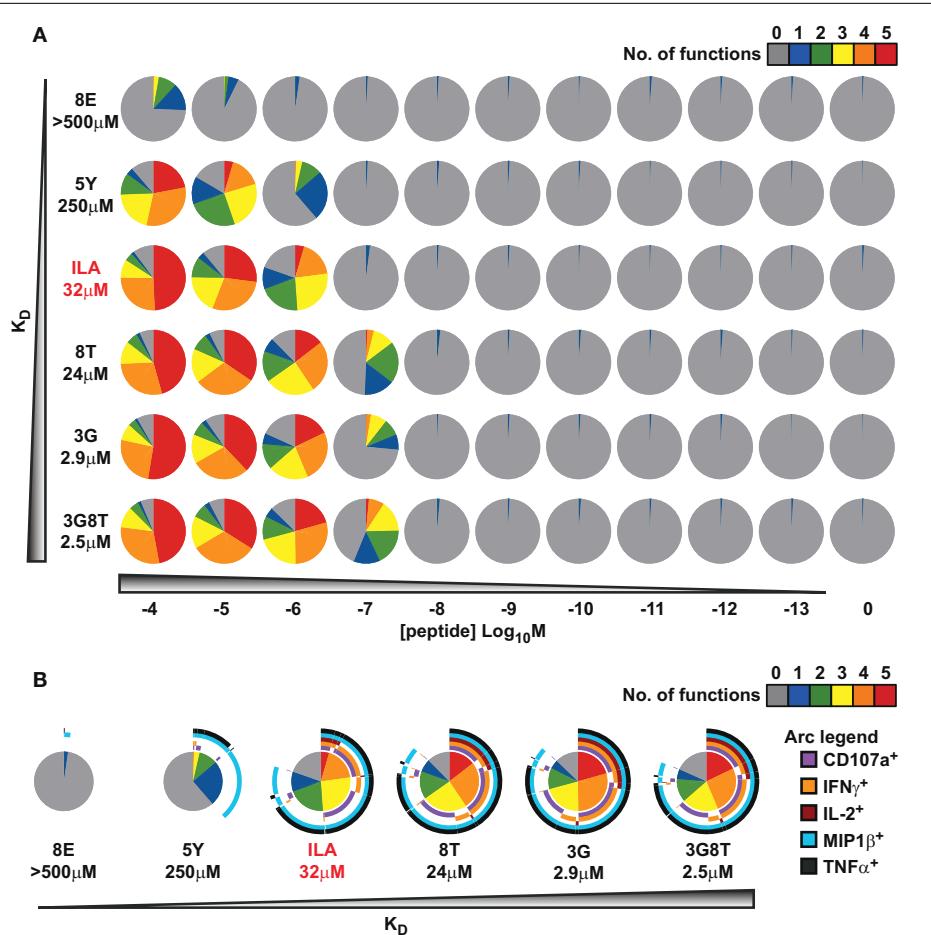


FIGURE 3 | Functional variation at the cell population level in response to six different peptide ligands. The CTL clone ILA1 was stimulated for 6 h with peptide-pulsed C1R-A2 targets as indicated. Five functional read-outs (CD107a, MIP-1 β , TNF- α , IL-2, and IFN- γ) were measured via flow cytometry. The 8E peptide was included as an

extremely weak ligand. **(A)** Overview of functional profiles. Pie chart segments represent the fraction of cells expressing the number of functions indicated in the key. **(B)** Detailed analysis of functional profiles at $[\text{peptide}] = 10^{-6} \text{ M}$. Pie charts are extended with arcs defining expressed functions as indicated in the key.

Table 1 | Biophysical parameters.

Ligand	On-rate ($\text{M}^{-1} \text{s}^{-1}$)	Off-rate (s^{-1})	K_D (M)
3G8T	19,500	0.049	2.5×10^{-6}
3G	16,000	0.047	2.9×10^{-6}
8T	4,000	0.095	2.4×10^{-5}
ILA	4,100	0.13	3.2×10^{-5}
5Y	1,300	0.32	2.5×10^{-4}

The kinetics of the TCR/pMHC1 interaction for the 5Y and 3G8T ligands are reported here for the first time; it should be noted that the 5Y data are subject to a comparatively greater potential measurement error due to their rapidity.

MHC1/CD8 BINDING MODULATES FUNCTIONAL SENSITIVITY

The experiments described above were repeated using C1R-A2null cells as APCs, which cannot productively engage the CD8 coreceptor (38). It is well-attested that the MHC1/CD8 interaction modulates the TCR/pMHC1 association rate (k_{on}), the TCR/pMHC1

dissociation rate (k_{off}), and the receptor-level triggering threshold (T_R) (27, 38, 39). Multipliers can be used to model these effects. For example, k_{off} is replaced throughout by $\gamma_{\text{off}} k_{\text{off}}$ where γ_{off} represents the effect of abrogating the MHC1/CD8 interaction and $\gamma_{\text{off}} > 1$ since CD8 stabilizes the TCR/pMHC1 interaction (38–40). The parameter γ_{off} was set to 2.16, the value reported by Wooldridge et al. (38).

Since the prevailing kinetic regime in the experiments described here is MHC limitation, the parameter γ_{on} for the association rate could not be estimated and was set to 1. This leaves a single free parameter, γ_R , for the data obtained with C1R-A2null APCs. Data fits are shown in Figure 5, with $\gamma_R = 1.14$ (the least-squares estimate) for the receptor threshold T_R . This value indicates that the MHC1/CD8 interaction decreases the TCR triggering threshold by 13%.

DISCUSSION

T-cells can exhibit a variety of cellular responses to TCR-mediated stimulation, some of which are elicited far more readily than

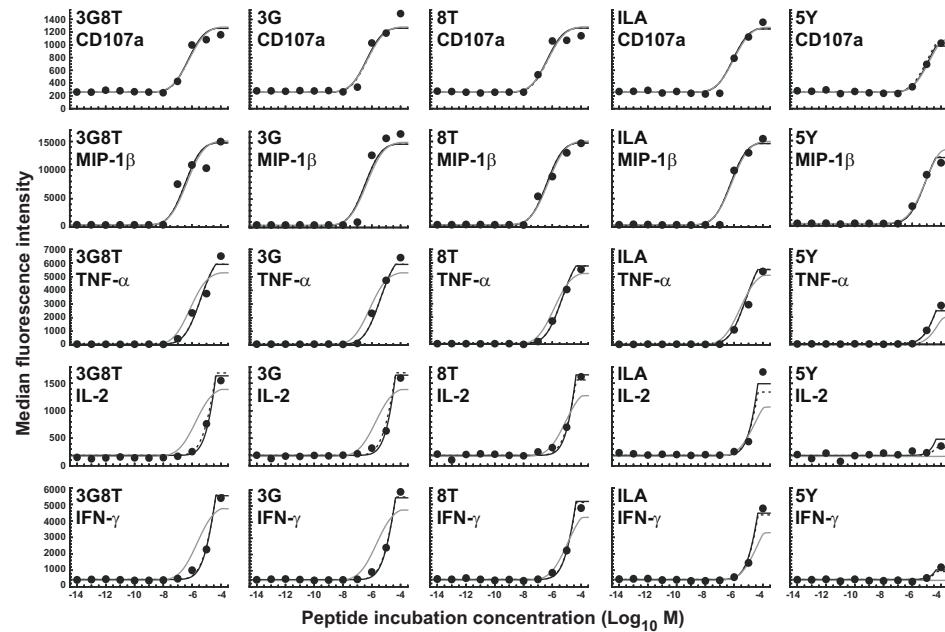


FIGURE 4 | Dose-response curves for five peptide ligands and five cellular responses. Solid black curves correspond to the dual-threshold model in which both the receptor-level and the cellular-level thresholds have different values for different cellular responses. Solid gray curves correspond to the receptor-level threshold model in which the receptor-level

thresholds have different values for different cellular responses, but the cellular activation threshold is constant. Dashed curves correspond to the cellular-level threshold model in which the cellular activation thresholds have different values for different cellular responses, but the receptor-level threshold is constant.

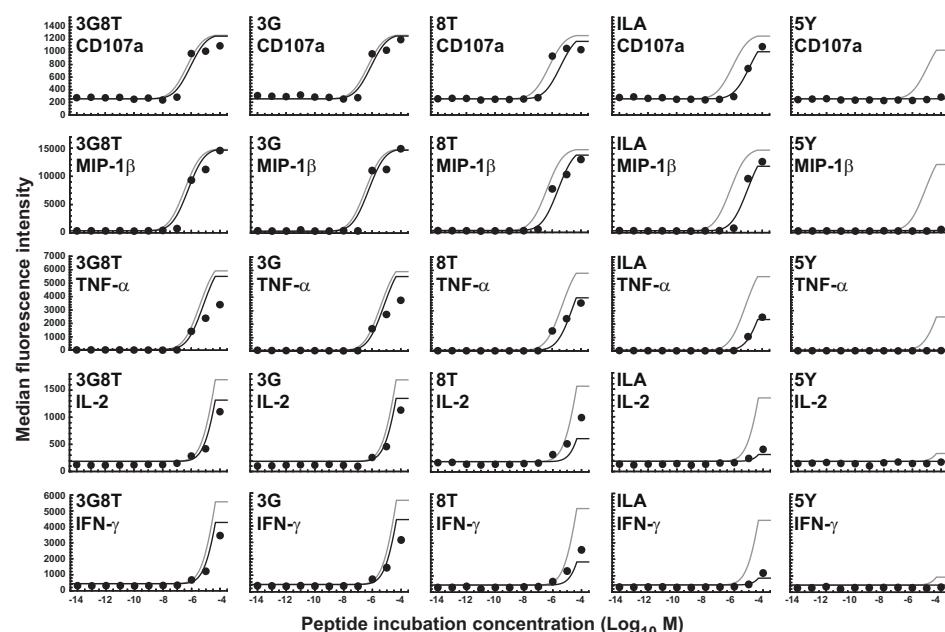


FIGURE 5 | Dose-response curves for five peptide ligands and five cellular responses in the absence of the MHCI/CD8 interaction. All curves correspond to the cellular-level threshold model in which the cellular activation thresholds have different values for different cellular responses, but the receptor-level threshold is constant. Black curves are the fits to the data

shown. Gray curves correspond to the situation in which the MHCI/CD8 interaction is present. Parameter values are as in the previous figure. Two new parameters (γ_{off} and γ_R) have been introduced and estimated to account for the absence of the CD8 effect; γ_{off} was set to 2.16 and γ_R was estimated as the sole remaining free parameter.

others. In particular, different responses require different levels of stimulation in terms of ligand densities present on the APC surface. Such threshold differences might be postulated at the receptor level (different response pathways branch off at different stages in the sequence of signaling events), and/or at the cellular level (the TCR signal received by the T-cell needs to exceed different threshold values for different responses). The present study suggests that the cellular-level hypothesis is the most parsimonious explanation consistent with the data. Furthermore, the role of the CD8 coreceptor as a modulator of T-cell functional sensitivity is confirmed by the present findings, which indicate that the MHCI/CD8 interaction decreases the TCR/pMHCI dissociation rate as well as the duration of TCR/pMHCI contact required to activate the T-cell.

A key consideration for the interpretation of our results is the standardized nature of the system. Determinative cellular factors, such as TCR density, membrane constituency, and the expression of costimulatory/inhibitory molecules, were fixed across all conditions, which means that these extraneous factors can be discounted as discriminative because only ligand nature and concentration varied between stimulations. These built-in controls justify the simplifying assumptions from which the mathematical model was derived.

The present findings certainly do not exclude the existence of a receptor-level hierarchy. Indeed, the co-existence of hierarchies at both the receptor and cellular levels may explain the data equally well. However, the hypothesis that the response hierarchy resides solely at the receptor level is contradicted by the present findings. A threshold hierarchy at the level of cellular activation would imply that the functional responses are located downstream from signaling pathways that share a common starting point (the triggered TCR or signalosome). Further detailed molecular studies of the CD3-complex phosphorylation cascade would be required to rule out a receptor-level hierarchy definitively.

Our mathematical model elucidates the contrasting roles played by the dissociation rate and the affinity constant as determinants of T-cell functional sensitivity, with TCR and pMHCI molecular densities governing the limitation regime under which the kinetics operate. The disappearance of the optimum behavior as the system moves from the receptor-limited to the affinity-limited regime can be studied experimentally by increasing TCR density. This shift was first predicted by Van den Berg et al. (23) and confirmed experimentally by Gonzales et al. (41). Furthermore, the pattern of dependence on k_{on} and k_{off} shown in Figure 2B may account for the discrepancies between the findings of Kalergis et al. (42), who reported such an optimal dwell-time ($= k_{off}^{-1}$), and the findings of Holler et al. (43), who reported monotone dependence. The shift along the k_{on} axis may also explain the observations of Irving et al. (26), who observed that affinity modulates the dependence on k_{off} . In the experimental system studied here, the TCR is stimulated under MHC-limited conditions. This means that the optimum-type dependence on k_{off} prevails. This is shown in Figure 6, where the calculated TCR triggering rate (at 10^{-5} M) is plotted against k_{off} . The effect of k_{on} is so slight that the curves overlap almost perfectly for the five ligands. Even under MHC-limited conditions, however, k_{on} remains a crucial determinant of functional sensitivity, in view of the fact that the ratio of k_{off} and k_{on} determines the transition between affinity-limited and

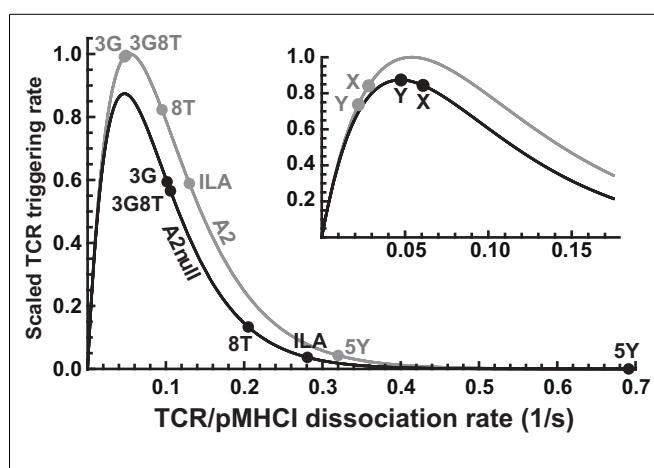


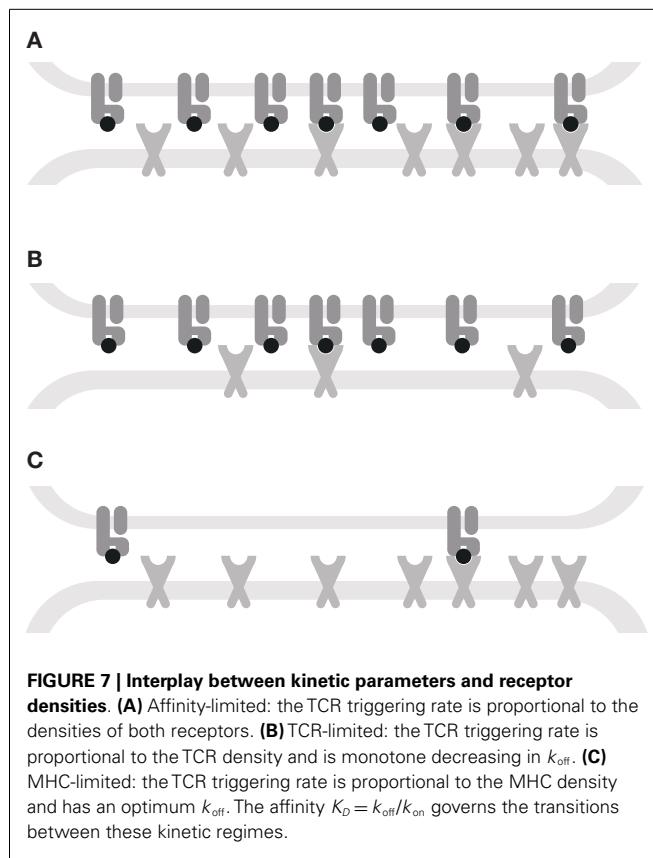
FIGURE 6 | Differential effects of CD8 modulation on functional sensitivity. TCR triggering rate, scaled with respect to the optimal ligand under MHC-limited conditions, as a function of k_{off} , comparing the wild-type MHCI/CD8 interaction ("A2") to the abrogated MHCI/CD8 interaction ("A2null"). The positions for the five ligands are indicated. The inset shows two hypothetical ligands: "X," which is unaffected by the absence of the CD8 interaction, and "Y," which is optimal under the MHC-null condition.

ligand-limited signaling, as illustrated schematically in Figure 7. Thus, the association rate and the dissociation rate play distinct roles in T-cell activation, consistent with reports of dominant effects associated with each of these biophysical parameters under different conditions (24, 44); these differences also explain earlier observations on the interplay of kinetic parameters and receptor numbers (41, 45, 46).

In the presence of the wild-type MHCI/CD8 interaction, the ligands 3G and 3G8T are virtually optimal, whereas abrogation of MHCI/CD8 binding leads to a reduction in functional sensitivity. In virtue of the non-linear character of the curve, the magnitude of the reduction varies considerably across ligands. Two parameters (γ_{on} and γ_R) suffice to capture these effects. The ILA ligand evokes a triggering rate approximately one order of magnitude less than optimal under C1R-A2null conditions. Nonetheless, the data show that strongly attenuated but discernible responses can be elicited at sufficiently high presentation levels, in keeping with Wooldridge et al. (14), who reported that a ligand could be physiologically significant even at an estimated ~ 2.1 orders of magnitude below the optimal ligand for a responding T-cell clone.

Although all ligands studied here become less potent when presented by C1R-A2null cells, the model predicts that this is not necessarily the case. Indeed, the functional sensitivity to a ligand marked "X" in the inset of Figure 6 is unaffected when the MHCI/CD8 interaction is abrogated. Moreover, ligand "Y" becomes optimal in the absence of the CD8 effect (so that the T-cell has a higher functional sensitivity to this ligand under CD8-null conditions). The existence of such ligands would imply that T-cells can "tune" to distinct cognate ligands by up- or down-regulating their CD8 coreceptors. This effect would greatly amplify the ability of T-cells to reconcile extensive cross-reactivity with the avoidance of self-recognition.

The hypothetical ligands "X" and "Y" are strongly heteroclitic. Peptides of this nature occur markedly less frequently than



homoclitic ligands, which makes them more challenging to detect and characterize. Nonetheless, work is ongoing to demonstrate the existence of such anomalous ligands, using combinatorial peptide library screening combined with importance sampling (14) in the presence of different extracellular contributions from the CD8 coreceptor (29).

The effect of CD8 on the value of the TCR triggering threshold is consistent with the conclusions of Van den Berg et al. (39) and may be due to the association of TCR/CD3 with protein tyrosine kinases such as p56lck, which expedites the immunoglobulin family tyrosine-based activation motif phosphorylation sequence (47). This agrees with the observation that the CD8 $\alpha\beta$ heterodimer is more potent as a coreceptor than the $\alpha\alpha$ homo-dimer (48, 49), perhaps due to interactions with other signalosome components mediated by the palmitoylated CD8 β chain (50), which interacts with myristoylated p56lck. Docking of CD8 to the TCR/CD3 complex may also be driven by initial activation (51). The kinetics of CD8 binding after TCR/pMHC contact was analyzed previously by Van den Berg and Sewell (52). More recently, Mukhopadhyay et al. (53) developed a model that explicitly accounts for the interplay between the CD3 phosphorylation cascade and p56lck, ZAP-70, and CD45.

The dissociation rates determined by SPR were assumed to be indicative of the normal interaction in this study. In reality, these values might be more true to the case where the MHC/CD8 interaction is absent. Assuming the latter would not dramatically alter the qualitative conclusions of this study, but would lead to a slight

adjustment in the interpretation of the parameter estimates. Most importantly, the value found for the TCR triggering threshold T_R would be adjusted down from 17.46 to 15.26 s.

Values based on SPR experiments are best regarded as 3-dimensional, representative of ligands in solution. However, the TCR/pMHC interaction takes place in a 2-dimensional environment, which essentially reduces spatial degrees of freedom of molecular motion and introduces dynamics related to the forces that constrain the molecules to this environment (54). Consequently, rate constants may be different in two dimensions as opposed to three dimensions. In particular, 2-dimensional dissociation rates can be substantially faster (55). The ratio between the 3-dimensional and 2-dimensional affinities is a length measure, denoted h and called the confinement length (56, 57). Wu et al. demonstrated that h is proportional to the range of motion available to the free forms of the interacting ligands along the spatial axis perpendicular to the two parallel membranes. Thus, the intermembrane separation provides an upper bound, and if the ranges of motion do not differ too much, it is reasonable to assume that the confinement length is roughly the same for all mutants involved.

The use of a fixed receptor duration might appear simplistic given the complexity of the events that are required to trigger a TCR/CD3 complex, but the law of large numbers provides indirect support for a simple assumption: the true waiting-time-till-triggering is a composite of a large number of stochastic durations which tends to regress it on the mean value. In fact, the number of terms is itself a random variate due to the possibility of alternative routes in the activation pathway of the complex (such processes are termed “compound stochastic”). The complexity illustrated in Figure 1 effectively underpins a simple assumption, viz. that there is a fixed, stereotypical receptor threshold T_R .

The parameters T_R and w_{act} are both subject to further modulation. By varying the levels of the CD8 coreceptor, surface molecules such as CD45, and cytosolic concentrations of kinases, phospho-rylases, and linker proteins in the immediate vicinity of the CD3 complex, the T-cell can adjust the effective value of the receptor triggering threshold T_R (29, 30, 38, 39, 58–61). The value of the cellular activation threshold w_{act} is modulated by costimulation (“signal 2”). Moreover, the T-cell can actively modulate its receptiveness to this signal by adjusting the relative levels of CD28 and CD152 (52, 62). Furthermore, the maintenance of tolerance requires continual dynamic tuning of the cellular activation threshold (63, 64). Conceivably, these mechanisms provide an additional “multiplier” that modulates w_{act} over and above the factors that set w_{act} to different values for different responses.

The data on intercellular response variability (Figure 3) support the assumption that the distribution of responsiveness in terms of MFI can be attributed to intercellular variation in the triggering threshold. However, the observed variability may also arise from differences in the numbers of TCR and pMHC molecules present in the T-cell/APC interaction area. Mathematically, this model is slightly more involved because interactions can now be distributed over different kinetic regimes. Nonetheless, apart from this technical difficulty, the resulting model is mathematically equivalent; this follows from the properties of the log-normal distribution. Thus, the analysis is not substantially affected by this

alternative explanation of the intercellular variability. Experimentally, the two hypotheses could be distinguished by following an individual T-cell over a series of interactions and responses. If cellular thresholds for the various responses remain largely constant, there would be little event-to-event variation according to the former hypothesis; in contrast, the receptor copy number hypothesis would predict considerable event-to-event variation.

In addition, there may be variability with respect to antigen presentation levels across the experimental APC population, due to differential antigen exposure and stochastic effects in antigen acceptance from the incubation medium. However, it can be shown that the resulting variance is not such as to make a substantial contribution to overall variability, for instance by following the argument presented in Appendix C of Van den Berg et al. (22).

When the TCR/pMHC1 ternary complex dissociates, the covalent modifications and multimeric aggregations that the CD3 complex undergoes during the gradual transition to signalosome status are reversed, both by thermal agitation and the action of phosphorylases. In the present model, it has been assumed that this “reset” occurs much more rapidly than the typical time required for the TCR/CD3 complex to encounter the next non-null pMHC1 ligand. However, given that the molecules have to drift away from each other through diffusion in the 2-dimensional arena of the T-cell/APC interface, there is a possibility of rapid rebinding to the same pMHC1 molecule before the CD3 complex has had sufficient time to “reset.” This rapid rebinding is equivalent to a diminishment of the effective off-rate; a basic model capturing this effect has been proposed by Aleksic et al. (65). Extending the present model with this effect is straightforward in principle, albeit at the cost of an additional parameter. Furthermore, the number of complexes with other pMHC1 species is ignored in equation (1). This is valid if the vast majority of all other ligands consists of null agonists. An analysis that takes the entire presentation profile (all pMHC1 species present) into account can be found in Van den Berg et al. (22, 23).

A further aspect of the cellular activation threshold that has been left implicit in the present treatment is the duration of the T-cell/APC interaction. If this contact is initiated at t_0 and is terminated at t_1 , the cumulative signal transmitted to the cytosolic

signaling machinery is given by an integral:

$$Q = \int_{t_0}^{t_1} W(\tau) d\tau. \quad (16)$$

In fact, this quantity Q is presumed to be directly compared to a threshold. However, $Q/(t_1 - t_0)$ is proportional to the activation threshold as defined in the present study. Indeed, variability in the duration of the contact is one of the sources of stochasticity underlying the log-normal response curve used to fit the dose-response curves.

A related phenomenon is that of TCR down-regulation (66–68), whereby triggered TCRs are gradually removed from the cell surface. This affects the integral in equation (16); Van den Berg et al. (23) discuss how the model can be extended to take this effect into account. TCR down-regulation can in fact be exploited by the T-cell to gauge both k_{on} and k_{off} independently. As indicated in Figure 2A, if the system starts in the MHC-limited regime, gradual removal of TCR molecules will result in a transition to the TCR-limited regime, where a sudden drop of the instantaneous signal results. At the point of change-over, $R_T \approx Z_T$. This means that the T-cell can, in principle, glean a rough estimate of the biophysical rate parameters k_{off} and k_{on} by combining information from the gross signal and the number of down-regulated TCRs at the transition point. This mechanism can only function in the ligand-limited regime, not in the affinity-limited regime.

In conclusion, the present study confirms that a response hierarchy exists with respect to the strength of TCR stimulation required to elicit various cellular responses. Moreover, a combination of experimentation and mathematical modeling indicates that this hierarchy resides at the cellular level rather than at the level of the individual receptor molecules. Functional sensitivity is generally enhanced by the CD8 coreceptor, although the theory indicates that the MHC1/CD8 interaction can depress TCR signaling for certain heteroclitic ligands.

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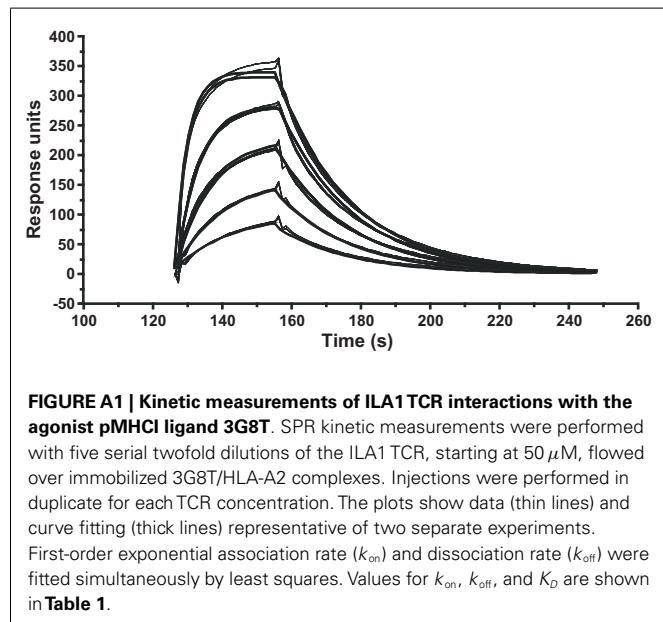
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APPENDIX





Structural and biophysical insights into the role of CD4 and CD8 in T cell activation

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T cell receptors (TCRs) recognize peptides presented by MHC molecules (pMHC) on an antigen-presenting cell (APC) to discriminate foreign from self-antigens and initiate adaptive immune responses. In addition, T cell activation generally requires binding of this same pMHC to a CD4 or CD8 co-receptor, resulting in assembly of a TCR–pMHC–CD4 or TCR–pMHC–CD8 complex and recruitment of Lck via its association with the co-receptor. Here we review structural and biophysical studies of CD4 and CD8 interactions with MHC molecules and TCR–pMHC complexes. Crystal structures have been determined of CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ in complex with MHC class I, of CD4 bound to MHC class II, and of a complete TCR–pMHC–CD4 ternary complex. Additionally, the binding of these co-receptors to pMHC and TCR–pMHC ligands has been investigated both in solution and *in situ* at the T cell–APC interface. Together, these studies have provided key insights into the role of CD4 and CD8 in T cell activation, and into how these co-receptors focus TCR on MHC to guide TCR docking on pMHC during thymic T cell selection.

Keywords: CD4, CD8, MHC, T cell receptor, structure, T cell activation

INTRODUCTION

Adaptive immunity depends on specific recognition by an $\alpha\beta$ T cell receptor (TCR) of an antigenic peptide bound to a major histocompatibility complex (pMHC) molecule on an antigen-presenting cell (APC). However, TCR–pMHC interactions alone do not efficiently trigger T cells, requiring the participation of the co-receptors CD4 and CD8 (1–4). These transmembrane glycoproteins mark different T cell subsets. CD4 is expressed on Th1, Th2, and Th17 helper cells, as well as on CD4 regulatory T cells (Tregs); CD8 is expressed on cytotoxic T lymphocytes (CTLs) and CD8 Tregs. CD4 and CD8 enhance T cell signaling by binding MHC class II (CD4) or MHC class I (CD8) molecules on APCs. The interaction of CD4 with MHC class II greatly reduces the number of antigenic peptides required for T cell activation (5) and substantially increases cytokine production by helper T cells (1). Likewise, the CD8–MHC class I interaction enhances the antigen sensitivity and response of cytotoxic T cells to pMHC ligands (6).

The principal role of the CD4 and CD8 co-receptors is to recruit the Src tyrosine kinase p65lck (Lck) to the TCR–pMHC complex following co-receptor binding to MHC, resulting in assembly of a TCR–pMHC–CD4 or TCR–pMHC–CD8 ternary complex (7–10). Recruitment of Lck occurs via its association with the cytoplasmic tail of CD4 or CD8. The accompanying increase in the local concentration of Lck promotes phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) in the cytoplasmic tails of CD3 subunits associated with the TCR in the TCR–CD3 complex, leading to the recruitment and activation of Zap-70. Activated Zap-70 phosphorylates LAT and SLP-76, which function as scaffolds to recruit other signaling molecules to the downstream T cell signaling apparatus that regulates T cell activation, proliferation, and

differentiation. The targeted delivery of Lck to TCR by CD4 or CD8 during thymic selection is believed to impose MHC restriction on the developing $\alpha\beta$ TCR repertoire (11). In support of this idea, $\alpha\beta$ TCRs in mice lacking co-receptors and MHC are not biased toward pMHC ligands, but instead display antibody-like recognition specificities (12, 13).

This review focuses on structural and biophysical studies of CD4 and CD8 interactions with MHC molecules. Crystal structures have been determined of CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ in complex with MHC class I, of CD4 bound to MHC class II, and, most recently, of a complete TCR–pMHC–CD4 ternary complex. In addition, the binding of these co-receptors to pMHC and TCR–pMHC ligands has been investigated both in solution and *in situ*. Collectively, these studies have provided critical insights into the role of CD4 and CD8 in T cell activation and in guiding TCR docking on pMHC during T cell development.

INTERACTION OF CD8 WITH MHC CLASS I

CD8 exists as two isoforms, CD8 $\alpha\alpha$ and CD8 $\alpha\beta$, which are expressed on different cell types and appear to have different functions. CD8 $\beta\beta$ homodimers have also been reported, but these lack MHC-binding activity and their significance is unknown (14). Whereas CD8 $\alpha\alpha$ is found on $\gamma\delta$ T cells, intestinal intraepithelial T lymphocytes, and natural killer (NK) cells, CD8 $\alpha\beta$ is expressed on $\alpha\beta$ TCR thymocytes and CTLs. The function of CD8 $\alpha\alpha$ remains obscure, but it has been implicated in the negative regulation of intestinal intraepithelial T lymphocytes (15). By contrast, CD8 $\alpha\beta$ is clearly required for positive selection of CD8 $+$ T cells in the thymus (16–18) and for activation of CD8 $+$ T cells in the periphery (19). In both CD8 $\alpha\alpha$ and CD8 $\alpha\beta$, Lck associates with the CD8 α

chain. In addition, ligation of CD8 $\alpha\beta$ by MHC, in the absence of TCR engagement, results in apoptosis of a proportion of double-positive thymocytes, which may be a mechanism for removing thymocytes that have failed positive selection (20).

Fluorescence resonance energy transfer experiments have shown that, during T cell activation, the TCR binds initially to pMHC, and that CD8 then binds to the same pMHC as the TCR, leading to formation of a TCR–pMHC–CD8 complex (21). This order of engagement ensures that the specific TCR–pMHC interaction dominates T cell recognition. As measured by surface plasmon resonance (SPR), CD8 binds very weakly to MHC class I in both mouse and human, with little influence of the MHC-bound peptide (21–23). In the mouse, the average binding affinity for CD8 $\alpha\beta$ ($K_D \sim 50 \mu\text{M}$) is similar to that for CD8 $\alpha\alpha$ ($\sim 70 \mu\text{M}$), with some variation depending on the particular MHC class I allele. The human CD8–MHC class I interaction is weaker still, averaging $\sim 150 \mu\text{M}$ (24–26). In both cases, these affinities are 10- to 100-fold lower than for most TCR–pMHC interactions.

In overall agreement with these SPR results, *in situ* measurements of the mouse CD8–MHC class I interaction gave an affinity several orders of magnitude lower than that of TCR for pMHC (27). These experiments used a micropipette adhesion frequency assay to measure the adhesion kinetics of live T cells interacting with pMHC ligands presented on surrogate APCs. The two-dimensional (2D) binding parameters derived from this technique are thought to more accurately reflect biological interactions in membranes than the three-dimensional (3D) parameters derived from SPR, in which fluid-phase receptors and ligands are removed from their cellular environment (28).

Although the affinity of CD8 for MHC class I is weak, recent 2D affinity measurements support the idea that CD8 contributes significantly to stabilizing the TCR–pMHC interaction at the T cell–APC interface (9). These experiments revealed that the TCR–pMHC–CD8 trimolecular interaction generates synergy over the simple sum of the individual TCR–pMHC and pMHC–CD8 interactions, and that this cooperativity amplifies peptide discrimination. Thus, in addition to its primary role of recruiting Lck to the TCR–pMHC complex, a secondary function of CD8 is to reinforce TCR binding to the pMHC ligand. Whether the CD4 co-receptor also promotes cooperative binding remains to be determined.

The micropipette adhesion frequency assay also revealed that the kinetics of the TCR–pMHC–CD8 trimolecular interaction at the T cell membrane proceeds in two stages (9). The first consists of TCR-dominant binding to agonist pMHC. This triggers a second stage involving an upregulation of CD8-dependent adhesion after a 1 s delay. The second stage requires Lck kinase activity to initiate CD8 binding to the same pMHC ligand engaged by the TCR, generating synergy. It remains to be established whether the TCR–pMHC–CD4 trimolecular interaction involves a similar sequence of events.

STRUCTURES OF CD8 BOUND TO MHC CLASS I

CD8 $\alpha\beta$ is a heterodimeric type I transmembrane glycoprotein, whose α and β chains are each composed of an immunoglobulin (Ig)-like domain connected by a long stalk to a transmembrane domain and a cytoplasmic tail, with Lck bound to the CD8 α tail.

By contrast, the CD8 $\alpha\alpha$ homodimer comprises only the α chain. Four structures of CD8 $\alpha\alpha$ or CD8 $\alpha\beta$ bound to MHC class I molecules have been reported: (1) the complex between human CD $\alpha\alpha$ and HLA-A*0201 (29); (2) the complex between human CD8 $\alpha\alpha$ and HLA-A*2402 (30); (3) the complex between mouse CD8 $\alpha\alpha$ and H-2K b (31); and (4) the complex between mouse CD8 $\alpha\beta$ and H-2D d (23).

In the CD8 $\alpha\beta$ –H-2D d complex (23), the CD8 $\alpha\beta$ heterodimer contacts only the $\alpha 3$ domain of the MHC class I heavy chain (Figure 1A). By contrast, CD8 $\alpha\alpha$ also contacts the $\alpha 2$ domain and β_2 -microglobulin ($\beta_2\text{m}$) in the CD $\alpha\alpha$ –HLA-A*0201 (29), CD8 $\alpha\alpha$ –HLA-A*2402 (30), and CD8 $\alpha\alpha$ –H-2K b complexes (31) (Figure 1C). The CD8 β subunit occupies a position equivalent to that of the CD8 $\alpha 1$ subunit in the three CD8 $\alpha\alpha$ –MHC class I structures, which places CD8 β proximal to the T cell membrane. The CD8 α subunit of CD8 $\alpha\beta$ is located in the same position as the CD8 $\alpha 2$ subunit, distal from the T cell and near the C-terminus of the MHC class I $\alpha 3$ domain (Figures 1A,C). Nearly all MHC class I residues that mediate key interactions with CD8 $\alpha\alpha$ or CD8 $\alpha\beta$ are non-polymorphic, which explains the largely allele-independent nature of CD8 binding.

For both CD8 $\alpha\alpha$ and CD8 $\alpha\beta$, the main binding interaction is with a protruding loop in the $\alpha 3$ domain of the MHC class I molecule (CD loop), corresponding to residues 220–228 (Figures 1B,D). This loop is flexible in the absence of CD8, but is stabilized by CD8 binding. In the CD8 $\alpha\alpha$ –MHC class I structures, the two CDR3-like loops of the CD8 α subunits clamp onto the central β -turn portion of the CD loop in an antibody-like manner, as do the two CDR3-like loops of CD8 $\alpha\beta$ (23, 29–31). In addition, CD8 $\alpha\alpha$ contacts the $\alpha 2$ domain and $\beta_2\text{m}$ through its CD8 $\alpha 1$ subunit, whereas the corresponding CD8 β subunit of CD8 $\alpha\beta$ contacts only $\alpha 3$. Of particular note in the CD8 $\alpha\beta$ –H-2D d complex is Gln226 of H-2D d , a highly conserved residue among MHC class I alleles and the only one that interacts with both CD8 α and CD8 β (Figure 1B).

ROLE OF THE CD8 STALK REGION IN CO-RECEPTOR FUNCTION

The Ig-like domains of CD8 α and CD8 β are tethered to the T cell membrane by long stalk regions comprising ~ 45 residues for CD8 α and ~ 35 residues CD8 β (32). These stalk regions, which are rich in threonine, serine, and proline residues, are heavily O-glycosylated at multiple sites in all species studied (33). The stalk of CD8 $\alpha\beta$ undergoes developmentally programmed O-glycan modification controlled by the sialyltransferase ST3 Gal-I, which catalyzes addition of sialic acid to core 1 O-linked glycans (34). In particular, immature CD4 $^+$ CD8 $^+$ thymocytes exhibit lower levels of CD8 sialylation than mature thymocytes (32, 34, 35). Decreased sialylation of the CD8 $\alpha\beta$ stalk was found to markedly increase the affinity of CD8 for MHC class I, as measured by MHC tetramer binding, thereby affecting T cell selection (34, 35). Enhanced CD8 $\alpha\beta$ binding at the CD4 $^+$ CD8 $^+$ stage facilitates elimination of autoreactive T cells in the thymus. However, once a double-positive thymocyte has differentiated to the CD8 $^+$ stage, O-glycan sialylation of the CD8 $\alpha\beta$ stalk reduces co-receptor affinity for pMHC, requiring a stronger TCR–pMHC interaction for T cell activation in the periphery (34, 35).

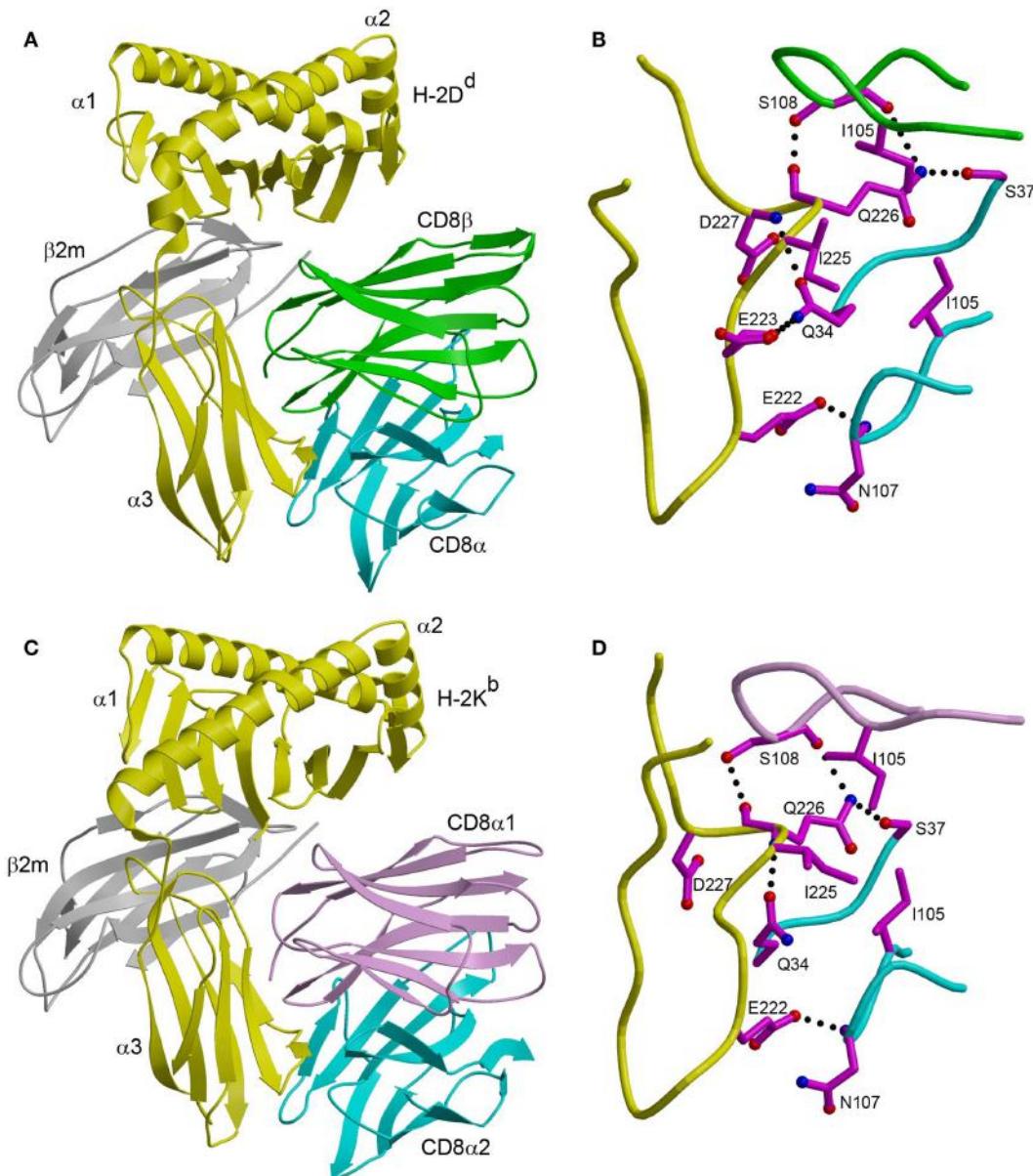


FIGURE 1 | Comparison of CD8 $\alpha\beta$ –H-2D d and CD8 $\alpha\alpha$ –H-2K b complexes.

(A) Ribbon diagram of the CD8 $\alpha\beta$ –H-2D d complex (Protein Data Bank accession code 3DMM) (23). MHC α chain, yellow; β_2m , gray; CD8 α , cyan; CD8 β , green. **(B)** Interaction between the CDR-like loops of CD8 $\alpha\beta$ and the H-2D d α 3 CD loop. The side chains of contacting residues are shown in

ball-and-stick representation with carbon atoms in magenta, nitrogen atoms in blue, and oxygen atoms in red. Hydrogen bonds are drawn as dotted black lines. **(C)** Ribbon diagram of the CD8 $\alpha\alpha$ –H-2K b complex (1BQH) (31). MHC α chain, yellow; β_2m , gray; CD8 α 1, pink; CD8 α 2, cyan. **(D)** Interaction between the CDR-like loops of CD8 $\alpha\alpha$ and the H-2K b α 3 CD loop.

The structural basis for reduced CD8 $\alpha\beta$ affinity for MHC class I upon sialylation of the stalk region is unknown. One possibility is that sialylation alters the association or orientation of the Ig-like domains of the CD8 $\alpha\beta$ heterodimer, reducing its capacity to bind MHC class I (34). However, Merry et al. (36) found that sialylation had little or no effect on the overall structure of CD8, insofar as sialylated and non-sialylated forms of soluble CD8 $\alpha\alpha$ exhibited indistinguishable hydrodynamic properties. This suggests that the results of Moody et al. (34) and Daniels et al. (35)

might be explained by avidity effects arising from aggregation of unsialylated CD8 on the T cell surface that increase MHC tetramer binding (36).

Irrespective of the underlying mechanism, the finding that developmentally regulated glycosylation of the CD8 $\alpha\beta$ stalk can modulate MHC binding at the cell surface demonstrates that the stalk has a specialized role in co-receptor function, beyond simply attaching the MHC-binding domains to the T cell membrane. The long CD8 $\alpha\beta$ stalk may also allow a possible *cis* interaction

between CD8 and MHC class I expressed on the same T cell (37), in addition to the established *trans* interaction between CD8 and MHC class I expressed on different cells. Interestingly, the stalk regions of other immune system receptors have also been recently found to play prominent roles in receptor function. For example, the long stalk regions of Ly49 NK cell receptors enable binding to MHC class I in *cis* or *trans* configurations (38). Additionally, the stalk domain of the activating NK receptor NKp30 is critical for NK cell killing and may contribute directly to binding its tumor cell ligand B7-H6 (39).

No 3D structural information is available for the stalk regions of CD8 $\alpha\beta$ or CD8 $\alpha\alpha$, since the stalks, when included in the constructs used for protein expression, were mostly or entirely disordered in the CD8–MHC class I crystal structures (23, 29–31). Although this has been interpreted to mean that the CD8 stalk is highly flexible, it should be emphasized that the stalks completely lacked glycosylation because the CD8 proteins were produced in bacteria. However, there is biophysical evidence that O-glycosylation may significantly restrict the flexibility of the stalks. Studies of mucins have demonstrated that O-glycans stiffen polypeptides through steric interactions between peptide-linked N-acetylgalactosamine residues and adjacent peptide residues (40, 41). Moreover, O-glycans in the CD8 stalk polypeptides were found to reduce the overall extension of the stalk from a theoretical maximum of 3.4 Å per residue to 2.6 Å per residue, indicating rigidification (36, 42). Therefore, O-glycosylation may limit the mobility of the CD8 head group relative to the T cell membrane, an important consideration in evaluating co-receptor interactions with TCR–pMHC during thymic selection and peripheral antigen recognition, as discussed below.

STRUCTURES OF CD4 BOUND TO MHC CLASS II

CD4 is a monomeric type I transmembrane glycoprotein consisting of four Ig-like extracellular domains connected by a short stalk to a transmembrane domain and a cytoplasmic tail that interacts with Lck. CD4 binds MHC class II with exceptionally low affinity compared to all other leukocyte cell–cell recognition molecules characterized to date, whose K_D s typically fall in the 1–100 μM range, as measured by SPR (43, 44). For the CD4–MHC class II interaction, K_D s have been estimated to range from $\sim 200 \mu\text{M}$ (for human CD4 binding to mouse MHC class II) (45) to $> 2 \text{ mM}$ (for human CD4 binding to human MHC class II) (43). These affinities are substantially weaker than those for CD8–MHC class I interactions, which range from $\sim 10 \mu\text{M}$ (for mouse CD8 $\alpha\beta$ binding to H-2D d) (23) to $\sim 150 \mu\text{M}$ (for human CD8 $\alpha\alpha$ binding to HLA-A*0201) (26). Presumably, evolution has calibrated the affinity of CD4 for MHC class II to enable peripheral T cells to respond efficiently to the very low abundance of foreign pMHC molecules on APCs (sensitivity), yet avoid activation by the far greater number of self-pMHC molecules (discrimination), which could cause autoimmunity.

The ability of CD4 to recognize highly polymorphic MHC class II molecules is central to its function as a co-receptor (1). In humans, MHC class II molecules are encoded by three separate loci (HLA-DR, -DQ, and -DP), while in mice they are encoded by two loci (I-A and I-E). For HLA-DR, most variability derives from the β -chain, with > 700 known alleles at the population level,

whereas there are only three α -chain variants. In contrast, both α - and β -chains of HLA-DQ and -DP are polymorphic (46). Two structures of CD4 bound to MHC class II molecules have been reported: (1) the complex between human CD4 (domains D1 and D2) and mouse I-A k to 4.3 Å resolution (47); and (2) the complex between human CD4 (domains D1 and D2) and human HLA-DR1 to 2.4 Å resolution (48). These structures readily explain the ability of CD4 to recognize multiple MHC class II alleles.

The CD4–I-A k and CD4–HLA-DR1 complexes display similar overall topologies, although only the higher-resolution CD4–HLA-DR1 structure permitted an atomic-level description of the CD4–MHC class II interface. In both complexes, CD4 binds MHC class II through its membrane-distal D1 domain, which contacts the membrane-proximal $\alpha 2$ and $\beta 2$ domains of the MHC class II molecule (Figure 2A). CD4 uses two discontinuous regions to engage MHC class II in a concavity formed by the $\alpha 2$ and $\beta 2$ domains (Figure 2B). The first region, composed of β -strands C' and C'', exclusively contacts the $\beta 2$ domain. The second region, comprising a short 3₁₀ helix within the loop connecting β -strands D and E, binds solely to the $\alpha 2$ domain.

Figure 2C shows sequence alignments of the α - and β -chains of selected HLA-DR, -DP, and -DQ alleles in the regions where HLA-DR1 contacts CD4. For the β -chains, 11 of 12 CD4-contacting residues are absolutely conserved in these human MHC class II molecules, and 1 is conservatively substituted (Val116Ile in HLA-DQ). For the α -chains, all three CD4-contacting residues (Glu88, Thr90, Leu176) are invariant across human MHC class II alleles. Hence, the remarkable cross-reactivity of CD4 is attributable to the targeting of non-polymorphic residues in the concavity formed by the $\alpha 2$ and $\beta 2$ domains of HLA-DR, -DP, and -DQ. For I-A k , 11 of 14 CD4-contacting residues are identical to those of HLA-DR1, while for I-E k 12 of 14 are identical (Figure 2C). All non-identical residues are conservatively substituted in both molecules. Therefore, CD4 almost certainly engages all human and mouse MHC class II molecules in the same manner as it does HLA-DR (48).

STRUCTURE OF A TCR–pMHC–CD4 TERNARY COMPLEX

The low affinity of CD4 for MHC class II presented a major technical obstacle to crystallizing a TCR–pMHC–CD4 ternary complex. To overcome this obstacle, *in vitro* directed evolution by yeast surface display was used to increase the affinity of CD4 for MHC class II (49). Affinity maturation by yeast display relies on expression of a library of mutants on the surface of yeast, followed by selection of variants with improved affinity (50). The D1 domain of human CD4 was subjected to *in vitro* random mutagenesis, and the resulting mutant library was displayed on yeast by fusion to agglutinin protein Aga2p (48). Because CD4 binds MHC class II very weakly (43, 45), the CD4 library was sorted by flow cytometry using HLA-DR1 tetramers, rather than monomers, to increase the avidity of the selecting ligand. In this way, a CD4 mutant that bound HLA-DR1 with $K_D = 9 \mu\text{M}$ was isolated, compared with no detectable binding for wild-type CD4, even at high concentrations (400 μM). The CD4 mutant exhibited similar affinity for HLA-DR4 ($K_D = 10 \mu\text{M}$), in agreement with the ability of CD4 to recognize all HLA-DR alleles, as discussed above.

The affinity-matured CD4 mutant contained two substitutions in the D1 domain: Gln40Tyr and Thr45Trp. In the mutant

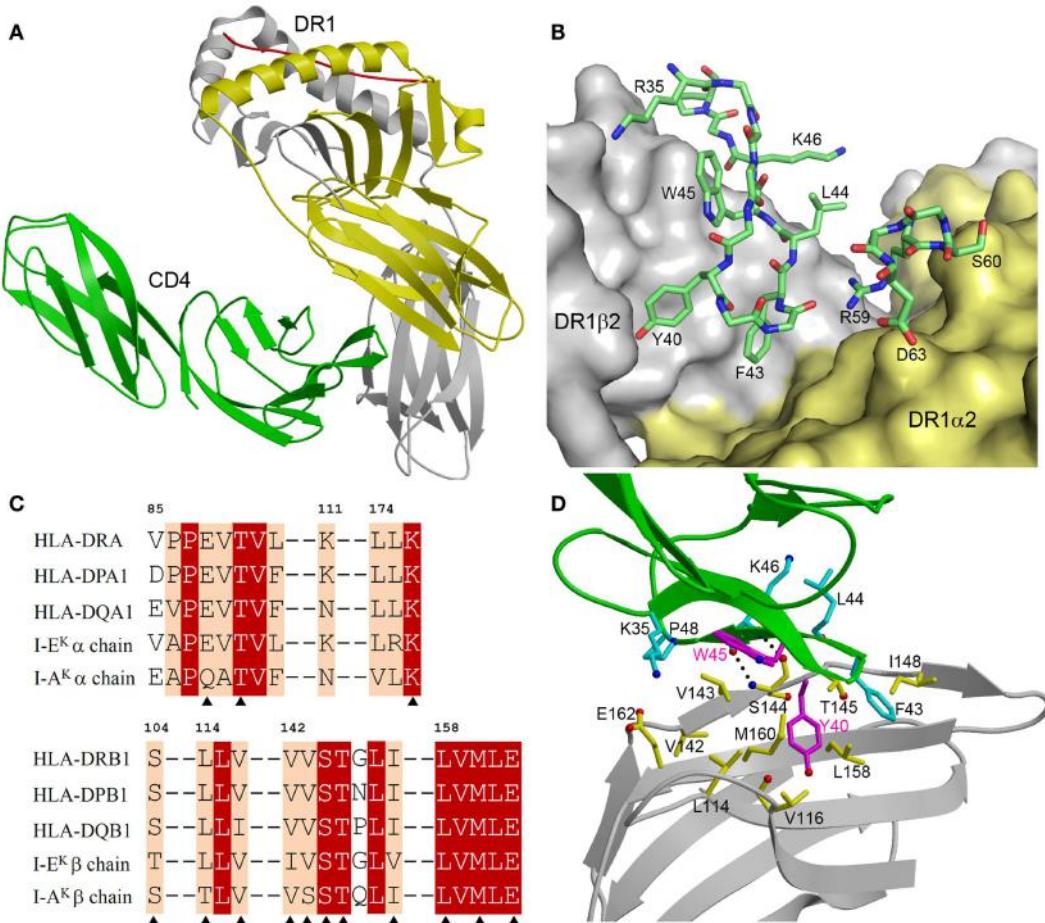


FIGURE 2 | Structure of a human CD4–MHC class II complex. (A) CD4 (green) contacts the α 2 (yellow) and β 2 (gray) domains of HLA-DR1 through its D4 domain (3S4S) (48). The MHC-bound peptide is red. **(B)** The CD4–HLA-DR1 binding interface. The two regions of CD4 (residues 35–48 and 59–63) that contact HLA-DR1 are shown in stick representation with carbon atoms in green, oxygen atoms in red, and nitrogen atoms in blue. The molecular surface of HLA-DR1 that interacts with CD4 is depicted with the α 2 domain in yellow and the β 2 domain in gray. **(C)** Sequence alignment of the CD4-contacting regions of the α - and β -chains of different human and mouse MHC class II alleles. Residues that contact CD4 in the

CD4–HLA-DR1 structure are marked with triangles. White characters on a red background show residues that are strictly conserved across human or mouse MHC class II molecules. Black characters on a tan background are conservatively substituted residues. **(D)** Close-up view of the interactions between an affinity-matured CD4 mutant (green) and the HLA-DR1 β 2 domain (gray). The side chains of interacting residues are shown in ball-and-stick representation with carbon atoms in cyan (CD4) or yellow (HLA-DR1), oxygen atoms in red, and nitrogen atoms in blue. The mutated Tyr40 and Trp45 residues of CD4 are in magenta. Hydrogen bonds are drawn as dotted black lines.

CD4–HLA-DR1 structure (48), CD4 Trp45 is located at the center of the interface with HLA-DR1, where its bulky side chain makes multiple hydrophobic contacts with DR1 β 2 Val143 (Figure 2D). Similarly, CD4 Tyr40 is surrounded by apolar DR1 β 2 residues Leu114, Val116, Leu158, and Met160, resulting in increased hydrophobic interactions at the mutation site. Together, the Gln40Tyr and Thr45Trp mutations improved shape and chemical complementarity with HLA-DR1, thereby stabilizing the CD4–HLA-DR1 complex.

The enhanced affinity of this CD4 mutant made possible the crystallization and structure determination of a complete TCR–pMHC–CD4 ternary complex involving a human autoimmune TCR (MS2–3C8), a self-peptide from myelin basic protein (MBP) bound to HLA-DR4, and CD4 (49) (Figure 3A).

The TCR–pMHC–CD4 complex resembles a pointed arch in which both TCR and CD4 are tilted rather than oriented vertically. The TCR and CD4 molecules each make an angle of $\sim 65^\circ$ with the T cell surface. The apex of the arch is formed by the α 2 and β 2 domains of HLA-DR4 and the D1 domain of CD4. MS2–3C8 engages MBP via the canonical docking mode of $\alpha\beta$ TCRs (51), in which the TCR adopts a central diagonal orientation over pMHC (52, 53). There are no direct contacts between TCR and CD4 (Figure 3A), in agreement with an earlier prediction (47).

The CD4 molecule bound to TCR–pMHC retains the overall extended conformation observed in different crystal forms of unbound CD4 (54), with some hinge-like variability at the D2–D3 junction. The limited segmental flexibility of CD4 implies that any

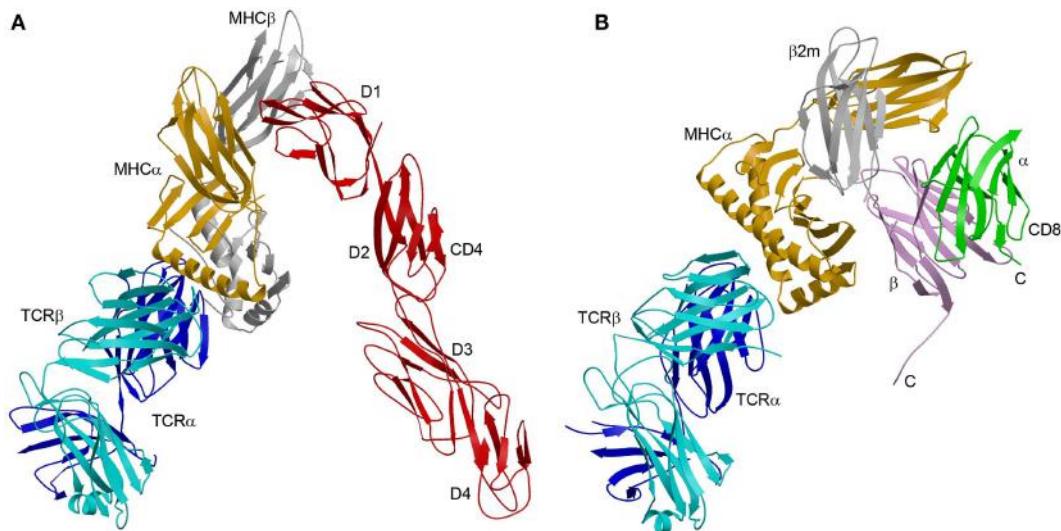


FIGURE 3 | Comparison of TCR-pMHC-CD4 and TCR-pMHC-CD8 ternary complexes. **(A)** Crystal structure of a TCR-pMHC-CD4 complex (MS2-3C8-MBP-DR4-CD4) oriented as if the TCR and CD4 molecules are attached to the T cell at the bottom and the MHC class II molecules are attached to an opposing APC at the top (3TOE) (49). TCR α chain, blue; TCR β chain, cyan; MHC α chain, yellow; MHC β chain, gray; CD4, red. **(B)** Hypothetical model of the TCR-pMHC-CD8 complex. The model was

constructed by superposing the CD8 $\alpha\beta$ -H-2D α complex (3DMM) (23) onto a TCR-H-2D β complex (3PQY) through the MHC class I molecule. A portion of the CD8 β stalk region was visible in the crystal structure and points toward the T cell membrane. The C-termini of the CD8 α and CD8 β chains are labeled. The orientation of the TCR-pMHC complex is the same as in **(A)**. TCR α chain, blue; TCR β chain, cyan; MHC α chain, yellow; β_2 m, gray; CD8 α , green; CD8 β , violet.

significant variations in overall complex architecture must arise from differences in TCR docking on pMHC.

The absence of direct contacts between TCR and CD4 explains how these molecules can simultaneously, yet independently, bind to pMHC. Importantly, the wide separation (~ 70 Å) between the membrane-proximal TCR $\alpha\beta$ module and CD4 D4 domain provides ample room for the placement of TCR-associated CD3 $\epsilon\gamma$, $\epsilon\delta$, and $\zeta\zeta$ subunits (Figure 4A), which transmit activation signals to the T cell (55). Although no crystal structure is available for the TCR-CD3 complex, mutational studies have identified docking sites for the ectodomains of CD3 $\epsilon\delta$ and CD3 $\epsilon\gamma$, which interact with the TCR through adjacent α DE and β CC' loops, respectively (56–58). Based on this information, CD3 $\epsilon\gamma$ and CD3 $\epsilon\delta$ would be situated inside the TCR-pMHC-CD4 arch, wedged between the TCR and T cell membrane (Figure 4A). In the organization of the TCR-CD3 complex proposed by Fernandes et al. (58), only CD3 γ and CD3 δ contact the TCR, whereas CD3 ϵ projects away from the receptor. The relative proximity of CD3 ϵ to CD4 in the TCR-pMHC-CD4 complex, compared to CD3 γ or CD3 δ , may confer preference to CD3 ϵ in the ITAM phosphorylation cascade upon Lck recruitment by CD4 (Figure 4A).

The ectodomain of CD3 ζ , which is only nine amino acids in length, has not been implicated in interactions with the TCR ectodomain, and so is not shown in Figure 4A. However, mutational analysis of the transmembrane regions of TCR and CD3 subunits has established that CD3 $\zeta\zeta$ is associated with TCR α in the T cell membrane (59).

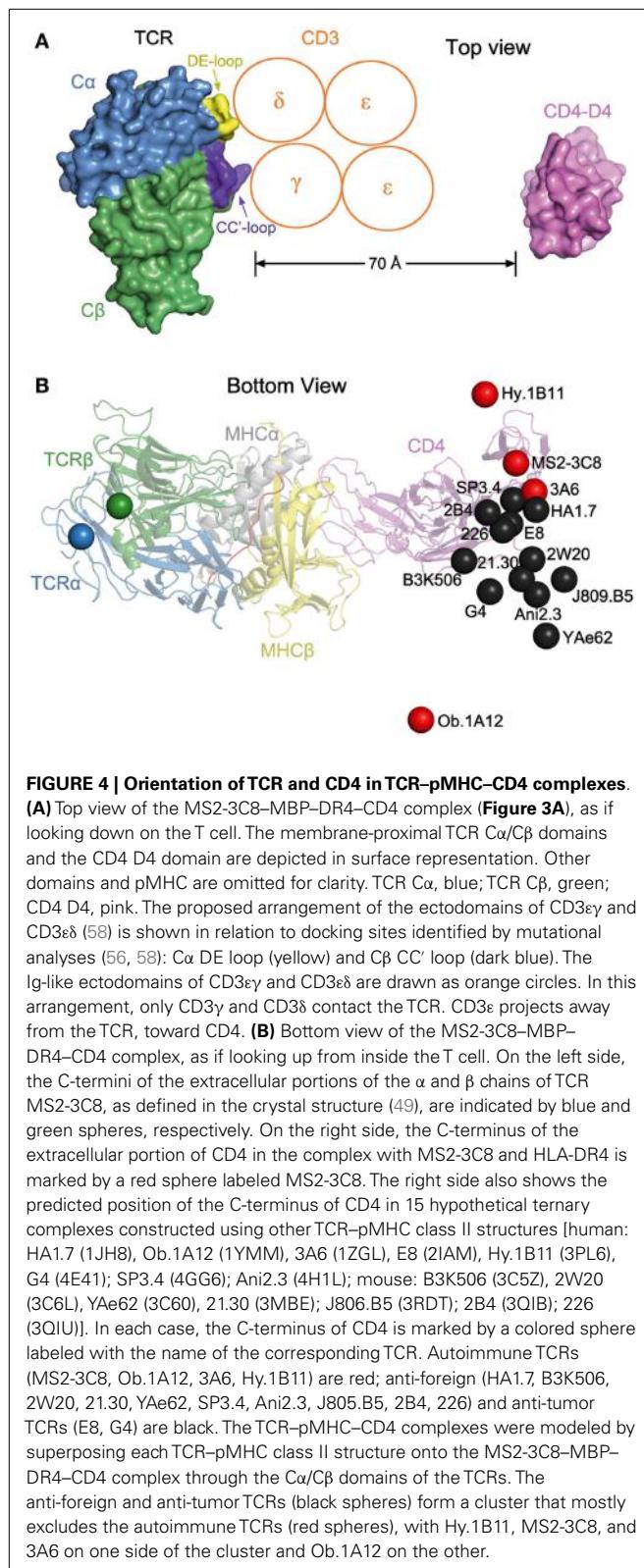
It has been proposed that, in resting T cells, CD3 ϵ ITAMs are sequestered in the membrane, and that activation results in ITAM exposure to Src kinases (60). If so, the TCR-pMHC-CD4 structure

suggests a possible mechanism by which this may occur. If the TCR shifts from an upright to a tilted orientation upon formation of the TCR-pMHC-CD4 complex (Figure 3A), this movement could potentially drive the CD3 ectodomains, situated inside the TCR-pMHC-CD4 arch, into the T cell membrane. This in turn could cause displacement of CD3 ϵ ITAMs from the membrane and their phosphorylation by Lck.

THE TCR-pMHC-CD4 COMPLEX AND MODELS FOR TCR TRIGGERING

There is considerable controversy over the mechanism of TCR triggering, and a variety of models have been proposed to explain how pMHC binding to TCR initiates signaling across the T cell membrane (55). Some of these models invoke dimerization (or oligomerization) of CD4 (54), MHC (61), or TCR (57) as a means of enhancing phosphorylation of CD3 ITAMs by increasing the proximity of associated tyrosine kinases. The plausibility of these models can be assessed in terms of the geometrical constraints imposed by the TCR-pMHC-CD4 structure.

The structure of human CD4 D1-D4 in unbound form showed that CD4 molecules form dimers through the D4 domain, at least in the crystal (54). This observation suggested that D4-D4-associated CD4 dimers might contribute to T cell activation by cross-linking TCR-pMHC complexes (54, 62). However, the TCR-pMHC-CD4 structure is incompatible with this idea. In a hypothetical model constructed by superposing the TCR-pMHC-CD4 structure onto the D4-D4-associated CD4 dimer, the distance between the C-termini of the D4 domains and the T cell surface is too far (~ 40 Å) to be spanned by the eight-residue stalk region of CD4 (49). Similarly, the finding that some HLA-DR



molecules crystallize as dimers (61, 63) suggested a mechanism for T cell triggering in which an MHC class II dimer cross-links two TCRs. However, the CD4-binding site on HLA-DR4 almost

completely overlaps the putative HLA-DR dimerization site, which would preclude formation of such MHC class II dimers (49).

Recently, it was proposed that TCRs can dimerize in the T cell membrane via α - α interactions, and that the resulting juxtaposition of two TCR-pMHC complexes facilitates signaling through the membrane (57, 64). Consistent with this model, the putative site of α - α dimerization is on the outside of the TCR-pMHC-CD4 arch, opposite the sites mediating TCR-CD3 interactions (Figure 4A) (49). As such, CD4 would not interfere sterically with TCR dimerization through the α domain. However, a survey of 22 TCR-pMHC crystal structures failed to reveal any α - α contacts consistent with biologically relevant TCR dimerization (65). More tellingly, the α domain contains two conserved N-linked glycans at positions that would preclude the hypothesized TCR dimerization via α - α interactions.

Several recent studies have demonstrated that physical force applied to the TCR-CD3 complex can activate T cells (66–68). This finding has led to the concept of the TCR as an anisotropic mechanosensor that converts mechanical energy into a biochemical signal upon specific pMHC ligation as a T cell moves over APCs during immune surveillance (66). While it is unknown how pulling on the TCR-CD3 complex can be transduced to the T cell interior, one possibility is that pMHC binding leads to a conformational change in the CD3 cytoplasmic tails, allowing ITAM phosphorylation by Src kinases. This process may be facilitated by the CD4 (or CD8) co-receptor, whose binding to the TCR-pMHC complex could promote dissociation of CD3 ITAMs from the cytoplasmic side of the T cell membrane and their exposure to Lck, as discussed above.

CO-RECEPTORS AND TCR-pMHC DOCKING ORIENTATION

The TCR-pMHC-CD4 complex provides a basis for understanding how the CD4 and CD8 co-receptors focus TCR on MHC to guide TCR docking on pMHC during thymic T cell selection. Structural studies of numerous (>25) TCR-pMHC complexes have demonstrated remarkable similarities in the overall topology of TCR binding to pMHC, regardless of MHC class I or class II restriction (52, 53). Typically, the TCR is positioned diagonally over the center of the composite surface created by the peptide and the MHC α -helices that flank the peptide-binding groove, with $\text{V}\alpha$ situated over the N-terminal half of the peptide, and $\text{V}\beta$ over the C-terminal half, although the exact angle and pitch of TCR engagement vary.

Two competing (though not mutually exclusive) hypotheses have been proposed to explain this roughly conserved diagonal binding mode. The first maintains that co-evolution of TCR and MHC genes has led to specific interaction motifs between the germline-encoded CDR1 and CDR2 loops of TCRs and the α -helices of MHC proteins (53, 69–73). According to the second hypothesis, TCR docking topology is guided by the CD4 and CD8 co-receptors during T cell development in order to achieve intracellular juxtaposition of co-receptor-bound Lck with CD3 ITAMs (11–13, 49, 71, 72, 74, 75). According this view, it is the need for co-receptor function during thymic T cell selection that restricts the geometry of TCR-pMHC recognition and eliminates from positive selection CD4 $^+$ CD8 $^+$ double-positive thymocytes expressing

TCRs unable to engage pMHC in a manner that generates a signal to induce maturation.

The arch-shaped TCR–pMHC–CD4 complex establishes anchor points for TCR and CD4 on the T cell membrane, thereby imposing constraints on the orientation of CD3 relative to Lck associated with CD4 on the cytoplasmic side of the membrane. **Figure 4B** shows the position of the C-terminus of CD4 observed in the complex with TCR MS2-3C8 and HLA-DR4, as well as the predicted position of the C-terminus of CD4 in hypothetical ternary complexes constructed using 15 other TCR–pMHC class II structures, both human and mouse. Except for the human autoimmune TCR Ob.1A12 (76), the C-termini of CD4 in these modeled complexes are grouped in a loose cluster that includes the C-terminus of CD4 in the MS2-3C8–MBP–DR4–CD4 complex. Differences in the position of the CD4 membrane anchor point are attributable to variations in the diagonal docking topology of the TCR–pMHC complexes, which places CD3 $\varepsilon\gamma$ and CD3 $\varepsilon\delta$ inside the TCR–pMHC–CD4 arch, opposite CD4 (**Figure 4A**). If the TCR–pMHC docking polarity were reversed (i.e., V α over the C-terminus of the peptide and V β over the N-terminus), CD3 $\varepsilon\gamma$ and CD3 $\varepsilon\delta$ would be positioned outside, rather than inside, the TCR–pMHC–CD4 arch. The much greater distance between CD4-bound Lck and CD3 ITAMs would likely hinder ITAM phosphorylation by Lck, thereby preventing positive selection of T cells bearing TCRs with the reversed polarity, or their activation in the periphery. We therefore propose that the diagonal docking topology of TCR–pMHC complexes reflects not only genetically encoded interactions with MHC (53, 69), but also the requirement to form a ternary complex with the CD4 or CD8 co-receptor that is geometrically competent to deliver a maturation signal to CD4 $^+$ CD8 $^+$ thymocytes during T cell selection.

Nonetheless, some flexibility must exist within the overall signaling complex to accommodate variations in TCR–pMHC docking geometry that affect the location of anchor points for TCR and CD4 on the T cell membrane (**Figure 4B**). Given the rigidity of the CD4 ectodomain (49), this flexibility most likely resides in interactions involving the flexible cytoplasmic tails of CD3 and CD4 with Lck, which itself can adopt multiple conformations (77). The flexibility of the cytoplasmic domains of CD3 is supported by circular dichroism analysis and disorder prediction algorithms (78). By both methods, the cytoplasmic domains of CD3 ζ , CD3 ϵ , CD3 γ , and CD3 δ were found to be intrinsically unstructured, random-coil proteins in both monomeric and oligomeric states, and in the presence or absence of lipids.

Of the 16 TCRs in **Figure 4B**, 4 are autoimmune (Hy.1B11, MS2-3C8, 3A6, Ob.1A12). These TCRs, which were isolated from different multiple sclerosis patients, recognize MBP self-peptides bound to HLA-DQ1 (Hy.1B11), HLA-DR4 (MS2-3C8), HLA-DR2a (3A6), or HLA-DR2b (Ob.1A12). T cells expressing these four autoimmune TCRs escaped negative selection in the thymus yet still retained the ability to productively engage self-antigens in the periphery. The remaining 12 TCRs recognize foreign antigens, such as influenza virus hemagglutinin (HA1.7) and moth cytochrome c (2B4, 226), or tumor antigens (G4, E8). In **Figure 4B**, the C-termini of CD4 in the 16 TCR–pMHC–CD4 complexes

sweep out an arc of \sim 70°, with autoimmune TCRs Ob.1A12 and Hy.1B11 at the two extremities. Strikingly, the 12 anti-foreign or tumor-specific TCRs form a relatively tight cluster that effectively excludes the four autoimmune TCRs, with Ob.1A12 on one side of the cluster and Hy.1B11, MS2-3C8, and 3A6 on the other. Because CD4-bound Lck would be positioned differently with respect to CD3 ITAMs inside the T cell, phosphorylation of CD3 ITAMs by Lck may be differentially affected in TCR–pMHC–CD4 complexes involving anti-foreign versus autoimmune TCRs. In this way, the geometry of the TCR–pMHC–CD4 complex could modulate TCR signaling, and thereby directly impact T cell development and autoimmunity.

IMPLICATIONS FOR THE TCR–pMHC–CD8 TERNARY COMPLEX

Although no structure of a TCR–pMHC–CD8 ternary complex has been reported, a hypothetical model may be constructed by superposing the CD8 $\alpha\beta$ –H-2D d complex (23) onto a representative TCR–pMHC class I complex through the shared MHC class I molecule (**Figure 3B**). The model is consistent with the idea that the shorter CD8 β stalk helps orient the cytoplasmic domains of CD8 $\alpha\beta$ for their role in signal transduction (23, 79). However, in the absence of structural information on the CD8 stalk, the model cannot establish anchor points for TCR and CD8 on the T cell membrane, as did the TCR–pMHC–CD4 structure (**Figure 3A**). While the TCR–pMHC–CD8 complex is probably not as conformationally constrained as the TCR–pMHC–CD4 complex, O-glycosylation of the CD8 stalk likely restricts its flexibility (36, 42), as discussed above. By limiting the mobility of the MHC-binding head group of CD8 in this way, O-glycosylation would impose constraints on the orientation of CD3 subunits relative to CD8-bound Lck, as does the rigid CD4 structure (**Figure 4A**).

Even with O-glycosylation, the proline-rich CD8 stalk is likely to be less stiff than the four tandem Ig-like domains of CD4. This flexibility may help compensate for variations in TCR–pMHC docking geometry to facilitate intracellular juxtaposition of CD8-bound Lck with CD3 ITAMs. The cytoplasmic tails of the CD3 subunits would provide additional flexibility within the overall TCR–pMHC–CD8 signaling complex, just as in the TCR–pMHC–CD4 complex.

FUTURE DIRECTIONS

Much more is known about the biophysics of CD8 interactions with TCR–pMHC at the T cell–APC interface than in the case of CD4. For example, whereas *in situ* studies have demonstrated that TCR and CD8 bind cooperatively to pMHC, and that this synergy amplifies peptide discrimination (9), there is no comparable information for CD4. Conversely, whereas the structure of a TCR–pMHC–CD4 complex has been reported (49), that of a TCR–pMHC–CD8 complex remains to be determined.

Until now, most studies of autoimmunity have emphasized reduced TCR affinity for self-pMHC as the main reason autoreactive T cells sometimes escape negative selection in the thymus (80). However, it is also possible that the altered docking topologies observed in autoimmune TCR–pMHC complexes (81) may

modulate T cell signaling by altering interactions with CD4 or CD8. In fact, autoimmune TCRs appear to segregate from anti-foreign TCRs in terms of the geometry of the corresponding TCR-pMHC-CD4 ternary complexes (Figure 4B). This hypothesis clearly merits further investigation, particularly in view of an emerging appreciation for the role of TCR docking geometry on T cell signaling (49, 71).

The TCR-pMHC-CD4 structure, in conjunction with mutational data on TCR-CD3 ectodomain interactions, suggests that CD3 ε and CD3 δ are located under the TCR-pMHC-CD4 arch, facing CD4 (Figure 4A). However, the current reality is that we only have nebulous ideas about the molecular architecture of

the TCR-CD3 complex. Direct structural analysis of the TCR-CD3 complex will be required to establish anchor points for CD3 and CD4 on the T cell membrane. This information, combined with the known pairings of the transmembrane helices of the TCR and CD3 chains from mutational analysis (TCR α -CD3 $\varepsilon\delta$, TCR α -CD3 $\zeta\zeta$, and TCR β -CD3 $\varepsilon\gamma$) (59), will reveal the intracellular organization of CD3 ITAMs relative to each other, and relative to Lck bound to the CD4 or CD8 co-receptor.

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Co-receptor CD8-mediated modulation of T-cell receptor functional sensitivity and epitope recognition degeneracy

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The interaction between T-cell receptors (TCRs) and peptide epitopes is highly degenerate: a TCR is capable of interacting productively with a wide range of different peptide ligands, involving not only cross-reactivity proper (similar epitopes elicit strong responses), but also polyspecificity (ligands with distinct physicochemical properties are capable of interacting with the TCR). Degeneracy does not gainsay the fact that TCR recognition is fundamentally specific: for the vast majority of ligands, the functional sensitivity of a given TCR is virtually null whereas this TCR has an appreciable functional sensitivity only for a minute fraction of all possible ligands. Degeneracy can be described mathematically as the probability that the functional sensitivity, of a given TCR to a randomly selected ligand, exceeds a set value. Variation of this value generates a statistical distribution that characterizes TCR degeneracy. This distribution can be modeled on the basis of a Gaussian distribution for the TCR/ligand dissociation energy. The kinetics of the TCR and the MHC I molecule can be used to transform this underlying Gaussian distribution into the observed distribution of functional sensitivity values. In the present paper, the model is extended by accounting explicitly for the kinetics of the interaction between the co-receptor and the MHC I molecule. We show that T-cells can modulate the level of degeneracy by varying the density of co-receptors on the cell surface. This could allow for an analog of avidity maturation during incipient T-cell responses.

Keywords: T-cell receptor, co-receptor CD8, degeneracy, functional sensitivity, ligand focusing, mathematical model

INTRODUCTION

Thymus-derived lymphocytes (T-cells) recognize peptide antigens via antigen-specific receptors (TCRs); in particular, CD8⁺ cytotoxic T lymphocytes (CTLs) recognize short peptides presented by major histocompatibility complex (MHC) class I molecules (1). Estimates of the human TCR diversity suggest that there are $\sim 10^8$ different antigen receptors in the naïve T-cell pool (2), which raises the question how such a limited TCR repertoire can provide effective immunity to perhaps over 10^{15} distinct pMHCs (2). The discrepancy suggests that even while TCRs are highly specific, a considerable degree of degeneracy remains (we use *degeneracy* as a term of art to cover both polyspecificity and cross-reactivity). The central importance of degeneracy was first pointed out by Mason (3) and later confirmed by others such as (4). Experimental and mathematical studies confirm that TCR recognition is highly degenerate: a single TCR may be able to recognize, at physiologically relevant degrees of functional sensitivity, over one million different peptides in the context of a single MHC I molecule (2, 5), an estimate that takes into account the binding specificity of the MHC molecule, but not the additional selection imposed by the stringency requirements of peptide cleavage and processing in the presentation pathway. The latter constitutes an epitope diversity filter that is instrumental in regulating the immunodiversity of salient epitopes (6). The issue of whether there exists an optimal level of TCR repertoire diversity was reviewed by

Nikolich-Žuglich et al. (7), and various authors have reviewed the functional repercussions at the systems level (8–10).

The interaction between TCR and pMHC I ligand can be modulated by the co-receptor CD8 in several ways: (i) promoting the association of TCR and pMHC I; (ii) stabilizing the TCR/pMHC I interaction; and (iii) enhancing the rate at which the TCR/CD3 complex attains signaling status by association of TCR/CD3 with protein tyrosine kinases such as p56^{lck} and adaptor molecules such as LAT and LIME (5, 11–16). The first of these three mechanisms modifies the affinity of the TCR/pMHC I interaction, whereas the third alters the time it takes for an engaged TCR/CD3 complex to attain full signalosome status. In particular, CD8 can enhance the TCR/pMHC I association rate by 50%, and reduce the TCR/pMHC I dissociation rate by at least 50% (16, 17), and CD8 modulates the rate of immune receptor tyrosine-based activation motif (ITAM) phosphorylation, by recruiting TCR/pMHC I complexes to membrane micro-domains at a rate which depends on the affinity of pMHC I/CD8 binding (16).

These findings suggest that CD8 not only controls degeneracy, but also differentially regulates functional sensitivity, that is, the T-cell can increase its sensitivity for one ligand, while reducing it for others. By varying the level of CD8 expression, the T-cell can increase its sensitivity to the disease-associated antigen, while at the same time decreasing its sensitivity to antigens associated with healthy conditions. This novel mode of co-receptor

action could be critical in ensuring that the TCR repertoire retains the ability to respond to antigenic challenges, while avoiding autoimmunity.

T-cell antigen recognition can be expressed in terms of its *functional sensitivity* (18). One of the main determinants of functional sensitivity is the rate at which a single agonist copy elicits TCR triggering. Functional sensitivity depends on bio-molecular parameters such as the TCR/pMHCI on-rate and off-rate. The molecular kinetics at the T-cell:antigen-presenting cell (APC) interface determine this relationship. This kinetic theory resolves the long-standing controversy over whether T-cell activation is governed by affinity or off-rate (cf. (19, 20)); the theory shows that *both* parameters play a role, but in the so-called MHC-limited regime, the off-rate is the main governing factor. However, the on-rate and the off-rate together determine whether or not the kinetics is MHC-limited (21–23).

The aim of the present study is to explore the kinetic basis of the role of CD8 in regulating degeneracy and functional sensitivity. Our model generalizes the classical kinetic proofreading model, as proposed by McKeithan (24), and later modified by others (21, 22, 25–27). The kinetic proofreading model assumes that the TCR needs to remain bound to a pMHCI in order for the TCR/CD3 complex to become fully activated, via several modification steps such as phosphorylation of several tyrosine residues on the TCR complex, and recruitment and subsequent activation of ZAP-70 (28).

The statistics of TCR degeneracy is modeled by relating the TCR/pMHCI mean interaction time with the dissociation energy according to Arrhenius theory. We show that by varying the total co-receptor density and key kinetic parameters, the T-cell can modulate the level of degeneracy. Furthermore, we compare our results with experimental data for HLA A*0201 mutants with altered binding affinity for CD8.

THEORY: TCR/pMHCI/CD8 KINETICS AND TCR DEGENERACY

We assume (i) that the TCR/CD3 complex on the T-cell surface becomes triggered (achieves signalosome status) during an interaction with a pMHCI ligand if it undergoes n ITAM phosphorylations, where n is a positive integer; and (ii) that the presence of a CD8 molecule bound to the pMHCI ligand affects the TCR/pMHCI association and dissociation rates, as well as the rate at which the TCR/CD3 complex progresses through the phosphorylation sequence. The co-receptor CD8 is associated with TCR/CD3 with tyrosine kinases such as p56^{lck}, which phosphorylates tyrosine residues within the ITAMs.

To determine the kinetics of the TCR/pMHCI/CD8 interactions, consider the four binding states of a pMHCI molecule, labeled (I–IV) in **Figure 1A**. The states in which the TCR and the pMHCI ligand are bound can be further subdivided into different states according to the number of phosphorylated ITAMs, as shown in **Figure 1B**. The transition rate between these states is n/T_R , where T_R is the average time required to go through the entire sequence. The notation T_R emphasizes that this quantity is the average time for the TCR/CD3 complex to become triggered. We call this the *TCR triggering threshold*. Typical values of TCR triggering threshold T_R are in the range of 5–15 s (29).

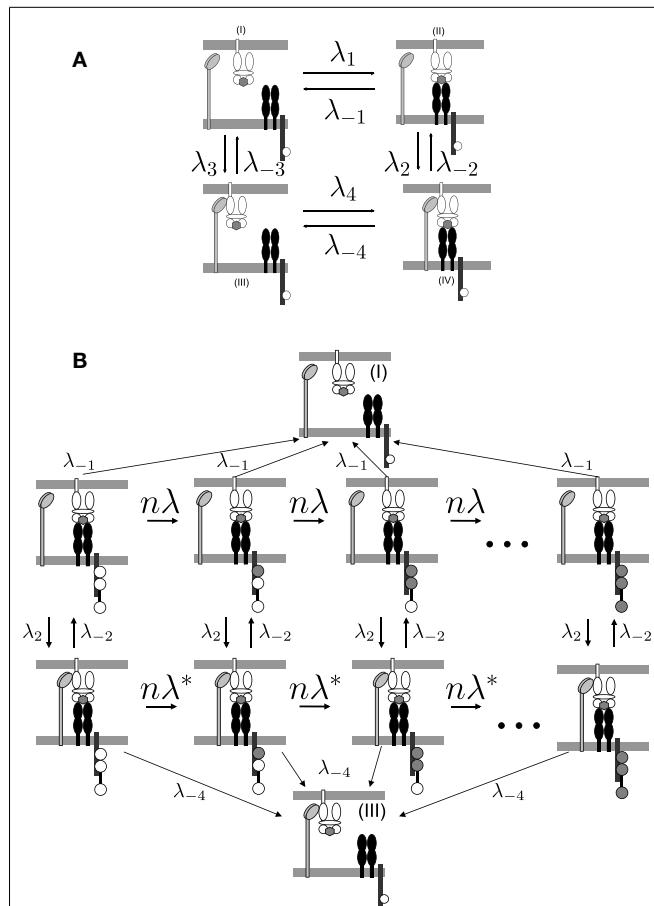


FIGURE 1 | TCR/pMHCI/CD8 interactions represented in the model.

(A) Kinetics of TCR/pMHCI/CD8 complex formation. TCRs and CD8s are located on the T-cell side and pMHCI on the APC side. Four possible states are shown: in (I) and (II), pMHCI is not bound to CD8 whereas in (III) and (IV), pMHCI is bound to CD8; in (I) and (III), pMHCI is not bound to TCR/CD3 whereas in (II) and (IV), pMHCI is bound to TCR/CD3. Transition rates between these four states are indicated with forward rates labeled as λ_i and backward rates labeled as λ_{-i} (where $i = 1, 2, 3, 4$). The white circles in the tails of the TCR/CD3 complex represent the ITAMs, which are present on the cytoplasmic tails of the γ , δ , the two ϵ , and the two ζ polypeptide chains of the CD3 complex; only one such tail is indicated here, for clarity. There are n such ITAMs, with only three shown here for the sake of clarity. This part of the kinetic scheme is assumed to be in equilibrium. **(B)** The TCR/pMHCI bound states (II) and (IV) are further subdivided into n states each depending on the number of phosphorylated ITAMs, resulting in a Markov chain. The triggered states are the right-most ones where all n ITAMs have been phosphorylated (indicated as filled-in circles). At each state a reversal (at rate λ_{-1} or λ_{-4}) to the unbound states (I) or (III), respectively, can occur. Transitions between the sequences occur at rates λ_2 and λ_{-2} . Left-to-right transitions within the sequences correspond to ITAM phosphorylation by kinases such as p56^{lck} and ZAP-70, at rate $n\lambda = nT_R$ in the sequence with CD8 unbound and at rate $n\lambda^* = n/T_R^*$ in the chain with CD8 bound. The Markov chain serves to calculate the TCR triggering probability and is not assumed to be in equilibrium.

KINETIC EQUATIONS

The model describes the kinetics of the interactions between the TCR, pMHCI molecules, and co-receptor CD8 in the contact area between a T-cell and an APC. This area is occupied by TCRs and

CD8s on the T-cell side and by pMHCI on the APC side, whereby CD8 binds pMHCI at a distinct site of the TCR/pMHCI complex. ITAMs are located on the ζ -chains of the CD3 complex associated with the TCR and are represented schematically in **Figure 1B**.

Interactions between TCR, pMHCI, and CD8

Key quantities in the model (summarized in **Table 1**) are the densities of free receptors on the T-cell and free ligands on the APC, which can also form complexes with the receptors. The surface densities of the TCR, CD8, and pMHCI molecules are subject to the following conservation laws:

$$M_T = M + M_R + M_X + M_{XR}, \quad (1)$$

$$X_T = X + M_X + M_{XR}, \quad (2)$$

$$R_T = R + M_R + M_{XR}, \quad (3)$$

which state that for each species, the total density must equal the sum of all bound forms plus the free density. We define pseudo-unimolecular association rates $\lambda_i (i=1, \dots, 4)$ as follows (see **Figure 1A**):

$$\lambda_1 = \Lambda_1 R, \quad \lambda_2 = \Lambda_2 X, \quad (4)$$

$$\lambda_3 = \Lambda_3 X, \quad \lambda_4 = \Lambda_4 R, \quad (5)$$

where $\Lambda_1 - \Lambda_4$ are the two-dimensional association rates (cm^2s^{-1}) for TCR/pMHCI or CD8/pMHCI binding. Two-dimensional dissociation constants (cm^{-2}) are defined as follows:

$$K_1 = \frac{\lambda_{-1}}{\lambda_1} R, \quad (6)$$

$$K_2 = \frac{\lambda_{-2}}{\lambda_2} X, \quad (7)$$

$$K_3 = \frac{\lambda_{-3}}{\lambda_3} X, \quad (8)$$

$$K_4 = \frac{\lambda_{-4}}{\lambda_4} R, \quad (9)$$

In order for a system of reactions to be in thermal equilibrium, each individual reaction must be at equilibrium (the principle of detailed balance):

$$\begin{aligned} \lambda_1 M &= \lambda_{-1} M_R, & \lambda_2 M_R &= \lambda_{-2} M_{XR}, \\ \lambda_3 M &= \lambda_{-3} M_X, & \lambda_4 M_X &= \lambda_{-4} M_{XR}, \end{aligned} \quad (10)$$

from which it follows that $K_1 K_2 = K_3 K_4$. Combining this with the conservation laws with we obtain:

$$\begin{aligned} M &= \frac{M_T}{C}, & M_R &= \frac{M_T \lambda_1}{C \lambda_{-1}}, \\ M_X &= \frac{M_T \lambda_3}{C \lambda_{-3}}, & M_{XR} &= \frac{M_T \lambda_1 \lambda_2}{C \lambda_{-1} \lambda_{-2}}, \end{aligned} \quad (11)$$

where

$$C = 1 + \frac{\lambda_1}{\lambda_{-1}} + \frac{\lambda_3}{\lambda_{-3}} + \frac{\lambda_1 \lambda_2}{\lambda_{-1} \lambda_{-2}}. \quad (12)$$

Table 1 | Model parameters and variables.

M	Free pMHCI density
M_R	TCR/pMHCI density without CD8 bound
M_X	pMHCI/CD8 density without TCR bound
M_{XR}	TCR/pMHCI/CD8 density
X	Free CD8 density
R	Free TCR density
M_T	Total pMHCI density
X_T	Total CD8 density
R_T	Total TCR density

These results can also be expressed in terms of standard affinity constants:

$$\begin{aligned} M_R &= \frac{M_T R}{K_1 + R + X K_1 / K_3 + R X / K_2}, \\ M_X &= \frac{M_T X}{K_3 + X + R K_3 / K_1 + R X / K_4}, \\ M_{XR} &= \frac{M_T X R}{K_1 K_2 + R K_2 + X K_4 + R X}. \end{aligned} \quad (13)$$

The co-receptor CD8 modulates the rate of TCR triggering. Three major modulatory functions of the co-receptor have been documented: modulation of TCR/pMHCI on-rate, TCR/pMHCI off-rate, and of the ITAM phosphorylation rate. These effects can be represented by dimensionless multipliers.

(i) enhanced TCR/pMHCI on-rate:

$$\Lambda_4 = \gamma_{\text{on}} \Lambda_1 \text{ where } \gamma_{\text{on}} \geq 1;$$

(ii) reduced TCR/pMHCI off-rate:

$$\lambda_{-4} = \gamma_{\text{off}} \lambda_{-1} \text{ where } 0 < \gamma_{\text{off}} \leq 1;$$

(iii) increased phosphorylation rate, which is equivalent to a reduced TCR triggering threshold T_R^*

$$\lambda = \gamma_R \lambda^*, \text{ where } \gamma_R \leq 1 \text{ and } \lambda = \frac{1}{T_R} \text{ and } \lambda^* = \frac{1}{T_R^*}. \quad (14)$$

It is sometimes convenient to combine the on-rate and off-rate effects into a single coefficient, as follows:

$$\gamma_{\text{kin}} = \frac{\gamma_{\text{off}}}{\gamma_{\text{on}}} = \frac{K_4}{K_1} = \frac{K_2}{K_3}, \text{ where } 0 < \gamma_{\text{kin}} \leq 1. \quad (15)$$

From equation (15), we have $\gamma_{\text{kin}} \leq \gamma_{\text{off}}$. We rewrite M_R , M_X , and M_{XR} , as given by equation (13), in terms of γ_{kin} :

$$\begin{aligned} M_R &= M_T \frac{R / K_1}{1 + R / K_1 + X / K_3 + R X / (K_1 K_3 \gamma_{\text{kin}})}, \\ M_X &= M_T \frac{X / K_3}{1 + R / K_1 + X / K_3 + R X / (K_1 K_3 \gamma_{\text{kin}})}, \\ M_{XR} &= M_T \frac{R X / (K_1 K_3)}{\gamma_{\text{kin}} (1 + X / K_3 + R / K_1) + X K_3 / (R K_1)}. \end{aligned} \quad (16)$$

To non-dimensionalize this system, we introduce the following dimensionless quantities:

$$\begin{aligned} x &= \frac{X}{K_3}, & r &= \frac{R}{K_1}, & x_T &= \frac{X_T}{K_3}, & r_T &= \frac{R_T}{K_1}, \\ \kappa &= \frac{K_1}{K_3}, & m_T &= \frac{M_T}{K_1}. \end{aligned} \quad (17)$$

It follows from equation (17) that $M_T/K_3 = \kappa m_T$. By equations (16) and (17), we have the following non-linear system of equations that determines the surface densities of free and bound receptors of all species:

$$\begin{aligned} x_T &= x + \kappa m_T \frac{x}{1 + x + r + xr/\gamma_{\text{kin}}} + \kappa m_T \frac{xr}{\gamma_{\text{kin}}(1 + x + r) + xr}, \\ r_T &= r + m_T \frac{r}{1 + x + r + xr/\gamma_{\text{kin}}} + m_T \frac{xr}{\gamma_{\text{kin}}(1 + x + r) + xr}. \end{aligned} \quad (18)$$

This system is readily solved numerically for x and r , given the parameters γ_{kin} , κ , r_T , and m_T .

The TCR triggering rate

The functional sensitivity of the TCR is represented in the present model as the rate at which TCR/CD3 complexes attain signalosome status. To calculate this TCR triggering rate, which we shall denote by W , consider the Markov chain as depicted in **Figure 1B**, which is best thought of as a system of two coupled linear Markov chains. The TCR/CD3 complex has to undergo n phosphorylations for the TCR to be triggered. When the co-receptor is not bound to the TCR/pMHCI complex, individual phosphorylation steps proceed at rate $n/T_R \equiv n\lambda$, whereas when the co-receptor is engaged, this rate is $n/T_R^* \equiv n\lambda^* \geq n\lambda$. The factor n arises simply as a matter of scaling, so that the average time to progress through the chain of phosphorylations equals T_R .

In reality, signalosome formation involves several other types of event besides ITAM phosphorylation, such as binding of ZAP-70, engagement of LAT, and so on. To avoid cumbersome notation we shall formulate the model as if ITAM phosphorylation were the only type of event; the essential theory is not materially affected by this simplification. We do not assume an equilibrium state for the Markov chain: the complex starts at zero phosphorylations at the beginning of every encounter with a pMHCI ligand and proceeds forward stochastically.

The pMHCI/TCR/CD3 complex may not attain the n th state (triggered state), but instead the pMHCI/TCR engagement may terminate, which happens at rate λ_{-1} when the co-receptor is not bound and at rate $\lambda_{-4} \leq \lambda_{-1}$ when the co-receptor is engaged. We assume that upon TCR/pMHCI dissociation the CD3 complex reverts to the basal state of zero ITAM phosphorylations sufficiently rapidly that the TCR/CD3 complex will be in this completely unphosphorylated state when the next encounter with a pMHCI molecule occurs. Essentially, this means that the CD3 complex is more susceptible to the action of phosphorylases and/or less susceptible to the action of kinases when the TCR is not engaged. A mechanistic explanation underpinning this assumption lies outside the scope of the present model.

The probability that the TCR/CD3 complex will undergo another ITAM phosphorylation is given by

$$\mathbb{P}_a^0 = \frac{n\lambda}{\lambda_{-1} + n\lambda + \lambda_2} \quad (19)$$

in the case where the co-receptor CD8 is not engaged and by

$$\mathbb{P}_a^* = \frac{n\lambda^*}{\lambda_{-4} + n\lambda^* + \lambda_{-2}} \quad (20)$$

when CD8 is engaged. The following expression gives the probability that the system switches from the chain with CD8 unbound to the chain with CD8 bound:

$$\mathbb{P}_b^0 = \frac{\lambda_2}{\lambda_{-1} + n\lambda + \lambda_2} \quad (21)$$

whereas the system will switch from CD8-unbound to CD8-bound with probability

$$\mathbb{P}_b^* = \frac{\lambda_{-2}}{\lambda_{-4} + n\lambda^* + \lambda_{-2}}. \quad (22)$$

TCR triggering requires completion of all steps before the TCR/pMHCI complex comes apart. We shall find an expression for the probability that the CD3 complex will ultimately attain completion when starting from i completed steps. Let \mathbb{P}_i^0 denote this probability for the case with CD8 unbound and \mathbb{P}_i^* for the case with CD8 bound. The TCR triggering probability is then found as \mathbb{P}_0^0 if CD8 is unbound when pMHCI docks the TCR, and \mathbb{P}_0^* if CD8 is bound. These triggering probabilities allow us to calculate the TCR triggering rate W . In particular, W can be expressed as the rate at which TCR/pMHCI complexes dissociate, times the probability that whenever a given TCR/pMHCI docking commences, the CD3 complex is ultimately triggered (the probabilities P_0^0 and P_0^*). In formula, this statement is represented as follows:

$$W = M\lambda_1 \mathbb{P}_0^0 + M_X \lambda_4 \mathbb{P}_0^* \quad (23)$$

which, by the principle of detailed balance, can be rewritten in terms of *dissociation rates*, as follows:

$$W = R\lambda_{-1} \mathbb{P}_0^0 + M_{X,R} \lambda_{-4} \mathbb{P}_0^*. \quad (24)$$

The law of total probability yields the following system of coupled difference equations:

$$\begin{bmatrix} \mathbb{P}_{i-1}^0 \\ \mathbb{P}_{i-1}^* \end{bmatrix} = \frac{1}{1 - \mathbb{P}_b^0 \mathbb{P}_b^*} \begin{bmatrix} \mathbb{P}_a^0 & \mathbb{P}_b^0 \mathbb{P}_a^* \\ \mathbb{P}_b^* \mathbb{P}_a^0 & \mathbb{P}_a^* \end{bmatrix} \begin{bmatrix} \mathbb{P}_i^0 \\ \mathbb{P}_i^* \end{bmatrix} \quad (25)$$

which can be solved to give

$$\begin{bmatrix} \mathbb{P}_0^0 \\ \mathbb{P}_0^* \end{bmatrix} = \frac{1}{(1 - \mathbb{P}_b^0 \mathbb{P}_b^*)^n} \begin{bmatrix} \mathbb{P}_a^0 & \mathbb{P}_b^0 \mathbb{P}_a^* \\ \mathbb{P}_b^* \mathbb{P}_a^0 & \mathbb{P}_a^* \end{bmatrix}^n \begin{bmatrix} 1 \\ 1 \end{bmatrix} \quad (26)$$

where we have used the boundary condition

$$\mathbb{P}_n^0 = \mathbb{P}_n^* = 1. \quad (27)$$

This boundary condition expresses the basic assumption that triggering is attained when the sequence has been completed. To render the equations dimensionless, we introduce the following parameters:

$$v = \frac{\Lambda_2 K_3}{\lambda}, \alpha = \frac{\lambda_{-1}}{\lambda}, \delta = \frac{\lambda_{-2}}{\lambda}. \quad (28)$$

The scaled (dimensionless) TCR triggering rate is then given by the following expression:

$$w = \alpha(\epsilon \mathbb{P}_0^0 + \zeta \gamma_{\text{off}} \mathbb{P}_0^*) \quad (29)$$

where

$$w = \frac{W}{K_1 m_T \lambda}; \quad (30)$$

$$\epsilon = \frac{r}{1 + x + r + xr/\gamma_{\text{kin}}}; \quad (31)$$

$$\zeta = \frac{xr}{\gamma_{\text{kin}}(1 + x + r) + xr}. \quad (32)$$

The scaled TCR triggering rate w depends on ten dimensionless parameters (**Table 2**).

STATISTICAL FORMULATION OF TCR DEGENERACY

To express TCR degeneracy mathematically, we consider the distribution of the triggering rate over the set of peptide ligands. This is just the set of w_{ij} -values for a given TCR clonotype i over pMHCI ligands j . The distribution can be represented by plotting the probability $\mathbb{P}(w_{ij} > \omega)$ as a function of ω . Such a graph shows how many randomly selected peptides would have triggering rate w larger than a set value ω . Let T_{ij} denote the mean dwell time of the TCR/pMHCI interaction for TCR clonotype i and pMHCI species j so that T_{ij}^{-1} is the TCR/pMHCI off-rate λ_{-1} (we have thus far suppressed subscripts for clone i and ligand j to keep notation uncluttered). Arrhenius theory furnishes the following relationship with the dissociation energy barrier ΔU_{ij} :

$$T_{ij} = T_0 \exp(\Delta U_{ij}), \quad (33)$$

where T_0 is the frequency factor and ΔU_{ij} is expressed in Boltzmann units. We assume that the dissociation energy barrier arises as a result of a large number of individual reaction steps at the TCR/pMHCI interface. If these combine additively, then the Central Limit Theorem implies that ΔU_{ij} has a Gaussian distribution. Letting $u_{ij} = \Delta U_{ij} - \ln\{T_R/T_0\}$ we have $u_{ij} \sim N(-\mu, \sigma^2)$, where $\mu > 0$ and σ are the underlying parameters of the normal distribution N . The assumption that the mean is negative is a consequence of the fact that for the vast majority of TCR/pMHCI complexes the mean dwell time is less than the typical time required to complete the chain of ITAM phosphorylations. It now follows that the dimensionless parameter α , associated with the TCR/pMHCI off-rate, is log-normally distributed.

RESULTS

We investigated the effect of variations of the total CD8 density on the functional sensitivity of hypothetical ligands with various

Table 2 | Dimensionless (scaled) parameters that govern functional sensitivity.

m_T	Scaled total pMHCI density
x_T	Scaled total CD8 density
r_T	Scaled total TCR density
α	Scaled TCR/pMHCI off-rate without CD8 bound
δ	Scaled pMHCI/CD8 off-rate with TCR bound
v	Scaled kinetic effect of pMHCI/CD8 interactions with and without TCR
κ	Ratio of dissociation constants K_1 and K_3
γ_{off}	Factor by which CD8 modulates TCR/pMHCI off-rate
γ_{kin}	Factor by which CD8 modulates the TCR/pMHCI affinity
γ_R	Factor by which CD8 modulates the TCR triggering threshold

TCR/pMHCI off-rates. All variables and parameters are dimensionless (scaled) in the model simulations. The scaled parameters are summarized in **Table 2** and their scaling is defined in equations (14), (15), (17), and (28).

LIGAND FOCUSING AND CD8-MEDIATED CONTROL OF DEGENERACY

Figure 2A shows the scaled functional sensitivity w as a function of scaled total CD8 density x_T with different scaled TCR/pMHCI off-rates α . A striking feature is the dip at $x_T \approx 50$, suggesting that at this CD8 density the T-cell is minimally responsive. As the scaled total CD8 density increases above $x_T \approx 50$, the scaled functional sensitivity increases for all hypothetical ligands.

It can be observed that a ligand with $\alpha = 2.5$ (solid line) and a ligand with $\alpha = 0.5$ (dotted line) show opposing changes in the scaled functional sensitivity: a ligand that is less potent at low CD8 becomes more potent at high CD8 and *vice versa*. Hence, changes in CD8 expression levels can differentially affect the potency of ligands, each of which is potentially a strong agonist. In effect, the T-cell can tune in on a specific ligand and thus control ligand promiscuity. We call this the principle of *ligand focusing*.

The corresponding degeneracy curves $\mathbb{P}(w > \omega)$ for different scaled total CD8 densities are shown in **Figure 2B**. The effect of the increase in co-receptor density on the sensitivity and degeneracy is indicated: sensitivity is a change in the horizontal direction (modulating the triggering threshold) and degeneracy is a change in the vertical direction (controlling cross-reactivity). As the scaled total CD8 density increases up to $x_T \approx 50$, the degeneracy curves $P(w > \omega)$ move to the left from the curve without CD8 ($x_T = 0$): degeneracy and sensitivity decrease. By contrast, as the scaled total CD8 density increases above $x_T \approx 50$, the degeneracy curves move to the right, which means that degeneracy and sensitivity both increase. The overall effect is that the T-cell becomes more degenerate as CD8 levels increase. Thus, in addition to the focusing effect, the co-receptor also governs the overall degeneracy of the T-cell.

A high degree of degeneracy can increase the risk autoimmune disease. On the other hand, too low a degree of degeneracy could compromise the immune system's ability to mount a timely and efficient response. To analyze these risks, suppose that the T-cell is activated if its integrated TCR triggering rate exceeds a certain value, termed *cellular activation threshold*. A simple model is to

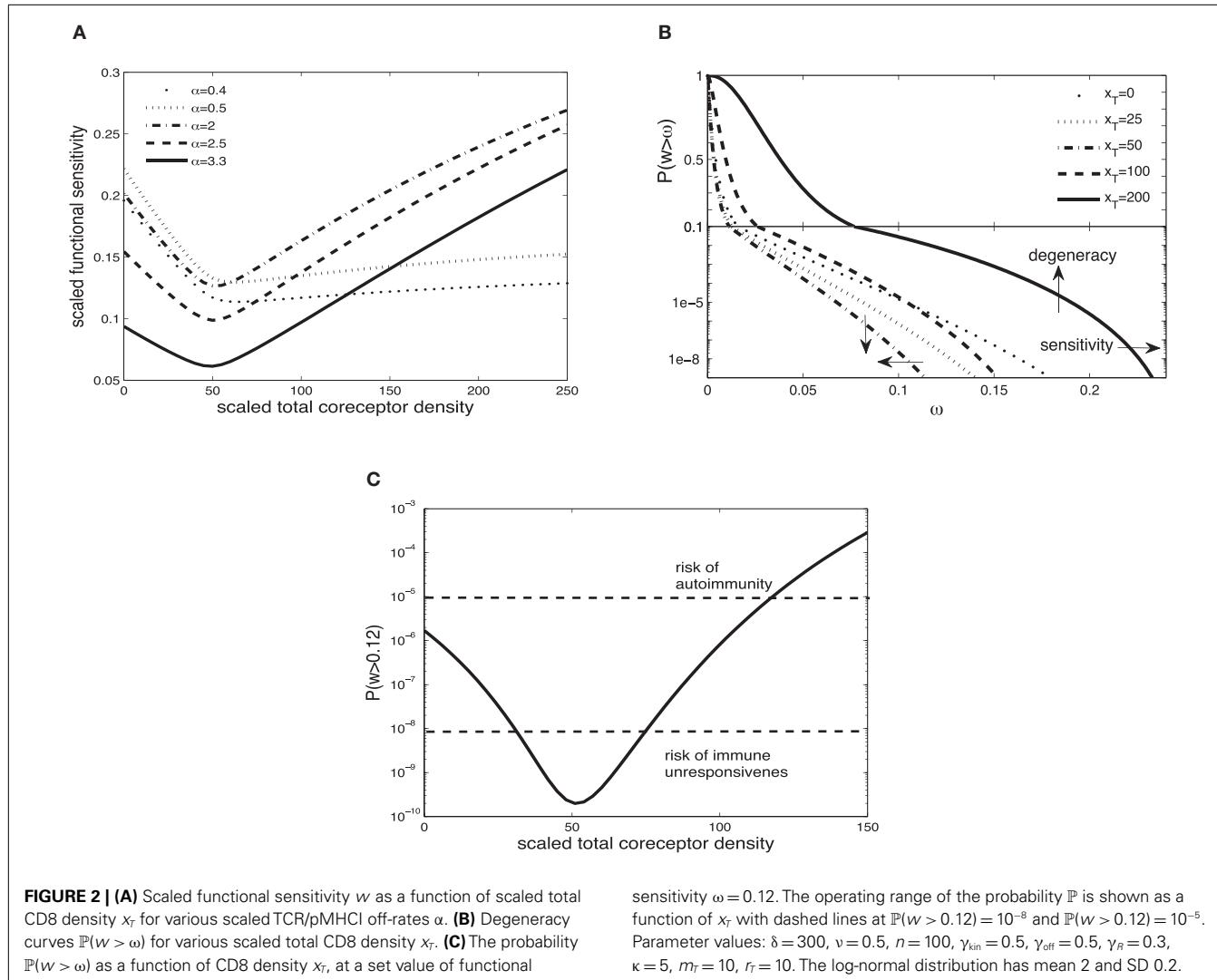


FIGURE 2 | (A) Scaled functional sensitivity w as a function of scaled total CD8 density x_T for various scaled TCR/pMHCI off-rates α . **(B)** Degeneracy curves $P(w > \omega)$ for various scaled total CD8 density x_T . **(C)** The probability $P(w > \omega)$ as a function of CD8 density x_T , at a set value of functional

sensitivity $\omega = 0.12$. The operating range of the probability P is shown as a function of x_T with dashed lines at $P(w > 0.12) = 10^{-8}$ and $P(w > 0.12) = 10^{-5}$. Parameter values: $\delta = 300$, $v = 0.5$, $n = 100$, $\gamma_{kin} = 0.5$, $\gamma_{off} = 0.5$, $\gamma_R = 0.3$, $\kappa = 5$, $m_T = 10$, $r_T = 10$. The log-normal distribution has mean 2 and SD 0.2.

assume that the T-cell is activated if

$$Z_j T_I w_{ij} > W_{act} \quad (34)$$

where Z_j is the presentation level of ligand j , T_I is the duration of the T-cell:APC interaction, and W_{act} is the activation threshold. For a given Z_j and T_I , there is a critical w_{ij} which is the minimum value required to satisfy equation (34). Suppose for instance that this corresponds to $w_{ij} = 0.12$. The corresponding probabilities $P(w > 0.12)$ are plotted in Figure 2C. Given the estimates of the TCR repertoire size, normal immune function is probably confined to an operating range of probabilities 10^{-8} to 10^{-5} . Figure 2C shows how the level of CD8 can regulate the responsiveness to remain within this band; if the activation probability drops below this range, the risk of not responding to a pathogen looms, whereas at much elevated activation probabilities, the risk of autoimmunity is heightened.

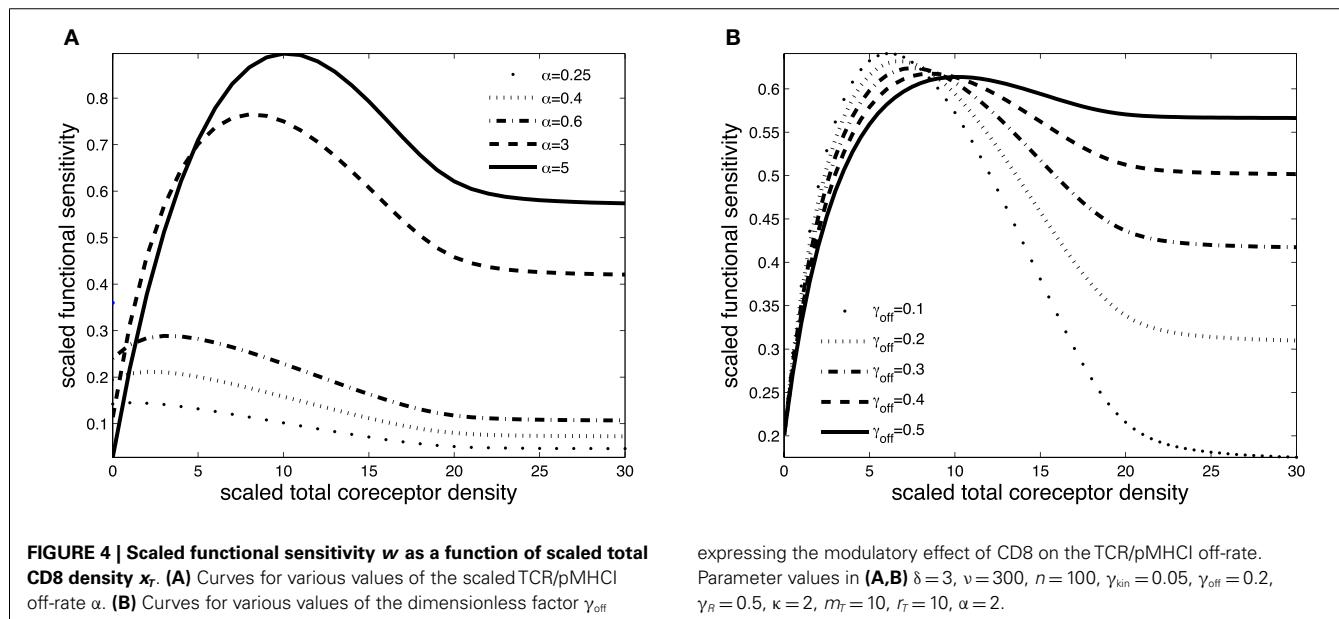
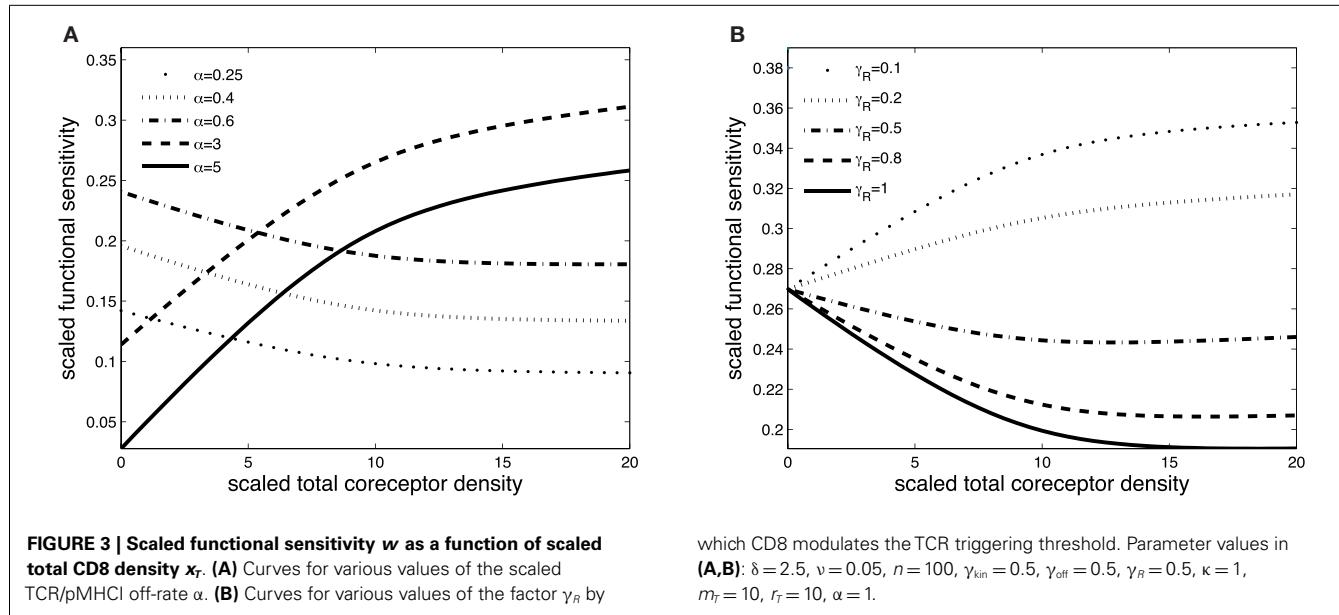
MODES OF CD8-MEDIATED MODULATION OF FUNCTIONAL SENSITIVITY

Figures 3 and 4 exhibit the co-receptor effect on functional sensitivity in two parameter scenarios, where we assume a weak

($v = 0.05$) or a strong ($v = 300$) kinetic effect of pMHCI/CD8 interactions, respectively. When the kinetic parameter v is small, increasing the levels of CD8 on the T-cell surface leads to enhanced functional sensitivity of ligands with $\alpha > 1$, as shown in Figure 3A. By contrast, ligands with low off-rates ($\alpha < 1$) become less potent when CD8 levels are increased. These opposite effects demonstrate CD8-mediated focusing on particular ligands.

Figure 3B shows the effect of increasing CD8 levels on a ligand that is optimal in the absence of CD8. When the modulatory effect of CD8 on the TCR triggering threshold is modest (γ_R is near 1), the ligand becomes less potent as levels of CD8 are increased. On the other hand, when CD8 has a strong effect on the TCR triggering threshold (low γ_R , since the time required to trigger the TCR/CD3 complex is shortened), increasing the levels of CD8 on the cell surface enhances the functional sensitivity to the ligand.

The effect of CD8 levels on functional sensitivity is shown in Figure 4A. For each ligand, there is an optimal CD8 level which depends on that ligand's TCR/pMHCI off-rate α . This shows that the T-cell can favor the signaling strength of a given ligand by



adjusting the density of CD8 molecules on its surface. As one would expect, the modulatory effect is most profound when the relative effect that the co-receptor exerts on the TCR/pMHCI off-rate is greatest; this is shown in **Figure 4B**. When CD8 alters the off-rate strongly, the functional sensitivity is strongly depressed when CD8 levels are increased beyond the optimal level, whereas for a moderate value of the modulatory multiplier γ_{off} , the functional sensitivity remains at near-optimal levels when CD8 levels are increased. The value of γ_{off} may be expected to be different for different TCR/ligand combinations. In particular, when CD8 makes a substantial contribution to the binding energy, the multiplier γ_{off} will be low, and the co-receptor role in governing ligand optimality will be more pronounced.

CO-RECEPTOR MODULATION OF DEGENERACY VIA ALTERED pMHCI/CD8 BINDING AFFINITY

Wooldridge et al. (5, 30, 31) have shown that (i) increased pMHCI/CD8 interaction results in enhanced recognition of pMHCI by cytotoxic T-cells and (ii) increased pMHCI/CD8 interaction impairs pMHCI recognition specificity, suggesting that the pMHCI-CD8 interaction is essential in regulating the balance between optimal T-cell cross-reactivity and T-cell antigen specificity (32). These findings suggest that an optimal pMHCI/CD8 strength exists that yields maximum pMHCI sensitivity without loss of specificity. Motivated by these results, we consider a hypothetical scenario in which pMHCI mutant molecules with altered binding affinity for CD8 modulate TCR degeneracy.

In keeping with (17), the following pMHCI mutants are considered: A245V representing weak pMHCI/CD8 affinity ($K_D = 498 \mu\text{M}$), wild-type ($K_D = 137.1 \mu\text{M}$), Q115E representing slightly enhanced affinity ($K_D = 97.94 \mu\text{M}$), and A2/α3k^b with enhanced affinity ($K_D = 10.87 \mu\text{M}$). These values are based on Surface Plasmon Resonance experiments and should be regarded as “three-dimensional,” relating to the ligands in solution. However, the TCR/pMHCI interaction takes place in the “two-dimensional” environment of the T-cell:contact area, which essentially reduces spatial degrees of freedom of molecular motion and, moreover, introduces dynamics related to the forces that constrain the molecules to this environment, such as rotations with respect to the membrane plane, membrane fluctuations, and the translational motion of the membranes themselves (33). Furthermore, cooperativity interactions, such as the involvement of the co-receptor, may be profoundly altered. As a result, the rate constants can be markedly different in the “two-dimensional” environment; in particular, two-dimensional dissociation rates can be substantially faster (28). The ratio between the three- and two-dimensional affinities is a length measure, denoted h and called the confinement length (34, 35). Wu et al. (33) demonstrated that h is proportional to the range of motion available to the free forms of the interacting ligands along the spatial axis perpendicular to the two parallel membranes. Thus the inter-membrane separation distance provides an upper bound, and if the ranges of motion are broadly comparable, we may assume that the confinement length is roughly the same for all mutants involved.

The two-dimensional dissociation constant for pMHCI/CD8 interaction without TCR bound, K_3 , appears in the scaled parameters κ , x_T , and v . Hence, by taking K_3 to be proportional to K_D for a given pMHCI mutant, we can simulate the impact of the altered pMHCI/CD8 binding affinity on TCR degeneracy and sensitivity. **Figure 5A** shows the scaled functional sensitivity w as a function of scaled TCR/pMHCI off-rate α for the four hypothetical pMHCI mutants.

The co-receptor CD8 can modulate the specificity of antigen recognition, as shown in **Figure 5B**. Each TCR degeneracy curve corresponds to a given pMHCI mutant, where the one with the strongest pMHCI/CD8 binding affinity (A2/α3k^b) is the most degenerate, with the largest antigen sensitivity. The three regions are a schematic representation of the overall pattern of CD8⁺ T-cell antigen specificity, as defined by Cole et al. (32). With increasing strength of pMHCI/CD8 affinity, as indicated by the arrow, the recognition efficiency of partially CD8-dependent ligands is enhanced and the spectrum of CD8⁺ T-cell antigen degeneracy becomes wider. Enhancing the kinetic effect of pMHCI/CD8 interactions (setting $v \gg 1$) results in the reversed pattern, as shown in **Figure 5C**.

Whereas in the MHC-limited kinetic regime, the behavior is as shown in **Figure 5B**, the degeneracy curves for the various mutants overlap in the TCR-limited regime. This suggests that excess levels of ligand, relative to the available levels of TCR molecules, can diminish the importance of the interaction between pMHCI and CD8. In principle, this endows the APC with a means to “override” the ligand focusing exerted by the T-cell, allowing a professional APC, such as a dendritic cell, to force a naïve T-cell, with which it has conjugated, to be maximally degenerate.

DISCUSSION

The co-receptors CD4 and CD8 are glycoproteins that modulate the interactions of the TCR with pMHCI and pMHCII molecules, by binding to invariant sites on these molecules (10). It is well-established that the co-receptors differentially regulate the responsiveness of the TCR to the ligand and thereby modulate TCR specificity (1). In particular, CD8 is known to affect both the on-rate and the off-rate of the TCR/pMHCI interaction (17, 36). This allows the co-receptor to differentially regulate the strengths of the various *potentially* strong agonists of the TCR. This accords with the finding that the strength of pMHCI/CD8 interaction is a determinant of T-cell degeneracy (5). This ligand focusing effect remains to be observed experimentally, to the best of our knowledge. Perhaps this is only to be expected inasmuch as the experimenter has to search for ligands that are sub-optimal under standard conditions but become better or worse agonists when CD8 levels are manipulated. Research is presently underway to identify such ligands and we anticipate that the phenomenon will be confirmed and eventually emerge as a pervasive “design principle” of cellular adaptive immunity.

Co-receptor-directed ligand focusing may allow the T-cell response to an antigen challenge to undergo an adaptive evolution that would be functionally analogous to affinity maturation in B-cell immunity. Moreover, CD8 modulation could allow for an elevated degeneracy among the earliest responding clones. This would ensure that at least one or more responding clones are activated sufficiently early in the course of the infection. Moreover, a gradual restriction of the degeneracy, coupled with an increase in functional sensitivity to the salient epitope, would then reduce the degeneracy of the response, which would gradually evolve from oligoclonal to one that is dominated by an optimally tuned single clone.

Disrupting the pMHCI/CD8 interaction impairs the ability of T-cells to recognize antigens. In particular, T-cell activation can be abrogated if the pMHCI/CD8 interaction is blocked (32), whereas increases in pMHCI/CD8 affinity have the opposite effect (37, 38). The contribution of CD8 to increase functional sensitivity appears to be crucial for weaker agonists (36). A comprehensive evaluation of clonal CD8⁺ T-cell degeneracy using combinatorial peptide libraries and APCs expressing mutant HLA A*0201 molecules with altered pMHCI/CD8 affinity has shown that the co-receptor enhances T-cell degeneracy by increasing the range of agonist ligands that can elicit T-cell activation (5). Furthermore, increasing the affinity of CD8 for HLA A*0201 by at least an order of magnitude resulted in the loss of cognate antigen specificity (5, 31). The affinity of the pMHCI/CD8 interaction may be directly linked to TCR degeneracy: increased pMHCI/CD8 interaction enhances CD8⁺ T-cell antigen sensitivity, but reduces CD8⁺ T-cell antigen specificity (32). This agrees with our main finding that variation of the co-receptor effect regulates the degree of T-cell degeneracy and antigen specificity.

A cornerstone of the present theory is that a certain amount of degeneracy is unavoidable, in view of the vast universe of possible peptides and the relatively modest number of TCR clonotypes that even a large mammal might be able to maintain in its standing repertoire. Moreover, salient epitopes, those associated with a disease state, and non-salient ones, such as self-peptides

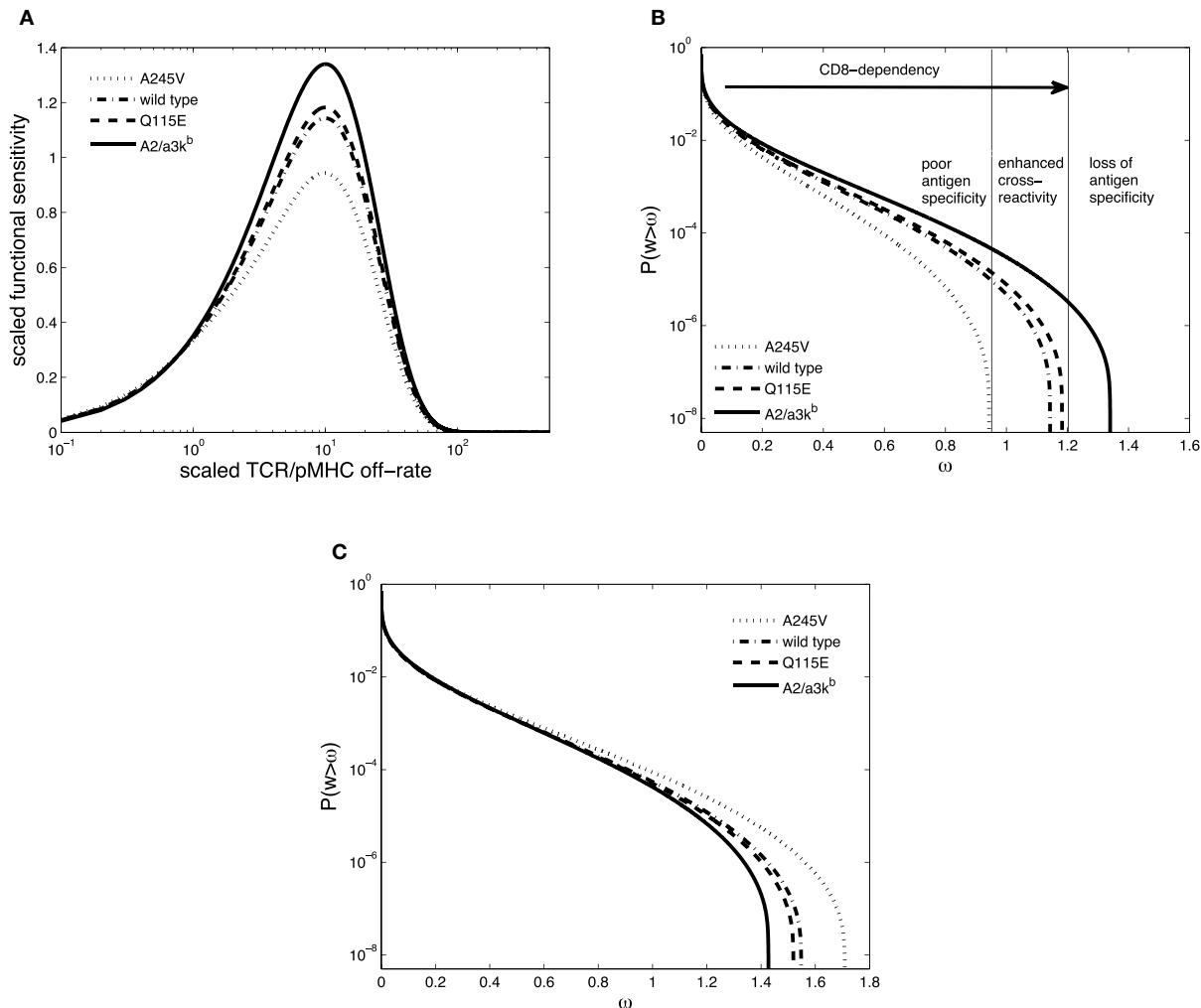


FIGURE 5 | (A) Scaled functional sensitivity w as a function of scaled TCR/pMHC off-rate ω . **(B)** Degeneracy curves $P(w > \omega)$ for HLA A*0201 mutants with altered binding affinity for CD8: A245V (dotted line), wild-type (semi-dashed line), Q115E (dashed line), and A2/a3k^b (solid line). The three regions represent the overall pattern of CD8⁺ T-cell antigen specificity and the

arrow indicates the strength of pMHC/CD8 interaction. **(C)** Degeneracy curves $P(w > \omega)$ for HLA A*0201 mutants with altered binding affinity for CD8. The parameter values are the same as in A except for $\nu = 10$. Parameter values in **(A,B)**: $\delta = 0.2$, $\nu = 0.05$, $n = 100$, $\gamma_{\text{kin}} = 0.5$, $\gamma_{\text{off}} = 0.5$, $\gamma_R = 0.2$, $\kappa = 1$, $m_r = 10$, $r_f = 10$, $x_f = 10$. The log-normal distribution has mean 5 and SD 0.5.

for which immune tolerance is required, will of necessity be “finely interleaved” subsets of the peptide universe (a mathematician would say that one subset is “dense” in the other), lest the tolerant subset forms a target for the rapidly evolving pathogens: the system cannot work if molecular mimicry is readily attained. From these two premises, it follows that a TCR must be degenerate, and also that this degeneracy must be susceptible to exquisite modulation. Against this line of reasoning a case could be made that the size of the ligand universe is effectively much smaller. For instance, if one considers n -mer peptides that are anchored to the MHC binding groove at a positions, and the region of the TCR that interacts with the peptide (roughly speaking, the CDR3 loop) makes contacts with c of the amino acid residues in the m -mer (so that $0 < m \leq n - a$), then there are $20^m(n - a)!/(m!(n - a - m)!)$ effectively distinct pMHC ligands as seen by the TCR. To give an extreme example, with $n = 9$,

$a = 2$, and $m = 1$, this works out as just $20 \times 7 = 140$ distinct ligands. Perhaps the estimate $m = 4$ is more realistic: this gives only $20^4 \times 35 = 5.6 \times 10^6$ functionally distinct ligands, which is of the same order as the TCR repertoire size. Whereas there may be some merit to this argument, its underlying image, essentially of CDR3 as a tape recorder head that interacts with only m amino acid residues and is indifferent to the $n - m$ other ones, is a gross oversimplification. The physical behavior of the m amino acid residues at the contact sites cannot fail to be influenced by the $n - m$ remaining ones (including the a anchor residues). This is overwhelmingly apparent not just from the basic principles of molecular dynamics, but also from the typical results obtained with combinatorial peptide library scans. A case in point is the finding that changes at the anchor position result in changes in the center of the peptide and therefore influence TCR binding (39, 40).

The present model indicates that intermediate levels of CD8 are associated with the lowest functional sensitivity. This suggests the following mechanism to maintain quiescent (naive) T-cells in a relatively unresponsive state. When the T-cell receives the appropriate stimuli, it either up-regulates or down-regulates the co-receptor and a specific subset of its potential agonists “comes into focus.” Such signals are known to be transmitted via cytokine profiles in the T-cell’s surroundings (15) as well as costimulatory receptor-mediated signals transmitted by professional APCs (41). When no harm is detected, the default response of the naïve T-cells would be to “de-tune” whenever a strong signal is registered. Detuning of T-cells via alterations of CD8 expression levels, under control of cytokine stimuli, has been reported (42) and the connection between functional sensitivity, tolerance, and CD8 expression levels is well-established (14, 43, 44). On the other hand, when harm is detected and transmitted via a pro-inflammatory cytokine profile, the T-cell’s response would invert and the tendency would become to “tune in” to any supra-threshold stimulation. On a molecular-cellular level, this would involve a scanning mechanism whereby the stimulus would make the cell enter a mode in which it gradually alters the CD8 expression level whilst the received TCR signal (which will shift in magnitude as the CD8 level changes) feeds back onto this pathway. To the best of our knowledge, the molecular details of such a regulatory pathway have not been elucidated to date. However, we believe that it is well within the regulatory capabilities of cellular signaling networks; we have previously discussed similar mechanisms in more depth (45, 46).

Whilst the model includes the key components of TCR triggering, many important aspects have been omitted. In particular, we have neglected the spatial dynamics of TCR, CD8, and pMHCI within the immunological synapse, where the relative concentrations of p56^{lck} and CD45 will determine how quickly partially

phosphorylated TCR/CD3 will reset to the basic state (47). A functional consequence of the exclusion of phosphorylases, for instance, could be the ability of the TCR/CD3 complex to be triggered over subsequent interactions with ligands that would in a normal context only have weak functional sensitivity.

In addition to kinetics of the interaction between TCR, MHCI, and CD8, we have only considered ITAM phosphorylation steps. It is well known that phosphorylated ITAMs orchestrate the activation of the Src-related protein tyrosine kinases which initiate TCR signaling. These kinases induce tyrosine phosphorylation of several polypeptides, including the transmembrane adaptors. Protein tyrosine phosphorylation subsequently leads to the activation of multiple pathways such as ERK, NF- κ B, and NFAT (48, 49). Moreover, negative regulation of TCR signaling is key to avoiding hyper-activation. Notwithstanding the additional layers of complexity which our simple model ignores, we believe that the system of two linked proofreading chains as presented here does capture, in a qualitative sense, the essence of TCR triggering.

In summary, the present findings suggest that the co-receptor CD8 can differentially modulate functional sensitivity to its potential agonists, thereby modulating TCR degeneracy in a tunable fashion. The ligand focusing mechanism would allow each T-cell to have a wide range of potential agonists, even while only one of these would be a ligand of high functional sensitivity at any particular moment in time.

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Corrigendum: Co-receptor CD8-mediated modulation of T-cell receptor functional sensitivity and epitope recognition degeneracy

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A corrigendum on

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It has been kindly pointed out to us by Dr. Omer Dushek of Oxford University that the thermodynamic constraints (arising from the principle of detailed balance) impose the following condition on the parameters:

$$v = \gamma_{\text{kin}}\delta \quad (1)$$

which means that the parameter v is fixed once γ_{kin} and δ have each been assigned a value. The objective of the paper was to exhibit the range of qualitative behaviors that is possible when pMHC1/CD8

kinetics interacts with TCR/pMHC1 kinetics and to show how varying levels of the co-receptor at the T-cell surface may be able to modulate the functional sensitivity of the T-cell to various ligands in a differential fashion. These qualitative phenomena remain very much the same when we choose parameter values that respect the constraint $v = \gamma_{\text{kin}}\delta$, as shown in the corrected figures that follow below (Figures 2–4). It is these qualitative patterns that are currently guiding experimental research to elucidate CD8-mediated ligand focusing in the T-cell system. The main thrust of the paper is therefore unaltered.

The kinetic scheme (Figure 1) and the equations are all unaltered, except for a typographical error in the subscript of a quantity appearing in one of the equations. Specifically, equation (23) in the published text should read:

$$W = M_R \lambda_{-1} \mathbb{P}_0^0 + M_{XR} \lambda_{-4} \mathbb{P}_0^*. \quad (2)$$

We apologize profusely for any inconvenience our oversight may have caused.

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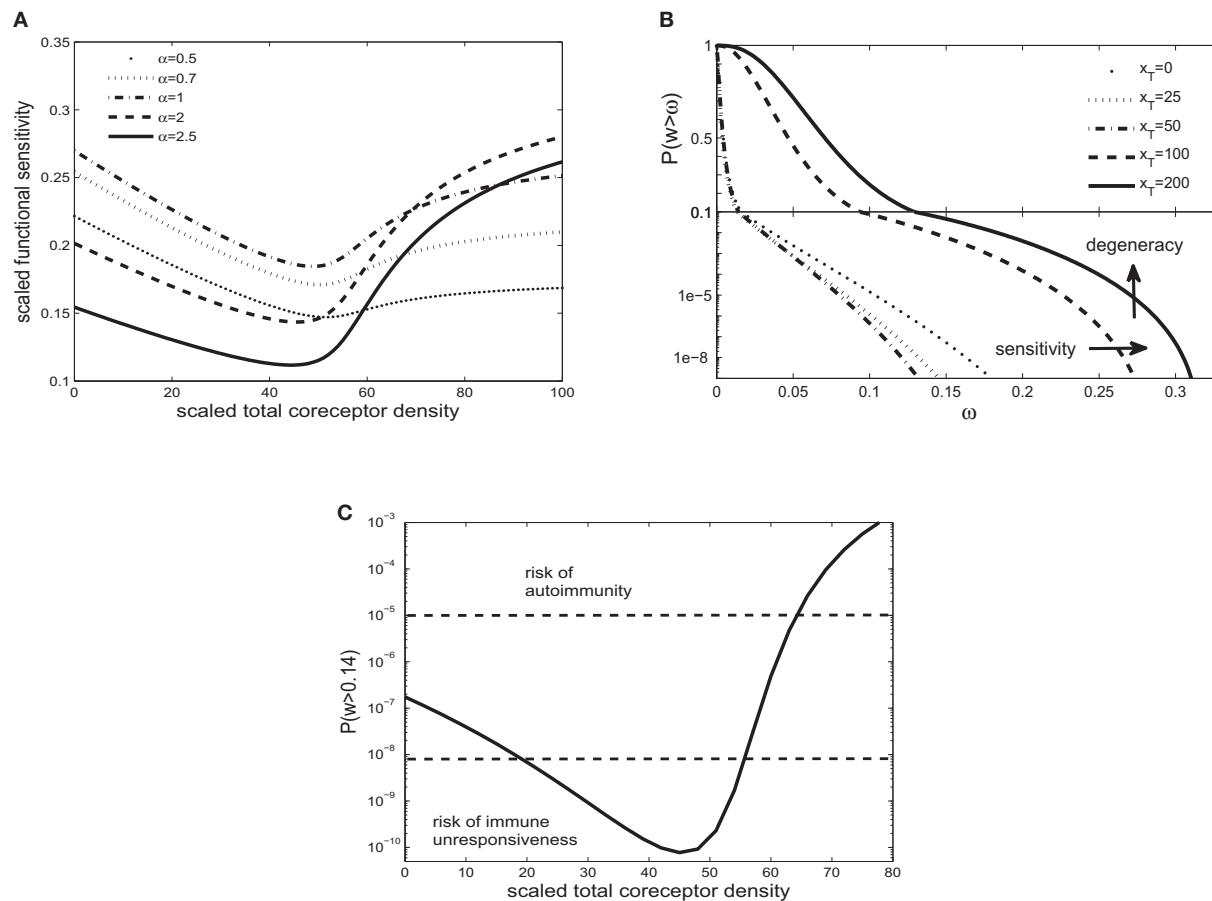


FIGURE 2 | (A) Scaled functional sensitivity w as a function of scaled total CD8 density x_T for various scaled TCR/pMHC1 off-rates α . **(B)** Degeneracy curves $\mathbb{P}(w > \omega)$ for various scaled total CD8 density x_T . **(C)** The probability $\mathbb{P}(w > \omega)$ as a function of CD8 density x_T , at a set value of functional sensitivity $\omega = 0.14$. The operating range of the probability \mathbb{P}

is shown as a function of x_T with dashed lines at $\mathbb{P}(w > 0.14) = 10^{-8}$ and $\mathbb{P}(w > 0.14) = 10^{-5}$. Parameters are as follows: $\delta = 300$, $n = 100$, $\gamma_{\text{kin}} = 0.1$, $\gamma_{\text{off}} = 0.5$, $\gamma_R = 1$, $\kappa = 5.5$, $m_T = 10$, $r_T = 10$. The log-normal distribution has mean 2 and SD 0.2. Changed: $\omega = 0.14$, γ_{kin} , γ_R , κ and α 's in **(A)**.

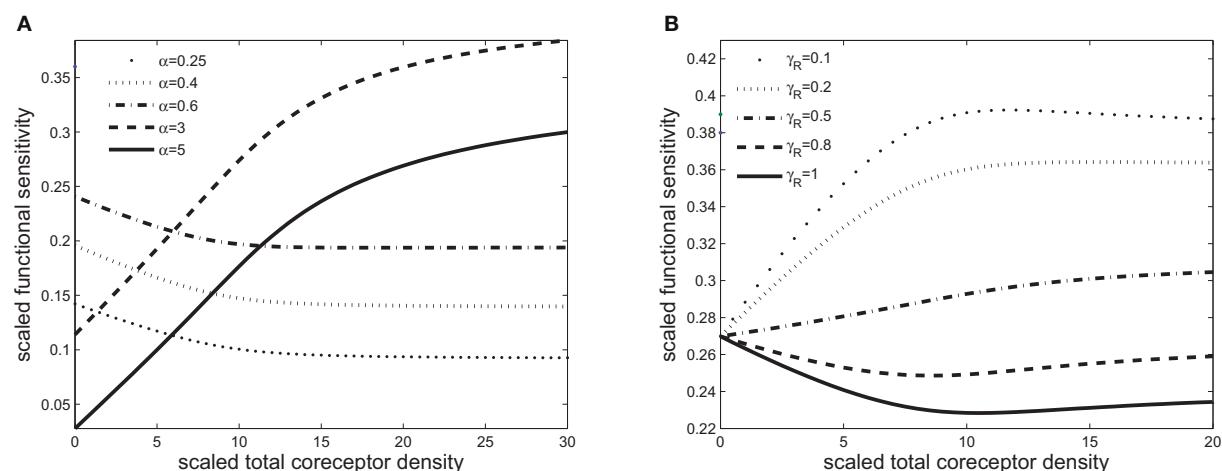


FIGURE 3 | Scaled functional sensitivity w as a function of scaled total CD8 density x_T . **(A)** Curves for various values of the scaled TCR/pMHC1 off-rate α . **(B)** Curves for various values of the factor γ_R by

which CD8 modulates the TCR triggering threshold. Parameters, unless stated otherwise, are as follows: $\delta = 2.5$, $n = 100$, $\gamma_{\text{kin}} = 0.5$, $\gamma_{\text{off}} = 0.5$, $\gamma_R = 0.7$, $\kappa = 1$, $m_T = 10$, $r_T = 10$, $\alpha = 1$. Changed: γ_R .

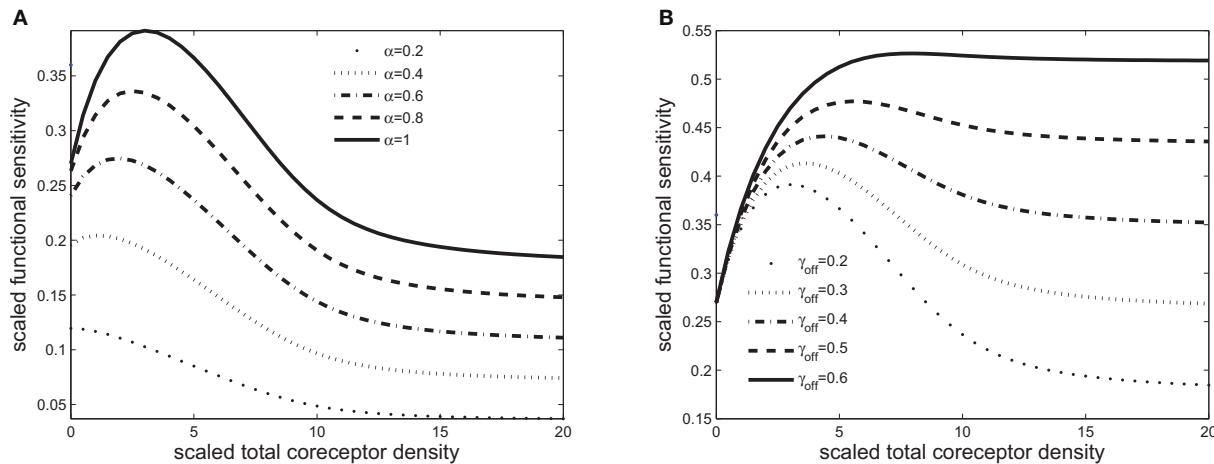


FIGURE 4 | Scaled functional sensitivity w as a function of scaled total CD8 density x_T . **(A)** Curves for various values of the scaled TCR/pMHC off-rate α . **(B)** Curves for various values of the dimensionless factor γ_{off} expressing the modulatory effect of CD8 on

the TCR/pMHC off-rate. Parameters, unless stated otherwise, are as follows: $\delta = 500$, $n = 100$, $\gamma_{\text{kin}} = 0.2$, $\gamma_{\text{off}} = 0.2$, $\gamma_R = 0.01$, $\kappa = 1$, $m_T = 10$, $r_T = 10$, $\alpha = 1$. Changed: δ , γ_{kin} , γ_R , α , κ , and α 's in **(A)** and γ_{off} 's in **(B)**.

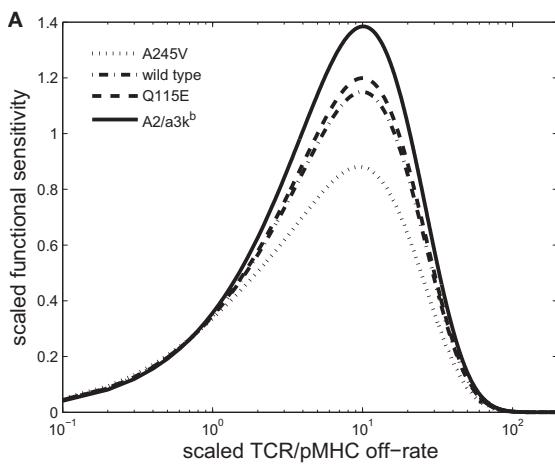
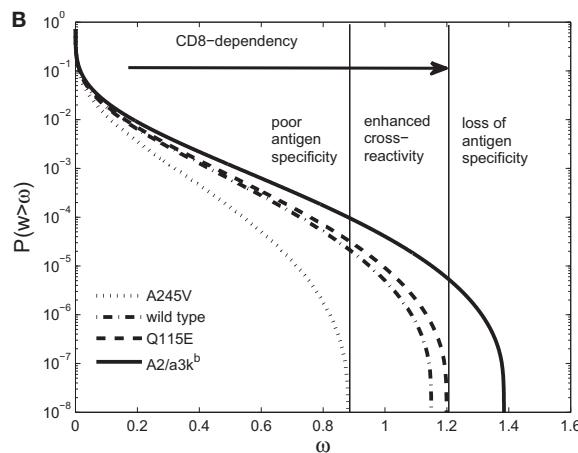


FIGURE 5 | (A) Scaled functional sensitivity w as a function of scaled TCR/pMHC off-rate α . **(B)** Degeneracy curves $\mathbb{P}(w > \omega)$ for HLA A*0201 mutants with altered binding affinity for CD8: A245V (dotted line), wild-type (semi-dashed line), Q115E (dashed line), and A2/a3k^b (solid line). The three regions represent the overall pattern of CD8⁺ T-cell antigen specificity and the arrow indicates the strength of pMHC/CD8 interaction. Wild-type parameters are as follows: $\delta = 0.2$, $n = 100$,



$\gamma_{\text{kin}} = 0.5$, $\gamma_{\text{off}} = 0.5$, $\gamma_R = 0.2$, $\kappa = 1$, $m_T = 10$, $r_T = 10$, $x_T = 10$; the parameter δ is adjusted in proportion to the ratio between the mutant pMHC/CD8 affinity constant (i.e., dissociation constant) and the wild-type affinity, whereas the parameters κ and x_T are adjusted in inverse proportion to the mutant/wild-type pMHC/CD8 affinity (these adjustments follow from the scaling). The log-normal distribution has mean 5 and SD 0.5. Figure 5C has been deleted.



Individual MHCI-restricted T-cell receptors are characterized by a unique peptide recognition signature

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Effective immunity requires that a limited TCR repertoire is able to recognize a vast number of foreign peptide-MHCI (peptide-major histocompatibility complex class I) molecules. This challenge is overcome by the ability of individual TCRs to recognize large numbers of peptides. Recently, it was demonstrated that MHCI-restricted TCRs can recognize up to 10^6 peptides of a defined length. Astonishingly, this remarkable level of promiscuity does not extend to peptides of different lengths, a fundamental observation that has broad implications for CD8⁺ T-cell immunity. In particular, the findings suggest that effective immunity can only be achieved by mobilization of "length-matched" CD8⁺ T-cell clonotypes. Overall, recent findings suggest that every TCR is specific for a unique set of peptides, which can be described as a unique "peptide recognition signature" (PRS) and consists of three components: (1) peptide length preference, (2) number of peptides recognized; and, (3) sequence identity (e.g., self versus pathogen derived). In future, the ability to de-convolute peptide recognition signatures across the normal and pathogenic repertoire will be essential for understanding the system requirements for effective CD8⁺ T-cell immunity and elucidating mechanisms which underlie CD8⁺ T-cell mediated disease.

Keywords: MHCI-peptide length, T-cell crossreactivity, vaccination, autoimmunity, alloreactivity

INTRODUCTION

CD8⁺ T cells are important for the control of viral infection and the natural eradication of cancer. CD8⁺ T cells recognize short peptides presented at the target cell surface bound to major histocompatibility complex class I (MHCI) molecules (1). Recognition of peptide-MHCI (pMHCI) is mediated by the alpha beta T-cell receptor (TCR). Initially, it was thought that MHCI molecules predominantly presented peptides of between 8 and 10 amino acids in length. However, subsequent studies demonstrated that peptides of between 11 and 14 amino acids in length are also presented and can be highly immunogenic (2). In fact, many longer peptides can be immunodominant over completely overlapping shorter peptides that also bind to the same MHCI allele (3–6). Thus, it is now well established that MHCI can present peptides of between 8 and 14 amino acids in length. A vast array of peptides within this length range can be generated from permutations of the twenty proteogenic L-amino acids (1.7×10^{18}). Of course, not all of these peptide sequences occur in the current database of natural proteins. However, it is vital that the TCR repertoire is poised to recognize vast numbers of peptide sequences, even if not identified to date, in order to adapt and deal with changes in pathogen diversity.

The MHCI-presented peptide repertoire is constrained by factors such as the specificity of antigen processing pathway components (proteasome, ERAAP/ERAP1, tapasin, and TAP) and the HLA type of the individual (7,8). As a result, conservative estimates suggest that only ~1% of the peptide universe can be processed and presented at the cell surface in the context of MHCI (9). However, this still leaves a staggering 1.7×10^{16} peptides of different lengths to which a relatively small number of TCRs (~25 million) (10) must be able to respond appropriately. Therefore, in

order to understand the requirements for effective CD8⁺ T-cell immunity, it is important to understand how a limited TCR repertoire responds and adapts to the vast number of possible different epitopes across a length spectrum.

INDIVIDUAL TCRs CAN RECOGNIZE LARGE NUMBERS OF PEPTIDES OF A DEFINED LENGTH

It has been appreciated for many years that individual TCRs can recognize multiple ligands (11–18). Initial studies of TCR degeneracy were conducted using small numbers of peptide analogs (typically less than 200). The development of combinatorial peptide library (CPL) screening which was pioneered by Houghten et al. (19, 20) allowed for a more comprehensive analysis of TCR degeneracy and the ability to define much larger numbers of candidate peptide analogs (21). A more recent study examined ~4,000 peptides, although it must be appreciated that this number still only represents an extremely small portion of the entire peptide universe (18).

Estimates of TCR degeneracy were initially performed using data from peptide library screening. Elegant studies which were conducted by two independent groups estimated that individual MHCI-restricted TCRs can recognize $\sim 10^6$ peptides (22, 23). A similar analysis performed by Wilson and colleagues suggested that MHCI-restricted TCRs exhibit lower levels of degeneracy (24). However, approaches performed using small peptide sets or library screening alone preclude a comprehensive analysis of the entire peptide universe and as such, may result in an underestimate of TCR degeneracy. In order to address this possibility, a recent study developed an approach to quantify TCR degeneracy that can be used to probe the entire peptide universe of a defined length.

This approach involves a combination of sampling from motif-restricted peptide sets and sampling from the entire peptide universe with bias toward those peptides predicted to elicit a response (termed CPL-based importance sampling) (25). The approach was used to construct a degeneracy curve which revealed, in the initial application of this strategy, that an individual MHCI-restricted TCR (1E6) can recognize over one million different decamer peptides in the context of a single MHCI at physiologically relevant concentrations. Furthermore, it was possible to identify peptides that were up to 100-fold more potent than the cognate peptide for this particular TCR. To date, this study represents the most comprehensive quantification of MHCI-restricted TCR degeneracy (25). However, continued development of techniques that allow rapid and accurate quantification of TCR degeneracy are essential if we are to understand the role that this phenomenon plays in common T-cell mediated diseases.

The ability of individual TCRs to recognize large numbers of peptides provides an explanation for how a limited TCR repertoire can provide effective immune coverage against all possible foreign pMHCI that can be encountered. However this analysis was confined to a single autoimmune TCR which may represent the extreme end of the cross-reactivity spectrum. In order to understand the system requirements for effective CD8⁺ T-cell immunity, it will be important to measure how this phenomenon varies across the normal TCR repertoire. It is tempting to speculate that variations in levels of TCR degeneracy may contribute to the pathogenesis of disease. In fact there is emerging evidence to suggest that this is indeed the case. For example, several studies suggest that levels of TCR degeneracy can influence disease outcome during infection with viruses such as HIV-1 that have a high mutation rate (26–29). Overall more information is required about how TCR degeneracy varies across the normal TCR repertoire and the role that variations play in the pathogenesis of disease.

TCRs EXHIBIT AN EXPLICIT PREFERENCE FOR MHCI-BOUND PEPTIDE LENGTH

It is clear that TCRs can recognize a large number of peptides of a defined length and it would seem logical that this flexibility could extend to the recognition of peptides of different lengths, as this would maximize the ability of the TCR repertoire to provide broad antigenic coverage. In order to examine this possibility, a recent study utilized a series of combinatorial peptide libraries, ranging from 8 to 13 amino acids in length, to perform a comprehensive analysis of peptide length preference across a panel of CD8⁺ T-cell clones raised against a range of pMHCI antigens spanning different peptide lengths derived from both foreign and self antigens (30). An unexpected feature of the TCR/pMHCI interaction was discovered by showing that any given TCR exhibits an explicit preference for a single MHCI-bound peptide length (30). This MHCI-bound peptide length preference was identical to the length of the original peptide which had been used to derive each clone. Furthermore, this unexpected finding applied to all TCRs examined regardless of antigenic specificity and MHCI restriction. Importantly, peptide length preference was shown to be an intrinsic feature of the TCR and not determined by differential MHCI-peptide binding. A very small number of agonists of non-preferred length were identified, but the incidence of this

phenomenon was extremely rare and recognition was generally sub-optimal when compared to peptides of the preferred length. Interestingly, however, agonists of non-preferred length identified were entirely distinct in sequence, with a different amino acid residue at every position. As described above, CPL-based importance sampling revealed that the 1E6 TCR can recognize over one million different 10mer peptides at a functional sensitivity equivalent to or greater than index (25). In contrast, the 1E6 TCR was not capable of recognizing any 8, 9, 11, 12, or 13mer peptides within this functional sensitivity range. Thus, individual MHCI-restricted TCRs exhibit an explicit preference for a single MHCI-peptide length (30). The next challenge will be to explain how a repertoire of TCRs that display a stringent preference for peptide length can provide effective immune coverage against all peptide lengths that can be presented by MHCI. To address this question, it will be important to examine how peptide length preference varies across the entire TCR repertoire.

IMPLICATIONS OF MHCI-BOUND PEPTIDE LENGTH PREFERENCE FOR CD8⁺ T-CELL IMMUNITY

The TCR/pMHCI interaction is pivotal in all aspects of CD8⁺ T-cell immunity including thymic development, naive T-cell survival, and recognition of foreign pMHCI in the periphery. Peptide length determines the outcome of TCR/pMHCI engagement and, as such, has far-reaching implications (Box 1). Firstly, the findings predict that the thymic epithelial cell (TEC) (31, 32) surface must display peptides of multiple different lengths to ensure that an appropriately diverse CD8⁺ T-cell repertoire undergoes positive and negative selection before being released into the periphery. Once in the periphery, continual TCR engagement of low affinity self-derived pMHCI molecules is essential for naive T-cell survival (33, 34). Here, peptides of non-preferred length may actually play a very important role because signaling below the threshold for T-cell activation may be sufficient for the delivery of survival signals.

Box 1 | Implications of peptide length preference.

A: Implications for CD8⁺ T-cell immunity

- Thymic development: TECs must display peptides of multiple lengths
- Naive T-cell survival: mediated by recognition of defined length peptides
- Effective CD8⁺ T-cell immunity: requires “length-matched” clonotypes
- Protective vaccination: must elicit “length-matched” clonotypes

B: Implications for disease pathogenesis

- Identification of ligands that trigger CD8⁺ T-cell expansions in autoimmunity and hematological disease: length preference must be established
- Rare peptides of non-preferred length peptides with entirely disparate sequence: may represent a novel mechanism underlying molecular mimicry
- Alloreactivity: an important consideration in the identification of alloreactive ligands

Importantly, the findings imply that the CD8⁺ T-cell repertoire is compartmentalized with respect to peptide length preference, such that effective immunity can only be achieved by mobilization of the appropriate “length-matched” antigen-specific CD8⁺ T-cell clonotypes (30). To understand the system requirements for effective CD8⁺ T-cell immunity, it will therefore be necessary to examine how peptide length preference varies across the normal TCR repertoire and how different “peptide length footprints” overlap to provide effective coverage across all peptide lengths. The length preference phenomenon also informs our understanding of the requirements for effective peptide vaccination. For example, it was recently observed that the heteroclitic 10mer epitope ELAGIG-ILTV derived from MART-1/Melan-A, which is commonly used in melanoma clinical trials, primes a population of CD8⁺ T cells that is very poor at cross-recognizing the dominant naturally presented 9mer epitope AAGIGILTV (35, 36). These findings may explain, at least in part, the poor objective response rates observed in these trials (37). Accordingly, such findings suggest that peptide length is likely to be an important consideration in the provision of effective CD8⁺ T-cell immunity and the response to peptide vaccination.

IMPLICATIONS OF MHCI-BOUND PEPTIDE LENGTH PREFERENCE FOR DISEASE PATHOGENESIS

As well as playing a critical role in CD8⁺ T-cell immunity, the TCR/pMHCI interaction can result in inappropriate CD8⁺ T-cell activity in situations such as autoimmunity, hematological disorders, and transplant rejection (alloreactivity). Factors that influence the outcome of TCR/pMHCI engagement, such as peptide length, therefore impact on our understanding of disease pathogenesis. Transient, asymptomatic CD8⁺ T-cell expansions are relatively common and often associated with viral infections. However, in certain disease states, monoclonal/oligoclonal CD8⁺ T-cell expansions with a differentiated phenotype persist, suggesting an exaggerated response to an immunodominant antigen. Monoclonal CD8⁺ T-cell expansions are a characteristic feature of T-LGL leukemia (38), but can also be triggered by drugs [e.g., protein tyrosine kinase inhibitors (39)]. Oligoclonal expansions have been observed in various autoimmune diseases [e.g., multiple sclerosis (40, 41), rheumatoid arthritis (42), and aplastic anemia (43)]. To date, there has been no attempt to identify the origin of the ligands that drive these potentially pathogenic CD8⁺ T-cell populations. This is a priority for the future because it will yield information about the pathogenesis of these important diseases. In order to achieve this goal, the dominant pathogenic T-cell clone must be isolated and the peptide length preference defined. This will narrow the search and allow the selection of length-appropriate peptide libraries to enable ligand hunting.

Alloreactivity represents a major barrier to transplantation and, as such, there is a pressing need to understand the molecular and structural basis of this phenomenon (44, 45). This is especially important considering that substantial efforts are being made to increase the number of available donors despite the fact that, in the last decade, little progress has been made in understanding how to reduce the risks and severity of transplant rejection. It is well established that alloreactive T-cells are highly prevalent within the T-cell repertoire. However, the mechanisms that underlie this phenomenon remain unclear. Originally, it was thought

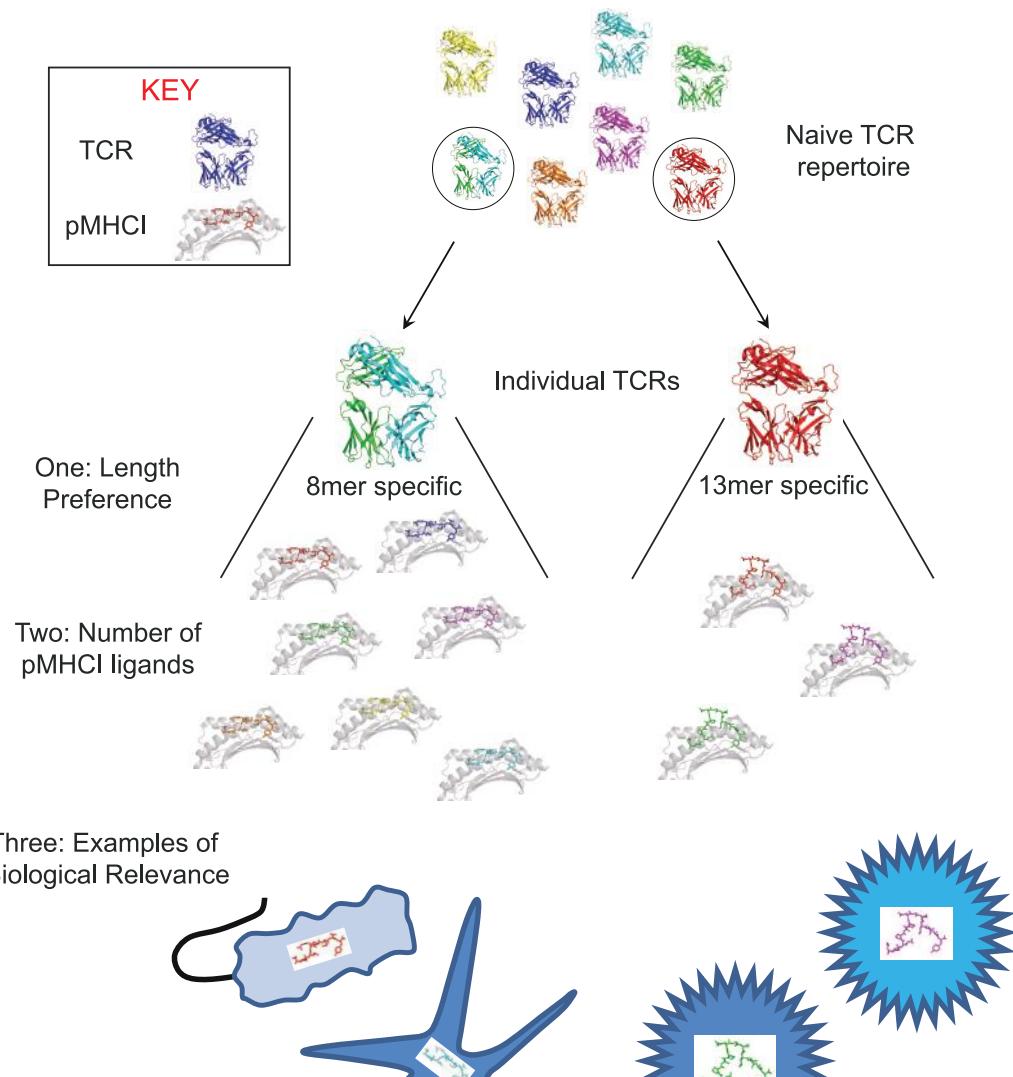
that alloreactivity was mediated by TCR recognition of foreign MHC (“MHC-centric”). More recently, however, it has become increasingly apparent that the alloreactive TCR/pMHCI interaction can be highly peptide-dependent (“peptide-centric”) (46–48). In fact, it appears that peptide specificity is profound when self-MHCI and foreign-MHCI are closely related (49). Further studies are required to determine whether alloreactive TCR/pMHCI interactions exhibit the levels of promiscuity that typify autologous TCR/pMHCI interactions. It will also be important to examine the possibility that alloreactive TCR/pMHCI interactions may exhibit a peptide length preference. This will facilitate the identification of alloreactive ligands and allow characterization of the peptide repertoire recognized in the context of non-self MHCI (30).

INDIVIDUAL MHCI-RESTRICTED TCRs ARE CHARACTERIZED BY A UNIQUE PEPTIDE RECOGNITION SIGNATURE

Taken together, recent observations suggest that every TCR is specific for a unique set of peptides, which can be described as a unique “peptide recognition signature” (PRS) (Figure 1). The PRS of individual TCRs comprises three different components: (i) an explicit preference for a single MHCI-peptide length; (ii) the number of peptide sequences recognized at the preferred length, which can be very large (up to $\sim 10^6$ different peptides); and, (iii) the sequence identity of the recognized peptides, many of which will be biologically relevant. The sequence identity of the peptides recognized by an individual TCR is the most important component of the PRS (Figure 1). Many of the peptide sequences recognized by individual TCRs can appear in a large number of self proteins or pathogen derived proteins. If these sequences can be naturally processed and presented at the cell surface then the TCR will have the opportunity to mount a response to them *in vivo*. If a TCR can respond to naturally processed sequences from more than one pathogen then this will allow a single TCR to provide immunity to more than one infection. This phenomenon [often referred to as “heterologous immunity” (50, 51)] is extremely important for the provision of effective immunity and an example of the immense benefits that can be gained from TCR degeneracy. However, the ability to be able to recognize both self and pathogen derived pMHCI has the potential to be highly pathogenic and provides a mechanistic basis for “molecular mimicry,” which is the widely hypothesized cause of autoimmune disease (52). Although good examples of molecular mimicry exist in mouse models of disease there is still a lack of concrete evidence for this in human autoimmune disease (52). It is very important that this question is answered in the context of human disease. The ability to de-convolute the PRS of TCRs involved in autoimmune disease will be of immense interest in the future.

APPROACHES THAT CAN BE USED TO DE-CONVOLUTE THE PRS OF INDIVIDUAL TCRs

CD8⁺ T cells play a major role in the pathogenesis of autoimmunity, hematological disease, and transplant rejection, as described above. In the future, the ability to de-convolute the PRS of pathogenically relevant TCRs will allow us to dissect the underlying mechanisms of common CD8⁺ T-cell mediated disease. This information can then be used to inform the rational design of



If a single TCR can recognize peptide sequences generated from both pathogens and self tissue such as oligodendrocytes:
AUTOIMMUNE DISEASE

If a single TCR can recognize peptide sequences generated from two distinct viruses:
HETEROLOGOUS IMMUNITY

FIGURE 1 | The peptide recognition signature of every TCR consists of three components. The figure depicts the three components of the peptide recognition signature. **One: peptide length preference:** this finding implies that effective CD8⁺ T-cell immunity can only be achieved if "length-matched" antigen-specific CD8⁺ T-cell clonotypes are mobilized during an immune response, **two: number of peptides recognized at the preferred peptide length:** which can be very large (up to ~10⁹). The ability of individual TCRs to recognize multiple pMHC1 is essential for the provision of effective immune coverage against the multitude of foreign pMHC1 that can be encountered, and, **three: sequence of the recognized peptides (which dictates the biological relevance of the peptide recognition signature):** the ability to be able to recognize both pathogen and self-derived pMHC1 could be the basis for "molecular mimicry," which

is the widely hypothesized cause of autoimmune disease. Evidence for this phenomenon exists in animal models of autoimmunity but convincing data in human disease is still lacking. By way of an example, the figure depicts the ability of the same TCR to recognize peptide sequences generated from both environmental pathogens and oligodendrocytes. Alternatively, if a TCR can respond to naturally processed and presented peptide sequences from more than one pathogen then this will allow a single TCR to provide immunity to more than one infection. It has been suggested that this phenomenon (often referred to as "heterologous immunity") is extremely important for the provision of effective immunity. By way of an example, the figure depicts the ability of the same TCR to recognize naturally processed and presented peptide sequences from two different viruses.

novel therapeutic strategies. As such there is a pressing need to design approaches that can be used to achieve this goal. There have been some elegant attempts to develop an approach that allows the identification of ligands recognized by CD8⁺ T-cell populations of potential pathogenic significance (15, 53, 54). However, an appreciation that every TCR is characterized by a unique PRS will facilitate this analysis. Firstly, it will be necessary to define the peptide length preference of the individual TCR of interest so that appropriate ligand hunting tools can be selected, such as length-matched peptide libraries. Secondly, the number of peptides that can be recognized by the individual TCR may influence the approach that is being used. For example, highly cross-reactive TCRs will be more challenging than those that exhibit a more focused phenotype. And finally, tools to identify the origin of the peptide sequences (i.e., pathogen versus self protein) will be essential. Emphasis should be placed on developing an approach that is rapid and can be scaled up to allow the analysis of large numbers of TCRs in a short space of time. If this can be achieved, then the ability to de-convolute PRSs across the normal and diseased repertoire is expected to yield large but very important databases of information for the future.

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CONCLUDING REMARKS

In summary, based on recently generated data, I suggest that every TCR in the naive repertoire is characterized by a unique PRS; all of which overlap to provide effective immune coverage against all possible foreign-MHCI-bound peptides that could be encountered. A comprehensive study to examine how the PRS of individual TCRs varies across the normal TCR repertoire will allow us to determine how a relatively limited number of TCRs can recognize the multitude of 8–14 amino acid length peptides that can be encountered in complex with MHCI, and thereby define the system requirements for effective CD8⁺ T-cell immunity. In addition, approaches that can be used to de-convolute the PRSs of individual pathogenically relevant TCRs are essential if we are to gain an understanding of the mechanisms that underlie common CD8⁺ T-cell mediated diseases.

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Immune parameters to consider when choosing T-cell receptors for therapy

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T-cell receptor (TCR) therapy has arrived as a realistic treatment option for many human diseases. TCR gene therapy allows for the mass redirection of T-cells against a defined antigen while high affinity TCR engineering allows for the creation of a new class of soluble drugs. However, deciding which TCR blueprint to take forward for gene therapy or engineering is difficult. More than one quintillion TCR combinations can be generated by somatic recombination and we are only now beginning to appreciate that not all are functionally equal. TCRs can exhibit high or low degrees of HLA-restricted cross-reactivity and alloreact against one or a combination of HLA alleles. Identifying TCR candidates with high specificity and minimal cross-reactivity/alloreactivity footprints before engineering is obviously highly desirable. Here we will summarize what we currently know about TCR biology with regard to immunoengineering.

Keywords: T-cell epitope, T-cell receptor, T-cell engineering

BACKGROUND

The $\alpha\beta$ T-cell receptor (TCR) is one of the most variable proteins known to science (1) with the human V(D)J recombination system capable of generating hundreds of trillion of unique $\alpha\beta$ TCR molecules (2). This incredibly vast receptor reserve is our immune systems' core defense against the torrent of hypervariable microorganisms and pathogenic challenges encountered over the course of life. During thymopoiesis, the TCR recombination machinery uses "cut-and-paste" transposition to incise and rearrange 174 variable (TRAV and TRBV), diversity (TRBD), joining (TRAJ and TRBJ), and constant (TRAC and TRBC) TCR gene segments on chromosomes 7 (TRB loci) and 14 (TRA loci) into around seven-and-a-half million distinctive gene combinations (2). This chromosomal recombination process generates only around 10% of total TCR diversity with the remaining 90% of diversity generated through further exonuclease activity and the addition of random, non-template-dependent nucleotides (N-nucleotides) across the V(D)J junction by the enzyme terminal deoxynucleotidyl transferase (TdT) (3). The collective sum of this recombination event is a theoretical 10^{15} – 10^{20} structurally unique $\alpha\beta$ TCR molecules (1, 4, 5). Due to size constraints (2), the human immune system only houses an infinitely small slice of the full repertoire. In an adult human, this equates to 10^{12} T-cells (6, 7) bearing around 2.5×10^6 unique $\alpha\beta$ TCR structures (6), with the upper bounds comprising 10^8 – 10^{11} unique $\alpha\beta$ TCR structures per individual (6, 7).

The $\alpha\beta$ TCR is a glycosylated, membrane-integral surface protein comprising one α -chain and one β -chain (2). The two chains fold and fuse via cysteine–cysteine disulfide linkers to produce a single, functional heterodimeric receptor (8, 9). The outward facing and solvent-exposed edge of the heterodimer bears six highly flexible complementarity determining region (CDR) loops. The CDR1 and CDR2 loops are encoded by the germline TRAV and

TRBV genes and generally function to fix the TCR to the major histocompatibility complex (MHC) platform. Conversely, the CDR3 loops are encoded by the somatically hypervariable V(D)J junction and classically function to engage the peptide (p) cradled in the MHC groove (8, 9), although variations on CDR binding geometry have been noted (9).

A TCR engages its cognate pMHC as a single, composite ligand, and docks in an approximately diagonal fashion that slightly varies in pivot and tilt from complex to complex (9, 10). One steady constant of TCR/pMHC engagement is that the CDR3 α loop is positioned toward the direction of the peptide N-terminus and the CDR3 β loop is positioned toward the direction of the peptide C-terminus and variation in this geometry has not been seen to date (9, 10), however extreme terminal focusing has been recently observed (11). The TCR/pMHC docking process can be very fluid and conformational changes to the TCR, peptide, and MHC have all been observed suggesting that both interfaces often adjust to each other to find a compatible binding solution (9). Biophysical data show that TCR binding is stratified based on function. TCRs that engage pMHC class-I (pMHC-I) targets bind strongly with a mean affinity three times stronger than TCRs that engage pMHC class-II (pMHC-II) targets (8). Likewise, TCRs are further stratified based on whether the antigen target is of self or foreign origin, with foreign-reactive TCRs binding cognate pMHC with a mean affinity 10 times stronger than TCRs that bind self pMHC (8).

In spite of the large number of TCR receptor "options" available in the naive repertoire, T-cell repertoires deployed against pMHC antigens often exhibit ordered and predictable TCR gene architecture [reviewed (2, 12)]. This phenomenon, termed TCR bias, can result in residue-identical memory clonotypes being found across multiple individuals sharing a common MHC allele. The mechanisms behind the appearance of these "public" T-cell

responses are still being determined (2) but is thought to involve both biases in recombination during thymopoiesis (13) and some optimal, structural-based, filtering event during antigen-driven selection (14–20). For this filtering event, it appears the peptide is the determining factor during repertoire formation (21) with TCR repertoire assembly not dependent on antigen source, presenting MHC allele or immunodominance hierarchy. Once a memory T-cell repertoire is established, it appears to remain relatively consistent both in terms of clonotype stability and clonotype frequency over decades of life (22, 23).

Adoptive cell therapy (ACT) using antigen-specific T-cells has proven to be a remarkably effective experimental treatment option for Epstein–Barr virus malignancies (24), cytomegalovirus infection (25), and melanoma (26). Given these promising results, many groups have turned toward TCR gene transfer as a simpler, faster, and more homogeneous technique for generating ACTs. Here, antigen-specific $\alpha\beta$ TCR genes are delivered into recipient T-cells using a γ -retroviral vector, lentiviral vector, or transposon [reviewed (26)]. Another parallel approach for TCR therapy is to engineer high affinity mutants from natural $\alpha\beta$ TCR “blueprints” using yeast display (27) or phage display (28). These TCR mutants can have their binding affinities amplified logarithmically to the low pM K_D range (28) while still retaining high specificity for antigen (28, 29). Affinity enhanced TCR can be used in two ways. First the mutants can be gene transferred into T-cells to increase antigen sensitivity and polyfunctionality (30). Second, the mutants can be used in soluble form to deliver therapeutic payloads to cells bearing the appropriate pMHC targets (31). Importantly, before considering a receptor for therapeutic ends, a number of parameters should be considered regarding the genetics and biology of the human TCR.

CONSIDERATION ONE: CROSS-REACTIVITY

The first parameter to considering when applying TCR therapy is the cross-reactivity profile of the candidate receptor. A theoretical proposal (32) predicted that the $\alpha\beta$ TCR must intrinsically encode a high degree of cross-reactivity in order to provide sufficient coverage against the huge constellation of pMHC complexes that could be encountered in nature. Through the use of combinatorial peptide libraries (CPLs), that comprise almost all possible peptides of a particular length, this theory was recently tested experimentally and proven (33, 34). In the context of a single MHC, a single $\alpha\beta$ TCR can recognize over one million different peptides as well or better than its cognate ligand (34). Whether this is the case for *all* TCRs is under active investigation. Very recent CPL data suggests that TCRs have sliding cross-reactive intensities (35) and, at least for pMHC-I-specific TCRs, an explicit preference for peptides of defined length. Thus, cross-reactivity for peptides outside a TCRs “programmed length preference” is unlikely.

Given the intrinsic cross-reactivity of TCRs, it is tempting to select for TCR that engage multiple target pMHC. Indeed, this “multiple birds with one stone” approach could dramatically boost therapeutic efficacy of a candidate TCR *in vivo*. However, caution is advised in this pursuit as it has recently been shown that multi-pMHC specific TCR can result in serious side effects (36). Here, a therapeutic TCR that recognized multiple MAGE-derived peptides resulted in neurological toxicity when administered to melanoma patients as TCR gene therapy. Off-target toxicity was thought to

be due to one of the MAGE peptides being expressed in the brain. This localized expression profile was not previously known.

Another parameter to consider (on top of the large numbers of proteogenic peptides T-cells can recognize) is the issue of “transformed self.” It is known that $\alpha\beta$ T-cells can engage proteogenic peptides containing post-translational modifications, such as phosphorylation (37), glycosylation (38), citrullination (39), and dimerization (40). Whether a given $\alpha\beta$ TCR also cross-recognizes large numbers of modified peptides is yet to be determined. In addition to classical pMHC-I and pMHC-II targets, $\alpha\beta$ TCR are also now known to bind a growing list of classical and non-classical MHC molecules which cradle an extraordinary diverse array of organic and inorganic compounds (41). TCR ligands can include proteogenic peptides in HLA-E (42), lipids in the cluster of differentiation 1 (CD1) molecule (41, 43), vitamin metabolites in MHC-I related (MR1) molecules (44), small molecule drugs in MHC-I (45), and the empty platform of human hemochromatosis protein (HFE) (46).

Precisely mapping the complete cross-reactive profile of a therapeutic TCR candidate across the thousands of classical and non-classical MHC alleles which present a combined universe of organic and inorganic compounds is currently possible but difficult. Basic approaches are available for assessment (Table 1). For instance, scanning a group of candidate TCRs across a CPL library can quickly rule out receptors with extensive pMHC cross-reactivity footprints. From these select receptors, blasting the raw CPL data across the human proteome may identify self peptides which could drive off-target activity *in vivo*. Candidate TCR with minimal cross-reactivity footprints as suggested by CPL scanning could then advance to *in vitro* testing on multi-cell subsets. Here, various cell types (monocytes, DCs, T-cells, B-cells, fibroblasts, epithelial cells, etc.) that express the HLA restriction allele of interest could be used as target cells to determine potential TCR cross-reactivity with self pMHC molecules. Target cells could be derived from primary sorted cells and/or cell lines.

CONSIDERATION TWO: ALLOREACTIVITY

As mentioned above, a significant degree of degeneracy in peptide recognition likely evolved to ensure that the TCR repertoire has the capacity to recognize the enormous variety of foreign peptides that are encountered throughout life. Furthermore, broadly reactive T-cells may aid primary and memory responses where memory T-cells for one pathogen are reactivated by a different infectious agent (47). However, limited specificity of self-MHC-restricted T-cells is also the basis of the alloresponse and its associated clinical problems.

T-cell allorecognition occurs when the immune system is presented with MHC molecules of a different allotype to that of the host. Alloreactivity becomes clinically significant in the case of solid-organ grafts or bone marrow transplants in which mismatched MHC molecules can potentially result in organ graft rejection or graft versus-host disease (GVHD). This response can be either direct, in which the T-cells mount an immune response to the foreign-pMHC, or indirect, a chronic self-MHC restricted response resulting from polymorphism in the processed antigen that can include peptides from allogeneic MHC molecules (48). It is estimated that up to 0.1–1% of T-cells are alloreactive toward a given allogeneic MHC molecule (49). However, the probability of

Table 1 | Pre-clinical testing options for therapeutic TCR candidates.

Parameter to consider when choosing a candidate TCR for therapy	Testing option/s
Could the candidate TCR cross-react with a peptide presented by an autologous classical and non-classical MHC molecule?	Scan the candidate TCR across different primary cell subset targets (monocytes, DCs, B-cells, T-cells) sorted from prospective patients. Scan the candidate TCR across PBMC and cell lines (monocytes, DCs, B-cells, T-cells, fibroblast, epithelial) from a library of HLA allele matched healthy donors. Scan the candidate TCR across peptide length-matched CPL to establish a metric of cross-reactivity potential.
Could the candidate TCR alloreact with a peptide presented by a mismatched MHC molecule?	Scan the candidate TCR across an extensive, fully HLA haplotyped cell line library. The cell line library should contain HLA alleles found at high frequency in the target population.
Are the germline sequences for the candidate TCR donor/patient matched?	Compare the TRAV, TRAJ, TRBV, and TRBJ sequences of the candidate TCR with patient TR loci. Polymorphisms in these genes may alter the effectiveness of the therapeutic TCR <i>in vivo</i> . Additionally, if the donor/patient TR alleles do not match, or if the patient has a key TR allele deleted, there is a possibility that a patient-derived immune response could be mobilized against the “foreign” TCR.
Could the candidate TCR steer functional phenotype of recipient T-cells when used in gene therapy?	Transduce the candidate TCR in naive T-cells <i>in vitro</i> or into mice with human immune system components. Prime the cultures with differing concentrations of cognate Ag and monitor cell fate decisions. Note temporal and final ratios in effector, memory and Tfh differentiation. Transduce the candidate TCR in memory T-cells <i>in vitro</i> . Prime the cultures with differing concentrations of cognate Ag and monitor if cell fate is altered when compared to phenotype pre-transduction.

a TCR reacting with any allogeneic MHC molecules is obviously much higher due to MHC polymorphism, and this is a potential problem for TCR therapy.

There are numerous reports of T-cell clones with dual specificity for an allo-MHC molecule and a nominal antigen complexed with self-MHC (50). The best characterized example is the response to the Epstein–Barr virus epitope FLRGRAYGL, that binds to HLA-B8, in which CTL clones were isolated that cross-reacted with one of three common alloantigens (HLA-B44, B14, or B35) (51, 52). Interestingly, the HLA-B44 alloreactive TCR [which has also been shown to alloreact with HLA-B*5501 (53)] is a public TCR that dominates the response to this viral epitope in most HLA-B8⁺ people (54, 55). By examining the response to this viral epitope in individuals who co-expressed HLA-B8 and one of the alloantigen targets, subdominant TCRs were identified that were not alloreactive (55, 56). Such TCRs would be the obvious choice for use in TCR therapy, and this approach could be used in other systems to identify non-alloreactive TCRs for therapeutic use where the dominant receptors are alloreactive.

Many other T-cell clones have been shown to cross-react with alloantigens, and work from Frans Claas's group has shown that up to 45% of virus-reactive T-cell clones from humans are alloreactive (50). Allo-HLA cross-reactivity was shown from T-cell clones raised against a range of viruses including cytomegalovirus, varicella-zoster virus, and influenza (50). These included both CD8⁺ and CD4⁺ clones alloreacting with MHC-I and MHC-II molecules, respectively, and surprisingly, they also included two distinct cytomegalovirus-reactive, MHC-I-restricted T-cell clones that recognized allogeneic MHC-II molecules (57).

The obvious way to manage the problem of T-cell alloreactivity in the context of TCR therapy is to perform preliminary *in vitro* screens of the TCR for cross-recognition of cell lines expressing a wide range of allo-HLA alleles. The limitation here is that it will be near impossible to screen against the huge variety of HLA molecules, given there are over 6,000 known class-I alleles and over 1,000 class-II alleles. Furthermore, alloreactive T-cells are generally also specific for one or more “self”-peptides presented by the allo-HLA molecule, and these may not be presented by cells from all tissues, or they could be derived from polymorphic gene products and are therefore not presented by all individuals or cell lines. For example, the EBV-reactive TCR described above is specific for a “self”-peptide derived from an ATP binding cassette protein ABCD3 which is presented by allo-HLA-B44 and shares only one residue with the viral peptide (58). This peptide appears to be presented at different levels in distinct tissues based on the recent observation that these T-cells recognize HLA-B44⁺ lymphoid cells but not epithelial and endothelial cells (59).

Although T-cell cross-reactivity with alloantigens has not proven to be a major problem in adoptive T-cell transfer clinical trials, it is an issue that should not be ignored in future trials of TCR therapy. Testing for cross-reactivity with one or more alloantigens is currently possible *in vitro* through target cell screening across large allo cell libraries (50, 53).

CONSIDERATION THREE: POLYMORPHISM

As with vaccines that elicit T-cell responses against a limited number of epitopes, TCR-based therapeutic approaches need to address the important issue of polymorphism in the genes involved in antigen presentation and those encoding for the target peptide

antigens. Viral antigens are particularly prone to accumulating escape mutations, and so TCRs that recognize regions of viral proteins that are critical for viral fitness and are therefore highly conserved (60) should be favored for TCR therapy. Genetic instability is also a common feature of cancer cells, often resulting in the selection of antigenic variants by T-cells which allow cancer cells to escape destruction (61). The simultaneous administration of multiple TCRs that target different epitopes should circumvent these problems to some extent. Another potential mechanism through which human genetic polymorphism could create problems is if a TCR, transferred into an unrelated recipient, cross-reacts with a polymorphic self-peptide which it had not encountered during thymic negative selection, leading to damage of healthy tissue.

HLA polymorphism is also a major consideration that restricts the potential value of individual TCRs to a limited subset of any given population. As mentioned above, a huge number of HLA alleles have now been identified and therefore TCR therapy will need to be personalized to ensure recognition of epitopes presented by relevant HLA alleles. TCRs that recognize antigenic peptides that are presented by multiple HLA alleles are also valuable candidates for TCR therapy. A degree of degeneracy in HLA-peptide binding has been demonstrated whereby multiple class-I alleles can share common sequence motifs due to homology of amino acids within the major pockets of the peptide binding cleft, and these groups of alleles are referred to as HLA supertypes. Based on HLA structural similarities and overlapping peptide binding motifs, nine major HLA supertypes have been proposed (62). Examples of TCRs that have the capacity to recognize individual peptides bound to multiple members of an HLA supertype have been described (63–66). These TCRs with promiscuous HLA restriction can often accommodate differences in the exposed HLA α -helix residues between the restricting MHC and foreign MHC antigens that present the same peptide.

As with the MHC genes, allelic sequence variation is also a feature of the TCR and this issue needs to be addressed in the context of TCR therapy. Several sequencing studies have revealed considerable polymorphism within the TRAV and TRBV gene segments (67, 68). In one study, the TCR loci from 40 individuals across four ethnic groups were fully sequenced, and more than 550 SNPs were found, with many being situated in coding/regulatory regions of functional TCR genes and several causing null and non-functional mutations. On average, the coding region of each TCR variable gene contained two SNPs, with many more found in the 5', 3' and intronic sequences of these segments. A total of 51 SNPs in the TRA locus and 72 SNPs in the TRB locus were found to result in amino acid changes (67, 68).

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The extensive variability within the TCR gene segments raises the interesting possibility that, unless the TCR genetics are matched between donor and recipient, some TCR gene products will be seen as foreign antigens and could elicit an immune response that limits the efficacy of transferred TCRs. Particularly strong immune responses could be expected in patients with deletions or inactivating polymorphisms that prevent expression of certain TRBV genes. There are seven frequently occurring inactivating polymorphisms in functional TRBV gene segments and a large (21.5 kb) insertion/deletion related polymorphism in the TRB locus encompassing two V gene segments (67–70). In the latter case, two functional variable β genes, *TRBV6-2/TRBV6-3* and *TRBV4-3*, are frequently deleted in all major ethnic groups (68, 70, 71). TCRs that are encoded by V genes that include common polymorphisms could perhaps be avoided for use in TCR therapy.

CONSIDERATION FOUR: FUNCTIONAL PHENOTYPE

Recent evidence suggests that different TCRs expressed by T-cell clones of the same pMHC specificity can have different effects on immune phenotype (72). When challenged with pathogen, clonotypically distinct naive T-cells were observed to give rise to differing ratios of Th1 and Thf progeny. These alternate differentiation programs were dependent on pMHC dwell time and/or Ag density. Interestingly, this data suggests that different TCR clonotypes of the same pMHC specificity may impart differential effects on total immune function through varying effects on macrophage activity and antibody secretion by B-cells. An additional complexity in this area is the observation that after priming, a single naive T-cell can have multiple fates when proceeding down the cell differentiation pathway (73, 74). Thus, determining exactly which differentiation program a candidate TCR induces is an important parameter when considering a receptor for therapeutic use.

CONCLUDING REMARK

The TCR is an extremely effective tool for targeting biological and non-biological molecules and vast opportunity exists to exploit these receptors therapeutically. However, TCRs are highly polymorphic by nature and intrinsically encode a considerable degree of differential functionality and cross-reactivity across a number of MHC and MHC-like molecules. These factors require that therapeutic TCR candidates are donor/patient matched and undergo the most comprehensive *in vitro* cross-reactivity testing we can perform with present technology. The goal of this testing should be the identification of receptor candidates with predictable cell differentiation “programs” and minimal and traceable cross-reactivity/alloreactivity footprints.

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Advances in T-cell epitope engineering

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INTRODUCTION

T-cells recognize small peptide fragments (p) cradled in multiple major histocompatibility complex (MHC) molecules, termed *human leukocyte antigens (HLA)* in humans. These membrane-integral pMHC molecules are present on surface of all nucleated cells and allow T-cells to detect aberrant intracellular activity, be this infection with micro-organisms or abnormal host biochemistry such as neoplastic division. Scanning of pMHC molecules occurs via the $\alpha\beta$ T-cell receptor (TCR), a clonotypic, heterodimeric, and membrane-integral molecule on the T-cell surface (Miles et al., 2011a). TCRs engage pMHC molecules via six highly flexible complementarity determining region (CDR) loops and, upon productive docking with a pMHC molecule, the TCR triggers a myriad of intracellular T-cell signaling cascades (Bridgeman et al., 2012). The binding strength (or affinity) between a TCR and a cognate pMHC is relatively weak across known biological systems with monomeric “dwell times” (or half-lives) typically measured in seconds or microseconds at physiological temperatures (Miles et al., 2010; Bridgeman et al., 2012; Smith et al., 2012). This is in contrast to numerous other biological interactions such as antibodies (van der Merwe and Davis, 2003), interleukins (Morton et al., 1994), lipoproteins (Misra et al., 2001), and structural membrane proteins (Matte et al., 2012) which have half-lives measured in hours-to-days. Overall, TCR/pMHC interactions are fleeting even by the dynamic standards of cell surface interactions (van der Merwe and Davis, 2003). The evolutionary rationale for this striking functional divide can only be speculated upon but likely pertains to the primary function of T-cells. T-cells must scan large numbers of pMHC on multiple cells in series in order to identify and eliminate threats quickly. Effective immunity requires that TCR scanning time must be minimal

and antigen coverage maximal. Theoretical arguments dictate that maximal immune cover of possible foreign pMHC requires each TCR to recognize huge numbers of different peptides (Mason, 1998; Sewell, 2012). This theory is now supported up by direct experimental evidence that shows a single TCR can cross-recognize millions of pMHC molecules as well or better than the native antigen (Sewell, 2012; Wooldridge et al., 2012; Ekeruche-Makinde et al., 2013). Curiously, this extensive T-cell cross-reactivity is strictly compartmentalized based on peptide length (Ekeruche-Makinde et al., 2013).

An interesting consequence of the low antigen affinity and high antigen cross-reactivity characteristics of TCRs is that many, and perhaps all, cognate antigens could potentially be improved upon. Through rational structural modifications of native blueprint antigens we now know it is possible to engineer “optimal fit antigens” which exhibit logarithmic increases in affinity and immunogenicity. Compared with the native antigens, if these optimal antigens prove more effective at stimulating antigen-specific T-cell populations during experimental priming then the compounds may fundamentally redefine how we think about vaccine design.

IMPROVING T-CELL EPITOPE

T-cell epitopes can be optimized by: (i) improving antigen affinity for MHC; (ii) improving antigen affinity for TCR; and (iii) improving antigen pharmacology through synthetic biology. Enhancing the stability of the epitope within the MHC cleft is the simplest engineering strategy as MHC anchoring preferences have been determined from MS elution data (Rammensee et al., 1995) resulting in MHC-binding databases and MHC-binding prediction algorithms (Rammensee et al., 1999; Wang et al., 2011). In theory, the replacement of buried

suboptimal anchor residues with optimal residues for MHC-binding will produce a more stable pMHC complex and improved recognition of peptide. Recent studies have shown that things are not straightforward and this simple strategy for antigen improvement requires careful evaluation. We now know that anchor residue-modified peptides can have minimal or no improvement on stability in the MHC cleft (Miles et al., 2011b). Additionally, previous work has shown that MHC-binding strength shows little correlation with immunogenicity (Assarsson et al., 2007). We have recently shown that anchor residue-modified peptides can substantially alter TCR binding in ways that are difficult to predict and thereby prime T-cells with altered TCR repertoires (Ekeruche-Makinde et al., 2012). These repertoire effects have clinical relevance as it was found that vaccination with anchor residue-modified peptides was less effective than vaccination with natural peptides at priming tumor-specific T-cells in patients (Speiser et al., 2008).

T-cell epitopes can also be improved by optimizing contact interface between the peptide and TCR. In its simplest form this can be achieved by scanning recognition of a monosubstituted analog library (MAL) (Burrows et al., 1992, 1995; Zaremba et al., 1997; Tangri et al., 2001; Kjer-Nielsen et al., 2003; Burrows, 2004; Bulek et al., 2012). MALs substitute one of all available proteogenic amino acids across each position of a peptide backbone. While this approach can rapidly identify optimal antigens it is expensive as it requires the manufacture a unique defined analog library for each epitope examined. Combinatorial peptide libraries (CPLs) provide more flexible approach for the identification of optimal ligands (Borras et al., 2002). These very large, mixture-based compound libraries are synthesized in positional scanning format so that just one position along the peptide

backbone is a fixed amino acid and all other positions are degenerate, with degenerate positions containing any one of 19 proteogenic amino acids (cysteine is excluded to reduce disulfide bond formation within the compound mixtures). Scanning a CPL across a T-cell clone can quantitatively map which residue/s are preferred by the TCR along a peptide backbone even if true antigen specificity of the clone is unknown. Replacing native residues with preferred residues identified by CPL can significantly increase epitope affinity and immunogenicity (see table below). The advantage of CPL scanning is that the same compound library can be used for any T-cell from any system although it is important to use a library of correct peptide length (Ekeruche-Makinde et al., 2012).

An alternative strategy for T-cell epitope optimization focuses on improving compound pharmacology by artifi-

cially engineering a compound to mimic a peptide (a peptidomimetic). The motive behind this strategy is that proteogenic amino acid-based polypeptides are susceptible to acid degradation and rapid proteolytic cleavage and have half-lives of less than 5 min in the presence of proteases (Guichard et al., 1994) or human serum (Stemmer et al., 1999). Replacing proteogenic amino acids with synthetic subunits generates resilience to proteases and has potential to vastly improve compound shelf life and *in vivo* bioavailability during prophylactic and therapeutic applications. Many synthetic subunits can be substituted for proteogenic amino acids to impede proteolysis. These include D-amino acids (Bartnes et al., 1997), β -amino acids (Webb et al., 2005), psi-bonded amino acids (Stemmer et al., 1999), and the shifting of the R group by one atom to create poly-*N*-substituted

glycines (peptoids) where the side chains are appended to the nitrogen atom of the peptide backbone (Gocke et al., 2009).

WHY OPTIMIZE T-CELL EPITOPES?

We know that the *number* of antigen-specific T-cells within the host has fundamental relevance for disease control. For example the number of virus-specific T-cells generated during primary infection shows an inverse correlation with viral load (Ogg et al., 1998; Bharadwaj et al., 2001) and, in multiple cancer vaccine trials, the number of tumor-specific T-cells circulating within a patient and at tumor site/s correlates with clinical response (Lonchay et al., 2004; Rosenberg et al., 2004). While T-cell numbers alone do not determine disease outcome *per se*, global numbers are central in tipping control toward the host. With this in mind, engineering potent new compounds aimed at amplifying defined subsections of cellular

Table 1 | Examples of T-cell epitope optimization.

Species	Disease	Model Ag	Epitope	MHC	Modification	Functional improvement	Reference
Human	EBV	EBNA 3A	FLRGRAYGL	B*0801	MAL-directed substitution	100 fold increase in sensitivity	Burrows et al. (1992)
Human	CMV	pp65	NLVPVMVATV	A*0201	CPL-directed substitution	1,000 to 10,000-fold increase in sensitivity	La Rosa et al. (2001)
Mouse	N/A	Ovalbumin	SIINFEKL	H-2Kb	β -amino acid insertion	500% more stable during serum digestion	Webb et al. (2005)
Mouse	LCMV	glycoprotein	KAVYNFATM	H-2Db	Psi bond insertion	20-fold more stable during protease digestion	Stemmer et al. (1999)
Mouse	keratitis	IgG2a	YFMYSKLRVQKSC	I-Ad	D-amino acid <i>retro-inverso</i>	As active <i>in vivo</i> as the proteogenic peptide	Mézière et al. (1997)
Human	cancer	MAGE/CEA	various	A*0201	MAL-directed substitution	Up to 10,000-fold increase in sensitivity	Tangri et al. (2001)
Human	cancer	gp100	various	A*0201	MHC-anchor substitution	More numbers of T-cells recovered after <i>in vitro</i> prime	Parkhurst et al. (1996)
Human	cancer	PSA	VISNDVCAQV	A*0201	MAL-directed substitution	Better able to induce T-cell activation	Terasawa et al. (2002)
Human	cancer	CEA	YLSGANLNL	A*0201	MAL-directed substitution	1,000-fold increase in sensitivity	Zaremba et al. (1997)
Human	HIV	Gag	TLNAWVKVV	A*0201	CPL-directed substitution	130% increase of T-cells recovered after <i>in vitro</i> prime	Blondelle et al. (2008)
Human	cancer	survivin	ELMLGEFLKL	A*0201	MHC-anchor substitution	Induces stronger T-cell responses in 30% of donors	Bernatchez et al. (2011)
Human	cancer	Melan A	AAGIGILTV	A*0201	CPL-directed substitution	500% increase in TCR/pMHC-binding affinity	Ekeruche-Makinde et al. (2012)
Human	diabetes	preproinsulin	ALVGDPAAA	A*0201	CPL-directed substitution	100 to 1,000-fold increase in sensitivity	Ekeruche-Makinde et al. (2013)

MAL, monosubstituted analog library; CPL, combinatorial peptide library.

immunity is the first step in manipulating the T-cell compartment for the purposes of rational vaccine design and therapeutic intervention. Such endeavors are particularly important for cancer (self) antigens which generally exhibit very low immunogenicity (Chatten and Bathe, 2010).

EXAMPLES OF T-CELL EPITOPE OPTIMIZATION

Table 1 presents examples of MHC-anchor based (Parkhurst et al., 1996; Bernatchez et al., 2011), MAL-based (Burrows et al., 1992; Zaremba et al., 1997; Tangri et al., 2001; Terasawa et al., 2002), CPL-based (La Rosa et al., 2001; Blondelle et al., 2008; Ekeruche-Makinde et al., 2012; Wooldridge et al., 2012), and synthetic ligand-based (Mézière et al., 1997; Stemmer et al., 1999; Webb et al., 2005) T-cell epitope optimization across mice and humans. Collectively, this work demonstrates that T-cell epitopes can be readily optimized across both foreign and self peptide “blueprints” to increase sensitivity to T-cells and/or increase compound stability. Both MAL-based and CPL-based platforms appear equally effective at generating optimized ligands and have been used to increase antigen sensitivity between 100- and 10,000-fold relative to the native epitope blueprint. It is of particular interest to note that it is not just self self-derived epitopes that can have their immunogenicities amplified logarithmically. One CPL study (La Rosa et al., 2001) generated optimal ligands based on the HLA-A*0201-binding epitope NLVPMVATV (A2-NLV) blueprint from human cytomegalovirus (HCMV) which were 10,000-fold more active. This achievement is striking given that the native A2-NLV epitope is one of the most immunogenic across the HCMV proteome (Khan et al., 2004). Thus, even very good T-cell antigens can be improved upon by modification in amino acid sequence. There is now accumulating evidence demonstrating that T-cells recognize a variety of molecular shapes presented by self MHC molecules (Bridgeman et al., 2012). These molecular shapes need not be made from L-amino acids as demonstrated by recognition of peptidomimetics. To date, such compounds have been made by rational design and have failed to exceed the potency of optimal peptide ligands. It is possible that the screening of combinatorial chemistries so that T-cells can select their recognition

preference may overcome this limitation. While these synthetic compounds are unable, at this time, to exceed the sensitivity of the model epitope during competitive titrations, they are considerably more resistant to digestion from proteases and serum and demonstrate remarkable biostability.

WHERE NEXT?

It is clear that optimal T-cell epitopes can be generated with relative ease but the big question of whether they really work as therapeutic and prophylactic vaccines remains unanswered. Future studies should determine whether optimal T-cell epitopes can outperform native ligands during *in vitro* priming from healthy and patient samples. We also need to determine what effects priming with optimal T-cell epitopes has on the responding T-cell repertoire, both in terms of polyfunctionality and TCR usage. Studies that directly test whether optimal T-cell epitopes can outperform native ligands during *in vivo* priming of naïve animals are required. Importantly, these responses must be tested in relevant *in vivo* models of infection. It is hoped that optimal epitopes can be found that amplify greater numbers of T-cells than the native ligand alone and that T-cells maintain the desired functional profiles required for effective immunity. The development of effective synthetic T-cell epitopes will be particularly exciting as these epitopes have many attractive characteristics. First, they can be engineered to show absolute resistance to temperature fluctuations and environmental degradation, effectively nullifying the need for cold chain storage and transport. Indeed, the cold chain transport of vaccines costs WHO–UNICEF around USD 11.5 billion every year (Wolfson et al., 2008). Second, the acid- and protease-resistant properties of synthetic compounds raise the possibility of orally active administration. Here, the needle-free nature of compound release would considerably decrease logistical problems with vaccine service delivery, significantly decrease systems costs and is likely to increase vaccine acceptance rates. T-cell sensitivity toward the first generation of “designed” synthetic epitopes has been slightly weaker when compared with proteogenic epitopes. However, we believe that there is ample scope to improve upon proteogenic epitopes by screening the right sorts of combinatorial chemistry. Our recent preliminary data with such a

synthetic epitope shows that it can produce strong responses when given orally and that the T-cells induced against this compound protect humanized mice against a lethal challenge with influenza. Further studies are required in order to see whether such an approach can be extended to other T-cell epitopes.

In summary, optimization of T-cell epitopes can be achieved using a number of different techniques. Whether the resultant compounds can be used as effective prophylactic or therapeutic vaccines in humans remains to be determined. However, such approaches may allow the precise targeting of the most effective T-cell clonotypes (Ekeruche-Makinde et al., 2012) *in vivo* and could have the potential to change the way we build vaccines and immunotherapies in the future.

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Re-directing CD4⁺ T cell responses with the flanking residues of MHC class II-bound peptides: the core is not enough

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Recombinant $\alpha\beta$ T cell receptors, expressed on T cell membranes, recognize short peptides presented at the cell surface in complex with MHC molecules. There are two main subsets of $\alpha\beta$ T cells: CD8⁺ T cells that recognize mainly cytosol-derived peptides in the context of MHC class I (pMHC-I), and CD4⁺ T cells that recognize peptides usually derived from exogenous proteins presented by MHC class II (pMHC-II). Unlike the more uniform peptide lengths (usually 8–13mers) bound in the MHC-I closed groove, MHC-II presented peptides are of a highly variable length. The bound peptides consist of a core bound 9mer (reflecting the binding motif for the particular MHC-II type) but with variable peptide flanking residues (PFRs) that can extend from both the N- and C-terminus of the MHC-II binding groove. Although pMHC-I and pMHC-II play a virtually identical role during T cell responses (T cell antigen presentation) and are very similar in overall conformation, there exist a number of subtle but important differences that may govern the functional dichotomy observed between CD8⁺ and CD4⁺ T cells. Here, we provide an overview of the impact of structural differences between pMHC-I and pMHC-II and the molecular interactions with the T cell receptor including the functional importance of MHC-II PFRs. We consider how factors such as anatomical location, inflammatory milieu, and particular types of antigen presenting cell might, in theory, contribute to the quantitative (i.e., pMHC ligand frequency) as well as qualitative (i.e., variable PFR) nature of peptide epitopes, and hence offer a means of control and influence of a CD4⁺ T cell response. Lastly, we review our recent findings showing how modifications to MHC-II PFRs can modify CD4⁺ T cell antigen recognition. These findings may have novel applications for the development of CD4⁺ T cell peptide vaccines and diagnostics.

Keywords: modified peptide, peptide flanking residue, peptide-major histocompatibility complex class II, T cell receptor, T cell repertoire, vaccine, crystal structure, MHC processing

INTRODUCTION

T cell immunity is mediated primarily by the membrane bound T cell receptor (TCR) that interacts with peptide epitopes presented by major histocompatibility molecules (pMHC) (1). This interaction governs T cell specificity and leads to downstream T cell activation. Classical MHC exists in two forms: MHC class I (MHC-I) and MHC class II (MHC-II), which differ in both their subunit composition and functional expression pattern. MHC-I presents peptides derived mainly from endogenous cytosolic proteins and is expressed upon the cell surface of most nucleated cells allowing cognate CD8⁺ T cells to scan cells for intracellular infections or abnormal proteins in cancerous cells (2, 3). In contrast, MHC-II is expressed mainly upon antigen presenting cells (APCs) e.g., dendritic cells and macrophages, that patrol the extracellular space, actively endocytosing potentially immunogenic proteins that are proteolysed and complexed with MHC-II (pMHC-II). Activated APCs enter the lymphatic system and travel to secondary lymphoid nodes allowing naive CD4⁺ T cells to interrogate cell surface expressed pMHC-II enabling CD4⁺ T cell activation and initiation of immune responses (3–5).

PEPTIDES PRESENTED BY MHC-I AND MHC-II HAVE DISTINCT STRUCTURAL CHARACTERISTICS

In spite of the differing subunit compositions of the two MHC classes, they are structurally very similar (Figures 1A,B). The peptide binding groove, in both cases, is comprised of two anti-parallel α -helices that form a channel in which the peptide can bind in an extended conformation, and eight anti-parallel β -sheets that provide specific peptide binding pockets in the base of the groove (Figures 1C,D) (3, 6). Peptides are selected according to their ability to bind to these MHC allele specific pockets within the floor of the peptide binding groove using peptide anchor residues. All of the currently available structural data suggest the TCRs bind to both pMHC-I and II with a fixed polarity (TCR α chain over the N-terminus of the peptide and the TCR β chain over the C-terminus) and make similar interactions with the bound peptide and MHC surface (Figures 1E,F). Thus, the overall mechanism by which TCRs interact with MHC-I and II to initiate T cell activation is closely matched.

Despite these similarities, MHC-I and II present peptides in a distinct manner that is governed by the composition of the

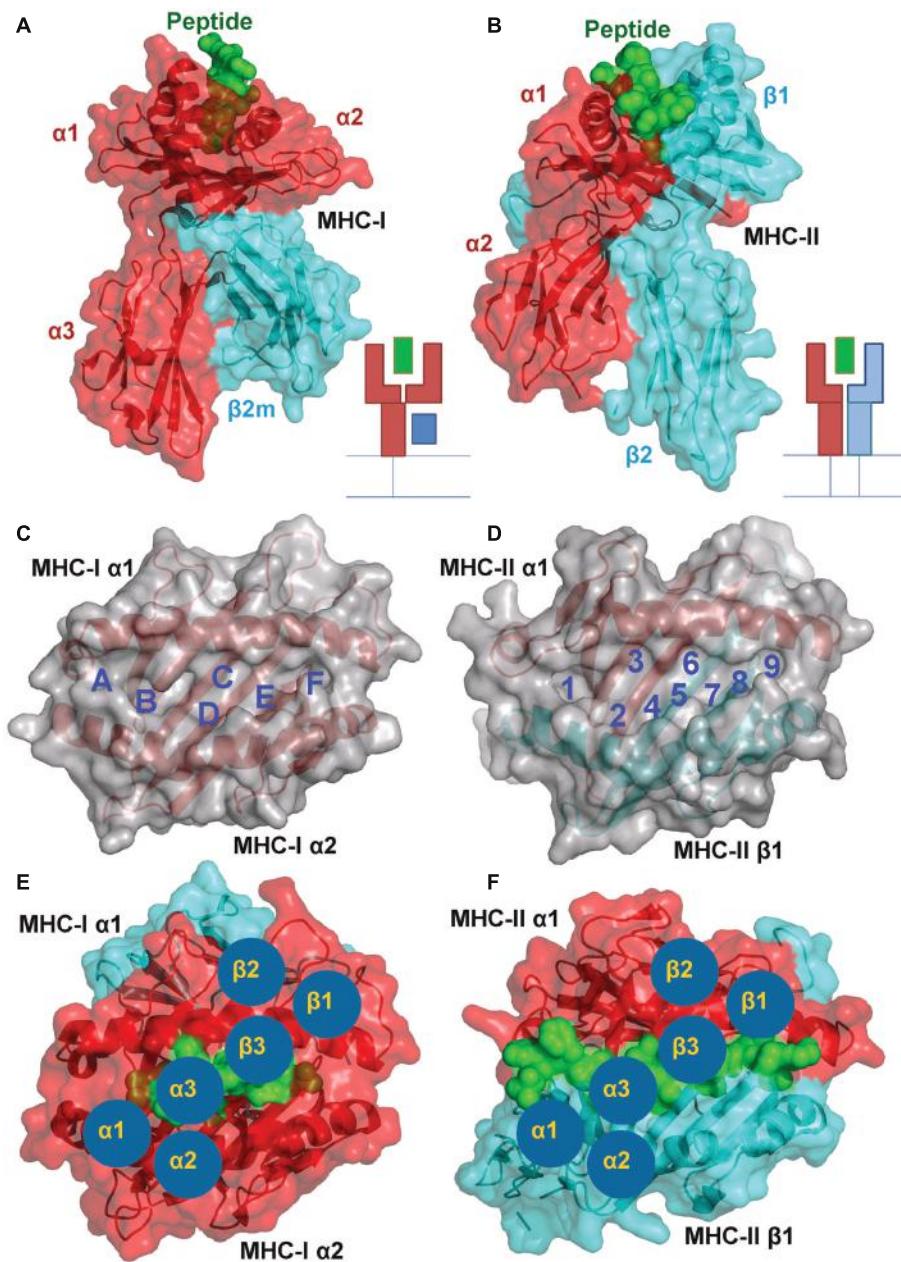


FIGURE 1 | A structural comparison of pMHC-I and pMHC-II. Although the subunit compositions of MHC-I (PDB: 1ZHL) (A) and MHC-II (PDB: 1KG0) (B) are different, the structural conformation they assume is very similar, illustrating their shared role in presenting antigenic peptides (green) to T cells. (A) MHC-I is comprised of three α -chain domains (1, 2, and 3 in red) and $\beta 2m$ (cyan), whereas (B) MHC-II is comprised of a two domain α -chain (red) and a two domain β -chain (cyan). A top down view of the MHC-I (C) and MHC-II (D) demonstrates the two molecules form similar peptide binding grooves comprised of two anti-parallel α -helices that form a channel in which the

peptide can bind in an extended conformation, and eight anti-parallel β -sheets that provide specific peptide binding pockets in the base of the groove. These pockets are lined with polymorphic residues that define the size and chemical characteristics of each pocket, and therefore the specific peptide binding motif and register that can be accommodated by different MHC alleles. TCR binding to pMHC-I (E) and pMHC-II (F) is also conserved. The three complementarity determining loops (CDRs) of the TCR (α -chain over the N-terminus of the peptide and the TCR β -chain over the C-terminus

MHC peptide binding groove. The closed conformation of the MHC-I $\alpha_1\alpha_2$ binding groove (Figure 2A) restricts peptide length to $\sim 8\text{--}13$ amino acids (most commonly 9 or 10mers) (3, 7). In contrast, the MHC-II $\alpha_1\beta_1$ binding groove comprises an *open-ended*

conformation (Figure 2B) that allows variable length peptides to bind. The core binding 9mer contains the motif for binding to the particular MHC-II heterodimer, but eluted and sequenced peptides often reveal families of processed peptides $\sim 12\text{--}20$ amino

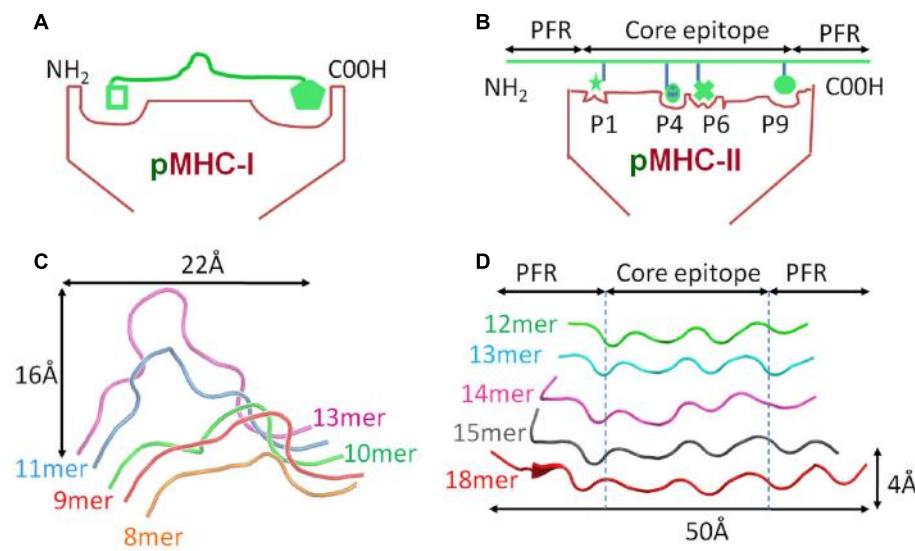


FIGURE 2 | Comparison of peptide conformations presented by MHC-I and MHC-II. Cartoon cross sections of the pMHC-I (A) and pMHC-II (B) binding grooves, show the key anchor sites in the floor of each groove determine which peptide can associate and the conformation it can assume. (C) The structural database of pMHC-I complexes shows that peptides presented by a MHC-I molecules (represented as ribbon cartoons) generally assume a central bulged conformation. As peptide length increases, the

“closed” nature of the pMHC-I binding groove forces the central residues of the peptide up out of the groove to accommodate the extra residues. (D) In contrast, the pMHC-II binding groove is “open” enabling longer peptide to extend out of the groove at form peptide flanking regions. Thus, peptides presented by MHC-II molecules (represented as ribbon cartoons) generally assume a much flatter conformation in the MHC-II binding groove, irrespective of the length of the peptide presented.

acids (referred to as nested sets) sharing the core binding region (3, 8, 9). MHC-I-restricted peptides usually bind to the MHC surface using anchor residues located at, or near, the N- and C-termini of the peptide. Depending on the length of the peptide, this binding mode squeezes the central peptide residues up so that they extend out the groove (central bulge), exposing peptide side chains for direct interaction with the TCR (3, 10). Longer MHC-I peptides can only be accommodated by forming a larger central bulge, which presumably constrains the length of the peptide beyond a certain threshold (Figure 2C; Table 1).

MHC-II restricted peptides contain a central binding motif of nine “core” amino acids that bind to the MHC-II groove via an extensive hydrogen bond network between the MHC-II groove and the peptide backbone (Figure 2B). Peptide side chains also form contacts with allelic specific pockets of the MHC-II binding groove. These pockets, usually P1, P4, P6, and P9, are lined with polymorphic residues that define the size and chemical characteristics of each pocket, and therefore the specific peptide binding motif and register that can be accommodated by different MHC-II alleles (Figure 1D) (11, 12). Amino acids that are outside of the “core” peptide region can extend out of the open MHC-II binding groove forming so called “peptide flanking regions” (PFRs) at both the N- and C-terminus (Figure 2D; Table 1). Thus, although pMHC-I and pMHC-II are similar in their overall structure and function, the nature of peptide presentation is generally distinct (e.g., bulged versus flat peptides). These differences present different challenges for TCR binding at the atomic level. For example, the flat binding surface and lack of a central peptide bulge may enable MHC-II restricted TCRs to adopt a more flexible binding mode compared to MHC-I restricted TCRs. In support

of this notion, the structures of a number of TCR-MHC-II complexes have shown that, although the binding mode can be very similar to the classical diagonal TCR-MHC binding mode (13–17), some MHC-II restricted TCR bind with highly unorthodox conformations (18, 19).

MHC-II RESTRICTED TCRS HAVE WEAKER BINDING AFFINITY COMPARED TO PMHC-I RESTRICTED TCRs

Biophysical studies have shown that TCR/pMHC affinity is relatively weak ($K_D = 100 \text{ nM}$ – $270 \mu\text{M}$), with fast kinetics, compared to antibody binding (usually nM–pM affinity) (20, 21). We have recently shown that TCR/pMHC-I binding affinities are, on average, five times stronger compared to equivalent TCR/pMHC-II interactions (i.e., viral pMHC-I restricted TCRs versus viral pMHC-II restricted TCRs) (Figures 3A,B), which has limited the usefulness of pMHC-II multimers for the identification, isolation and detection of antigen specific CD4⁺ T cells. This distinction in affinity was mainly due to a significantly faster on-rate for TCR/pMHC-I binding compared to that of TCR/pMHC-II, while the off-rate or half-lives of all of the TCR/pMHC interactions were relatively conserved, possibly indicating a more important role for off-rate in determining T cell activation. The consistent difference in binding affinity between TCRs restricted to either pMHC-I or pMHC-II is extraordinary when considering that the same pools of genes, on chromosome 9, encode the human TCR for both types of $\alpha\beta$ T cell (22). The TCR itself is expressed before positive selection, at which point immature T cells express both the CD4 and CD8 co-receptors (double positive). Once positively selected, immature thymocytes become single positive for either CD4 or CD8 (22). Until this point, the thymocyte, which has

Table 1 | Comparison of peptide conformations presented by MHC-I and MHC-II.

MHC	Protein	Peptide length (mer)	Peptide Sequence	Height (Å)	Width (Å)	PDB (Ref)
HLA-B*3501	HIV-1 NEF _{75–82}	8	VPLRPMTY	4	21	1A1N (56)
HLA-A*0201	Flu A Matrix _{58–66}	9	GILGFVFTL	6	22	1HH1 (57)
HLA-A*0201	MART-1 _{26–35}	10	EAAGIGILTV	8	22	2GT9 (58)
HLA-B*3501	EBV _{407–417}	11	HPVGEADYFEY	13	22	2FZ3 (59)
HLA-B*3501	M-CSF _{4–17}	14	LPAVVGGLSPGEQEY	16	22	1XH3 (60)
DRB1*0401, DRA1*0101	Collagen II _{261–273}	12	AYMRADAAAGGA	4	35	2SEB (61)
DRB1*0101, DRA1*0101	HA _{306–318}	13	PKYVKQNTLKLAT	4	37	1DLH (12)
DRB1*1501, DRA1*0101	MBP _{85–99}	14	ENPVVHFFKNIVTP	4	42	1BX2 (62)
DRB1*0101, DRA1*0101	MART-1 _{100–114}	15	APPAYEKLSAEQSPP	4	44	3L6F (63)
DRB1*0101, DRA1*0101	CLIP _{102–120}	19	KPVSKMRMATPLLMQALPM	4	50	3PDO (64)

already developed antigen specificity through its TCR, can theoretically have either cell fate (23). Considering these shared genetic and developmental processes, it is possible that the differences in MHC restricted TCR binding is conferred by the variations in the “antigenic landscape” of pMHC-I versus pMHC-II.

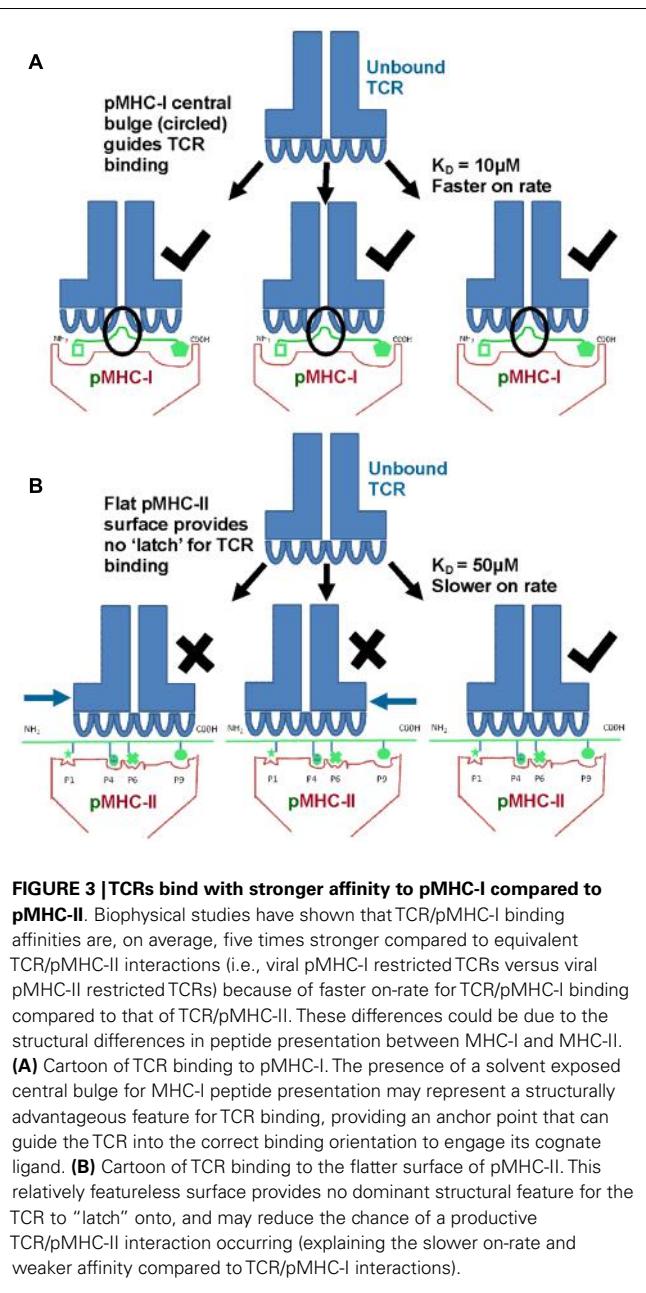
As discussed above, peptides presented by MHC-I molecules generally assume a central bulged conformation, often requiring conformational adjustments in the binding regions of the TCR during ligand engagement (Figure 2C) (24–26). An extreme example of this observation is a 13mer Epstein-Barr virus derived peptide presented by HLA-B*3508 which forms a “superbulge” extending nearly 20 Å out of the MHC-I binding groove (26). In contrast, MHC-II presented peptides generally assume a much flatter conformation in the MHC-II binding groove (13, 14) (Figure 2D). The presence of a solvent exposed central bulge for MHC-I peptide presentation may represent a structurally advantageous feature for TCR binding, providing an anchor point that can guide the TCR into the correct binding orientation to engage its cognate ligand (Figure 3A). Conversely, the flat, and relatively featureless surface of pMHC-II confers no dominant structural feature for the TCR to “latch” onto, and may reduce the chance of a productive TCR/pMHC-II interaction occurring (explaining the slower on-rate and weaker affinity compared to TCR/pMHC-I interactions) (Figure 3B). This notion is consistent with our recent observation that a MHC-II restricted TCR underwent minimal conformational adjustments during binding compared to most MHC-I restricted TCRs (27). The immunological significance of these topological and biophysical distinctions between MHC-I and MHC-II is still unclear. However, the difference in binding affinity between MHC restricted TCRs may represent a biophysical characteristic that relates to cellular function.

MHC-II ANTIGEN PROCESSING GENERATES VARIABLE LENGTH PEPTIDES

The “flat” surface of pMHC-II may contribute to the reduced affinity of MHC-II restricted TCRs. However, a striking difference in the peptides bound to MHC-II is the presence of non-bound PFRs, which may be available to interact with adjacent membrane proteins on the same or different cells. These PFRs can vary in length generating nested sets of peptides that are presented on the

surface of APCs (28, 29). One consequence of having a longer PFR is an increased binding affinity of peptides to the MHC-II (30–33), and therefore an increased probability of a meaningful interaction with a cognate T cell.

These variable PFRs are generated by proteolytic processes during the exogenous antigen processing pathway that has been reviewed in detail elsewhere (34, 35). Briefly, extracellular protein antigens are endocytosed by tissue resident APCs (Figure 4A). The pH of the endosome containing potential antigens progressively decreases, activating proteases which cleave captured proteins (Figure 4B). Newly synthesized MHC-II molecules reside in the endoplasmic reticulum (ER) in complex with a stabilizing chaperon, calnexin. To prevent premature peptide association with the MHC-II binding groove by ER derived proteins, the groove is “plugged” with a protein known as the MHC-II associated invariant chain (I_i) (36) (Figure 4C). Exocytic vesicles containing precursor I_i:MHC-II complexes then combine with endosomes containing exogenous peptide fragments forming the MHC-II compartment (Figure 4D). The acidic pH of the MHC-II compartment and presence of the chaperon, HLA-DM (37), allows peptide exchange between the class II-associated invariant-chain peptide (CLIP) and high affinity complementary peptides proteolysed in the endosomal compartment. Peptide selection, that presumably plays a strong role in determining the characteristics of PFRs, is also facilitated by HLA-DM in a process termed “peptide-editing” which ensures that only stable MHC-II peptide complexes are expressed and transported to the cell surface for potential TCR interactions (38, 39) (Figure 4E). Structural modeling of HLA-DM association with pMHC-II indicated that peptide editing was achieved through conformational changes around pocket 1 (P1) of the binding groove, a pocket crucial for the stability of the peptide-MHC-II complex (40). Such conformational changes induced by HLA-DM were thought to weaken the hydrogen bond network between the bound peptide and MHC-II molecule and facilitate peptide release (41). A recent co-complex structure of HLA-DM with HLA-DR α *0101; β *0101 (HLA-DR1) has confirmed this experimentally, revealing that HLA-DM binding induced a conformational change in the α -helix of the DR α chain in the peptide binding groove (42). This change enabled two HLA-DR1 residues, DR α phenylalanine 51 and DR β phenylalanine 89, to



bind to, and stabilize, the P1 binding pocket of the MHC-II binding groove, presumably blocking the association of weakly binding peptides. Thus, only the association of high affinity peptides with MHC-II results in displacement of these residues, enabling a revision in the conformation of MHC-II and the dissociation of HLA-DM (40, 42).

It is possible that the proteolytic events that occur before peptide-MHC-II loading govern the final pool of peptides available for selection during MHC-II peptide loading. However, it has also been suggested that the final pMHC-II, loaded with exogenous peptide, can be modified further in a process termed peptide trimming, whereby the length of the PFRs can be edited (Figure 4F) (43, 44). These processes demonstrate a remarkable degree of

complexity and control during MHC-II peptide selection that is still not fully understood. The antigen processing by cellular proteases and the generation of pMHC-II may also be influenced by cell extrinsic factors such as inflammatory cytokines, e.g., IFNs (45), as well as cell intrinsic factors reflecting the type/subtype of APC (46, 47). The cellular machinery involved in antigen processing and presentation is different between cell types (48), hence the determinants resulting from protein digestion may vary depending on cell type or subtype (e.g., B cell versus macrophage; CD8⁺ versus CD8⁻ dendritic cells etc); and context (e.g., inflamed versus non-inflamed tissues, anatomical location). It is conceivable that a range of determinants presented in a lymph node may differ from those presented at the primary site of infection in both a quantitative fashion, i.e., the number of pMHC-II complexes per cell, and qualitative fashion i.e., length and type of PFRs and hence offer a local control of CD4⁺ T cell responses accordingly.

MODULATING CD4⁺ T CELL RESPONSES VIA ALTERED PEPTIDE FLANKING RESIDUES

There is convincing evidence that PFRs can modulate T cell function (49). A study of a HIV-I p24 (GAG) epitope, presented by HLA-DR1, revealed that antigen specific T cell activation was enhanced with longer flanking residues. Structural analyzes showed that the C-terminal flank could form a hairpin turn, raising the possibility that MHC-II PFRs may form more complex conformations that could directly impinge on TCR binding (50). Thus, the open ended nature of the MHC-II binding groove, that allows long peptides to extend beyond the binding region at both the N- and C-terminus (Figures 2 and 3), may play a direct role during T cell antigen recognition. In support of this notion, it has been demonstrated that removal of C-terminus PFRs from the immunodominant epitope in hen egg lysosome₅₂₋₆₁ (HEL) significantly altered the immunogenicity of the epitope, reducing T cell sensitivity (9).

Our previous work, using sequence analysis of eluted peptide ligands from a range of allelic variants of MHC-II molecules, has identified allele-transcending enrichments in PFRs at the peptide C- and N-terminus (51, 52). These data show that a range of different modifications to PFRs could modulate specific CD4⁺ T cell responses including amino acids with biochemically distinct side chains (52, 53). The identification of these PFR amino acid enrichment patterns suggests that they play a role during CD4⁺ T cell activation and can modulate antigen recognition. Further studies using antigen specific CD4⁺ T cell clones demonstrated that PFR modifications could enhance CD4⁺ T cell activation (53). Although a wide range of different amino acid substitutions in PFRs could generate stronger CD4⁺ T cell responses, we observed that basic residues at the peptide C-terminus, or acidic residues in the N-terminus, were most commonly enriched and generated enhanced CD4⁺ T cell responses across different MHC-II alleles and different peptides. Studies focusing on the C-terminal PFRs, in which the basic amino acid, arginine was substituted into the C-terminal flank (at position 10 or 11) of known T cell epitopes from haemagglutinin (HA) and myelin basic protein (MBP), demonstrated that these alterations led to a significant increase in CD4⁺ T cell responses (52, 53). Screening T cells which recognized

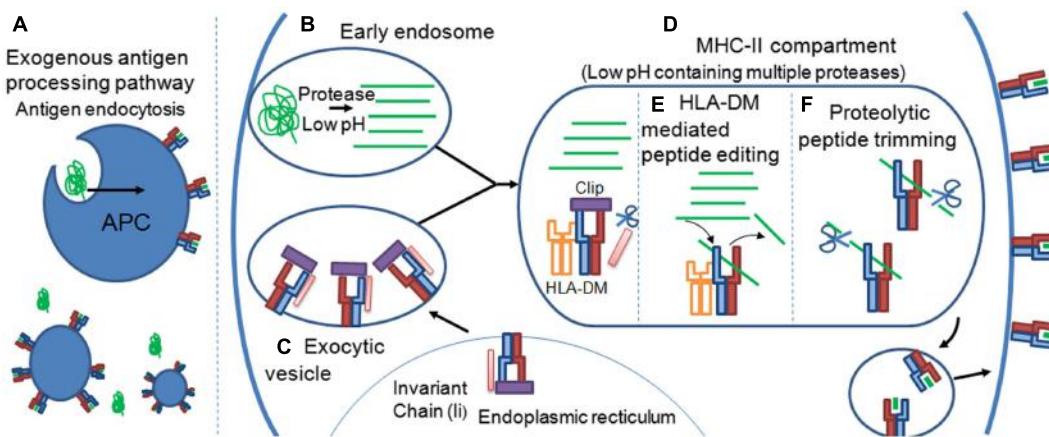


FIGURE 4 | Peptide flanking regions are determined during the MHC-II antigen processing pathway. (A) Exogenous antigen processing pathway. Antigen endocytosis. (B) Early endosome. (C) Exocytic vesicle. (D) MHC-II compartment (Low pH containing multiple proteases). (E) HLA-DM mediated peptide editing. (F) Proteolytic peptide trimming. APC, antigen-presenting cell; ER, endoplasmic reticulum; HLA-DM, HLA class II-associated invariant chain-binding protein; MHC-II, major histocompatibility complex class II; PFR, peptide flanking region; TCR, T cell receptor.

peptide (CLIP) within the binding groove of the MHC-II molecule. The acidic pH of the MHC-II compartment and presence of the chaperon, HLA-DM, allows peptide exchange between CLIP and high affinity complementary peptides proteolysed in the endosomal compartment. (E) Peptide selection, that presumably plays a strong role in determining the characteristics of PFRs, is also facilitated by HLA-DM in a process termed “peptide-editing” which ensures that only stable MHC-II peptide complexes are expressed and transported to the cell surface for potential TCR interactions. (F) The final pMHC-II, loaded with exogenous peptide, can also be modified further in a process termed peptide trimming that may play a role in governing PFR length. pMHC-II molecules are then transported to the cell surface for interrogation by CD4⁺ T cells.

this same MBP-derived epitope with a combinatorial library also revealed a preference for C-terminal basic residues (54).

However, in all of these examples, the mechanism for the effect of PFRs on T cell responsiveness had remained elusive. Two possibilities can be considered. Firstly, PFR modifications may alter the stability of pMHC-II molecules (a notion that has been experimentally observed (30–33), altering their expression levels at the surface of APCs. However, we have demonstrated that, although the substitution of basic residues in the C-terminus increased T cell activation, they actually reduced peptide/MHC binding (52). Secondly, if the TCR can directly contact the PFRs, then modifications in the PFR could alter TCR binding affinity and subsequent T cell activation. In order to investigate the second possibility, we conducted biophysical experiments by surface plasmon resonance using cloned TCRs specific for an influenza epitope (HA_{305–320}) presented by HLA-DR1 (53). The substitution of arginine into either position 10 (HA_{10R}) or 11 (HA_{11R}) of HA_{306–318} generated approximately twofold increase in TCR binding affinity (Figures 5A,B). Intriguingly, analysis of the TCR clonotypic repertoire of peptide-expanded influenza-specific CD4⁺ T cells from HLA-DR1⁺ donors in response to HA_{305–320} or arginine altered variants (HA_{10R} and HA_{11R}) demonstrated a marked alteration in TCR usage, with a striking focusing of the response when using the peptides which are known to increase TCR binding i.e., number of clonotypes for HA > HA_{10R} > HA_{11R}. The structure of HLA-DR1-HA_{306–318} in complex with the MHC-II restricted TCR, HA1.7, has been solved by X-ray crystallography (55). This structure demonstrated that the TCR could not directly contact the short side chains of either Alanine at P10, or Threonine at

P11 in the universal HA_{306–318} epitope (Figure 5C). The closest proximity between the TCR and either P10 or P11 of the HA_{306–318} peptide was over 8 Å, which was beyond the limits for atomic contacts. However, structural modeling of the substitution of arginine, which has a long acidic side chain, at either P10 or P11 indicated this gap could be closed allowing additional interactions to form between the peptide and the TCR (Figure 5D) (53). These potential new contacts could offer an explanation for the stronger binding affinity between the HA1.7 TCR and the P10 or P11 substituted DR1-HA_{306–318} epitope.

CONCLUSION

The function of classical MHC molecules is presentation of peptide epitopes to the cell mediated arm of adaptive immune response. However, the subtle differences that exist between the two classical forms of MHC, with respect to antigen processing and structural architecture, significantly alters the nature of the peptide each class of MHC can present upon the cell surface. In particular, the closed binding groove of MHC-I forces bound peptides to bulge in the center, compared to the open binding groove of MHC-II that allow PFRs to form. *In vitro* experiments using a variety of antigens in mice and human systems, including HA, GAG, MBP, and HEL, have demonstrated that the PFRs of an epitope can profoundly affect CD4⁺ T cell function. Generation and selection of different PFRs might be governed according to the anatomical location, inflammatory milieu and particular types of APC involved during antigen processing. Thus, a key question that remains is whether particular changes in PFRs occur through a random, stochastic process, or whether changes are purposefully

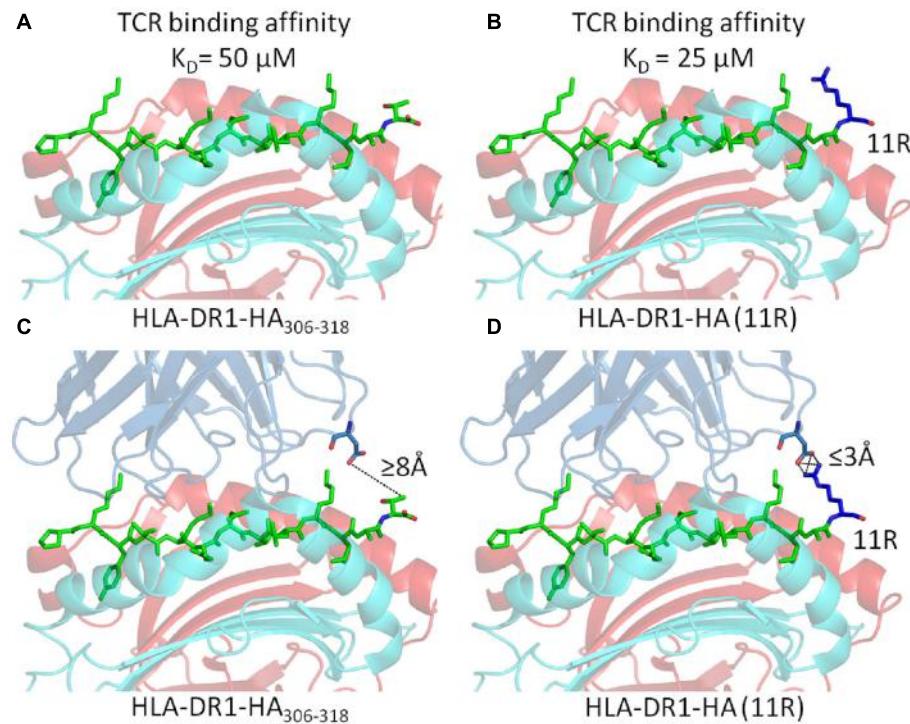


FIGURE 5 | Substitution of Arginine substitutions in the C-terminal flanking region of the native Flu1 peptide increases binding affinity. **(A,B)** Substitution of arginine at position 11 (blue) in the HA₃₀₅₋₃₂₀ epitope generates around a twofold increase in TCR binding affinity. **(C,D)** Cartoon representation of the interaction between the TCR and C-terminal PFR (modeled from PDB: 1FYT). **(C)** The TCR β -chain is

beyond the limits for atomic contacts with HA₃₀₅₋₃₂₀ P11 (dotted line). **(D)** Modeling shows that a new interaction, possibly a salt bridge, could be formed between the TCR β -chain and arginine (blue) substituted at position 11 of the HA₃₀₅₋₃₂₀ peptide. This new interaction could explain the increase in affinity observed for cognate TCR binding to the HA₃₀₅₋₃₂₀ peptide and HA_{11R}.

intentioned to control or alter the nature of a specific immune response. Irrespective of the answer to this question, our recent data revealed that experimental PFR modifications could enhance TCR/pMHC-II affinities closer to the range typically observed for TCR/pMHC-I interactions. This exciting observation suggests that augmentation of pMHC-II antigens through C-terminal PFR modifications might be a useful strategy to enhance MHC-II

restricted TCR binding affinity and CD4⁺ T cell responsiveness, with attendant implications for vaccination and other immune system interventions.

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Analysis, isolation, and activation of antigen-specific CD4⁺ and CD8⁺ T cells by soluble MHC-peptide complexes

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T cells constitute the core of adaptive cellular immunity and protect higher organisms against pathogen infections and cancer. Monitoring of disease progression as well as prophylactic or therapeutic vaccines and immunotherapies call for conclusive detection, analysis, and sorting of antigen-specific T cells. This is possible by means of soluble recombinant ligands for T cells, i.e., MHC class I-peptide (pMHC I) complexes for CD8⁺ T cells and MHC class II-peptide (pMHC II) complexes for CD4⁺ T cells and flow cytometry. Here we review major developments in the development of pMHC staining reagents and their diverse applications and discuss perspectives of their use for basic and clinical investigations.

Keywords: T cells, T cell receptor, tetramers, MHC, flow cytometry, coreceptor

INTRODUCTION

TCR $\alpha\beta^+$ thymus derived T lymphocytes constitute the core of adaptive cellular immunity in higher organisms and play essential roles in their protection against pathogen infection and neoplastic transformations (1). CD8⁺ T cells differentiate to cytotoxic T lymphocytes (CTL) capable of efficient killing of cells displaying pMHC I complexes containing 8–10 residues long peptides, derived from a pathogen or cancer (2). CD8 $\alpha\beta$ coreceptor of CTL substantially enhances their antigen recognition, namely by strengthening the pMHC I ligand binding and bringing CD8-associated p56^{lck} to the TCR/CD3 complex, thus promoting TCR signal induction (3). In most cancer vaccines and immunotherapies, tumor antigen-specific CD8⁺ T cells take center stage (4, 5). Antigen-specific CD8⁺ T cells can be reliably detected, analyzed, and sorted by means of soluble pMHC oligomers (6, 7). Peptide-MHC complexes have limited life spans at 37°C and therefore MHC I molecules provide a dynamic display of endogenously produced peptides. pMHC complexes can be obtained by refolding of denatured heavy and light (β 2m) chains in the presence of a peptide of interest (8). This procedure is simple, inexpensive, and yields highly pure pMHC complexes. Because the peptides bind to MHC I molecules in a canonical manner, in which both termini are firmly anchored in well-defined positions, these complexes are homogenous and structurally well-defined (9).

On the other hand, CD4⁺ T cells differentiate from a common precursor (TH0) in different types of effector cells, such as T help cells (Th1, Th2), or regulatory T cells (Treg). Efforts to detect antigen-specific CD4⁺ T cells by soluble pMHC II multimers fared less well than their class I counterparts for diverse reasons. MHC II molecules consist of non-covalently linked alpha and beta chains, both of which are transmembrane spanning proteins and their variable domains (alpha 1 and beta 1) form an open ended peptide binding groove that accommodates exogenous peptide usually

10–15 residues long (10). In contrast to MHC I, MHC II molecules, in particular HLA II molecules, are stable without a peptide, which allows loading of “empty” MHC II molecules with a peptide of interest (11). Newly synthesized MHC II molecules associate with invariant chain (li), which assists their refolding, prevents binding of endogenous peptides in the endoplasmic reticulum, and directs them to the endosomal/lysosomal pathway where they are loaded with peptides generated from exogenous antigens by lysosomal proteolytic activity (12). To prepare soluble recombinant MHC II molecules, their transmembrane and cytoplasmic domains are replaced by leucine zippers to prevent dissociation of the alpha and beta chains (13). With a few exceptions pMHC II complexes cannot be obtained in good yields by refolding and/or by mammalian expression systems, but can be well expressed by insect expression systems, such as *Drosophila* S2 cells or SF9 cells infected with baculo virus (14, 15). While binding of soluble pMHC I complexes to CD8⁺ T cells is greatly strengthened by CD8, CD4 does not stabilize pMHC II binding to CD4⁺ T cells (16). Together with the fact that CD4⁺ T cells usually express lower affinity than CD8⁺ T cells, this accounts for frequent poor pMHC II tetramer staining and missing of a substantial fraction of antigen-specific CD4⁺ T cells; this is often aggravated by poor reagent quality, namely incomplete or disperse peptide loading (17).

CHRONOLOGICAL OVERVIEW OF SOLUBLE pMHC CLASS I COMPLEXES AND THEIR USE FOR T CELL ANALYSIS

Soluble recombinant pMHC complexes are valuable tools for investigations, analysis, and isolation of antigen-specific T cells (Figure 1). The smallest pMHC complex is a monomer, which according to SPR studies binds recombinant TCR with dissociation constants (K_D) in the range of 1–1000 μ M, and dissociation kinetic rates in the range of 1–1500 s⁻¹ (18), which precludes analysis by flow cytometry or fluorescence microscopy.

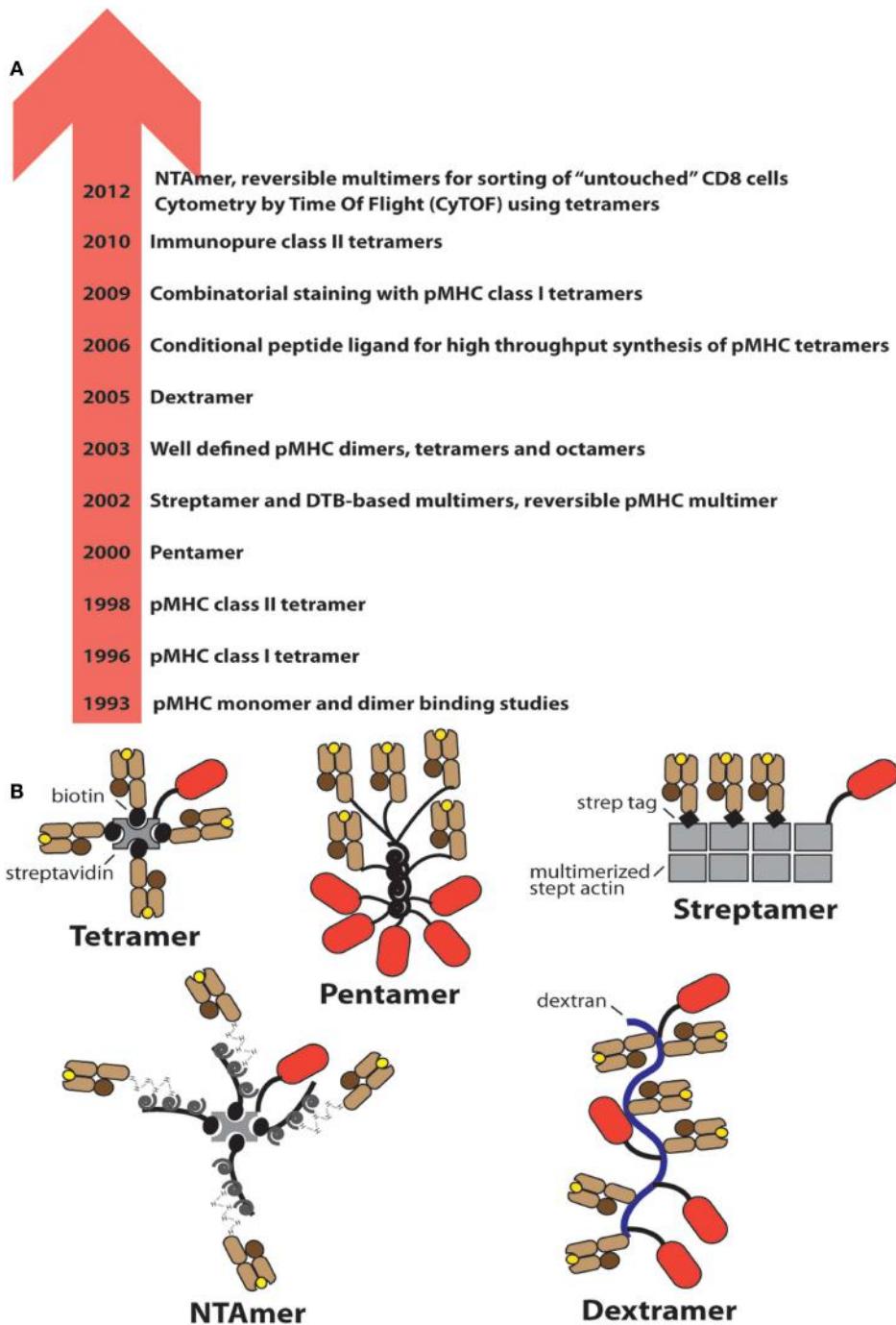


FIGURE 1 | Evolution of soluble pMHC complexes and their applications for T cell analysis and sorting. (A) Chronological listing of milestones in the use of pMHC complexes for the detection and analysis of antigen-specific CD8⁺ and CD4⁺ T cells. **(B)** Cartoons of the most frequently used pMHC oligomers; the red oval represents PE.

Nevertheless, the binding and dissociation of monomeric pMHC complexes was extensively studied on CD8⁺ CTL by means of TCR photoaffinity labeling (19, 20). These studies revealed that coordinate binding of CD8 to TCR-associated pMHC I complexes greatly strengthens pMHC I binding, mainly by decreasing

the TCR-pMHC dissociation (3). It is noteworthy that although pMHC binding data measured by SPR are markedly different than those measured on living CD8⁺ T cells, they correlate well with CD8⁺ T cell activation. The likely explanation for this is that intercellular TCR-pMHC interactions precede

CD8 co-engagement, i.e., CD8 is recruited to sufficiently stable TCR–pMHC complexes (21).

Also in 1993 pMHC I dimers were described, with the capacity to fully activate CD8⁺ CTL clones, whereas monomers were inactive (22). The ability of pMHC I and later also pMHC II dimers to inhibit antigen-specific T cells *in vitro* and *in vivo* was shown to be a promising means to block autoimmunity (23, 24). Peptide-MHC dimers built on chemical linkers were described only many years later (see below).

In 1996 the first pMHC I “tetramer” was described and its usefulness demonstrated for the detection and analysis of virus-specific CD8⁺ T cells (25). The subunit pMHC I monomers carry a biotinylation sequence peptide (BSP) C-terminal of the alpha3 domain that is amenable to biotinylation with the biotin transferase BirA (26). Reaction of biotinylated pMHC I monomers with phycoerythrin-streptavidin (PE-SA) (or allophycocyanin-SA) typically results in mixtures of pMHC I conjugates of different stoichiometry and relative orientations and hence are better termed multimers (27). Our analysis indicated that such multimers contained species carrying up to 12 pMHC I monomer (unpublished results). Real tetramers can be prepared by using “tetra-grade” PE-SA and exhibit substantially reduced staining (see below).

In 1998 the first pMHC II multimers were described (28), which performed less well than their pMHC I counterparts, often exhibiting erratic, unreliable staining performance and according to one study approximately half of all antigen-specific CD4⁺ T cells, especially self-antigens-specific ones, are not detected by conventional multimers (29). Only in 2010 molecular defined pMHC II multimers were described and their superior staining performance demonstrated (30, 31) (Figure 1).

In 2000 “Pentamers” (by ProImmune) were introduced that comprise five pMHC I complexes and five PE. Despite a lower valency compared to conventional multimers, the staining performance of both reagents seems to be similar (32). In 2002 meeting the increasing need for sorting of antigen-specific CD8⁺ T cells namely for *in vitro* studies or adoptive cell transfer experiments, reversible multimers, based on biotin analogs, were introduced, which allow sorting of untouched CD8⁺ T cells (33, 34). A third type of reversible multimers, the NTAmers, based on multivalent Ni²⁺-NTA-His-tag complexes, followed nearly a decade later (35). In 2003 well-defined homologous pMHC dimers, tetramers, and octamers were described that contained linkers of defined lengths and configurations (36, 37). Binding and activation studies on cloned murine CTL and on CD8⁺ T cells from melanoma patients revealed that the binding avidity and CD8⁺ T cell activation critically depends not only on the valence, but also on pMHC–pMHC distances and configurations (38). In 2005 Dextramers (by Immudex) were introduced, which consist of long dextran fibers conjugated with multiple PE and pMHC I molecules (39) (Figure 1). While these studies contributed to improve analysis of antigen-specific T cells by means of pMHC oligomers, only recent developments have substantially increased their usefulness for comprehensive immune monitoring. For example an original new strategy allowing production of a wealth of different pMHC I complexes was introduced in 2006, i.e., the UV irradiation induced peptide exchange on pMHC I complexes containing a photoreactive peptide (40). This technique is useful especially

for combinatorial multimer staining, independently established by two groups in 2009 (41, 42). Combinatorial multimer staining allows parallel detection of multiple specificities, which provides more information on precious, often small blood samples. One strategy uses binary color coding, i.e., antigen-specificity is defined by a unique combination of two colors (41), whereas the other strategy uses all possible color combinations, which affords higher number of color combinations, but also stronger reduction in fluorescence intensity (42). Three years later CyTOF was introduced, i.e., cytometry combined by time of flight measurements (43). The cells are labeled with lanthanide labeled pMHC multimers, resolved in a cytometer, converted in plasma, and its lanthanide content analyzed by mass spectrometry. Presently about 30 channels, i.e., different mass tags, are available, which provides more possibilities for parallel detection than flow cytometry (44). In 2012 also NTAmers were described, which are pMHC multimers built on NTA-Ni²⁺-His-tag interactions that are nearly as stable as biotin–SA bonds, but upon addition of imidazole instantly decay in their constituents. By using Cy5 labeled pMHC molecules, NTAmers allow measurements of pMHC monomer dissociation kinetics on CD8⁺ T cells (Figure 4).

WELL-DEFINED SOLUBLE pMHC COMPLEXES: TOOLS TO STUDY CD8⁺ T CELLS LIGAND BINDING AND ACTIVATION

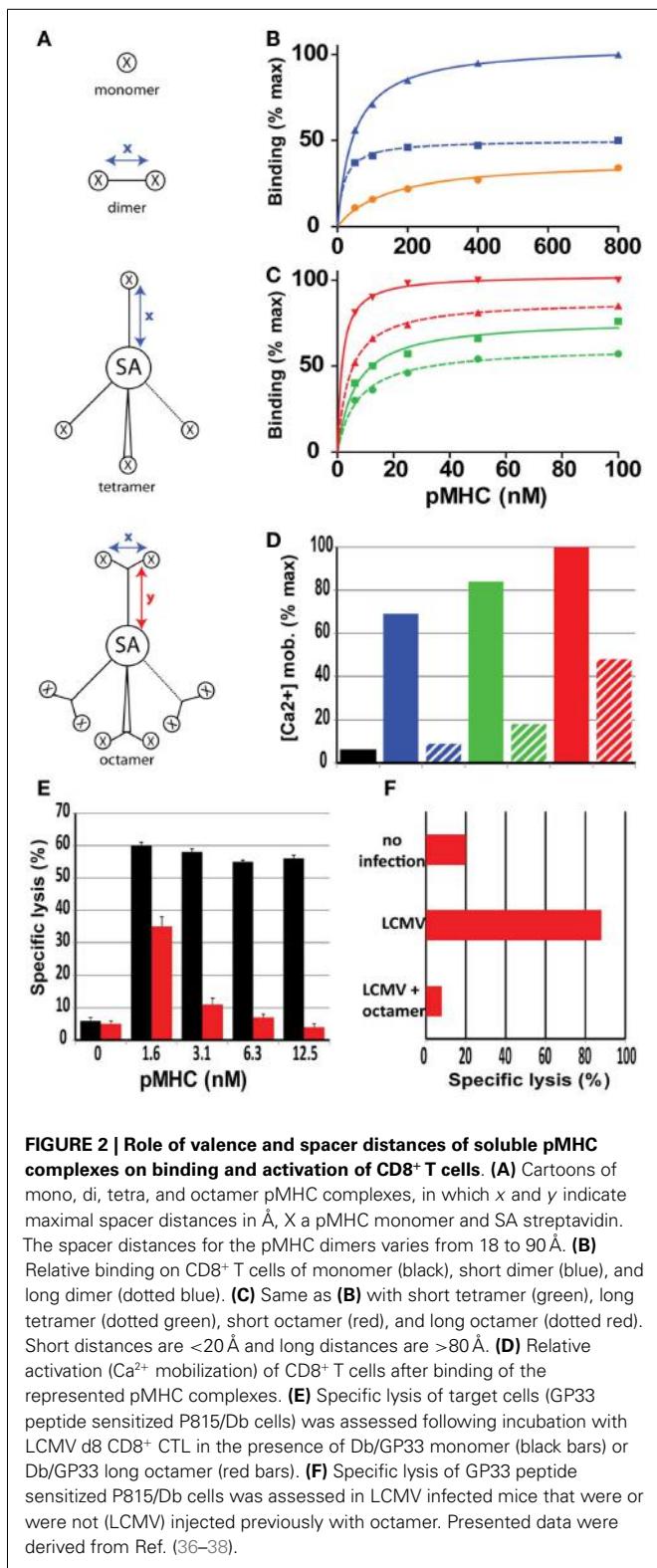
While most soluble pMHC complexes used for detection and isolation of antigen-specific T cells are heterogeneous, complexes of defined valence, configuration, and pMHC–pMHC distances were made and tested on CD8⁺ T cells, which provided valuable insights on how they bind and are activated by pMHC complexes (Figure 2).

pMHC I MONOMERS

Due to the weak and transient nature of pMHC monomer binding to CD8⁺ (and CD4⁺) T cells, on cells binding studies require special techniques, such as TCR photoaffinity labeling (3, 19, 45, 46). To this end CTL clones were derived from mice immunized with a photoreactive derivative of the malaria circum sporozoite peptide PbCS_{252–260} [SYIPSAEK(ABA)I, ABA: 4-azido-benzoic acid] that specifically recognize this peptide derivative. These binding studies on living CD8⁺ T cells revealed that the coreceptor CD8 $\alpha\beta$ strongly strengthens TCR–pMHC interaction by coordinate binding to TCR-associated pMHC molecules (3). This increase in binding avidity varies in the range of 4- to 20-fold, depending of the clone and temperature and was mainly accounted for by decreased TCR–pMHC dissociation kinetics (45–47). The kinetics of pMHC monomer binding on living CTL exhibited a prominent temperature dependence, namely a dramatic decreases of dissociation rates with decreasing temperatures and biphasic association binding kinetics at 37°C, but not in the cold (3). Soluble monomeric complexes do not activate CD8⁺ T cells unless these are adhered to a substrate; however, it was demonstrated that peptides of soluble pMHC monomers can be transferred to CD8⁺ T cells CTL-associated MHC I molecules by an unknown mechanism and that this can engender CTL–CTL interactions (48, 49).

pMHC DIMERS

The first pMHC dimers described were fusion proteins consisting of pMHC monomers fused to IgG and provided initial new



insights in pMHC dimer binding to CD8⁺ T cells, namely that naïve, effector, and memory T cells exhibits substantially different binding and activation/inactivation properties (24, 50). More recently soluble pMHC I and II dimers were prepared that contain

synthetic linkers of different length and flexibility (37). Testing of homologous K^d/PbCS(ABA) dimers containing linkers of 12–130 Å showed that the binding avidity (K_D) and the maximal binding (B_{\max}) were maximal for very short cross-linkers and gradually decreased with the spacer length of the linker to nearly 50% reduced K_D and B_{\max} values at ≥80 Å (37) (Figure 2B). Dimers containing short flexible linkers very strongly activated CTL, which frequently induced CTL death via irreversible mitochondrial damages (38) (Figure 2D). By contrast dimers containing >80 Å long, rigid linkers induced no significant CTL activation and were able to inhibit CTL activation (36). Moreover, pMHC I (and II) mixed dimers were used to investigate the role of endogenous pMHC complexes for T cell activation. Antigen-specific T cell activation can be triggered by just a few cognate complexes, but for this high sensitivity, endogenous complexes are needed that although unable to trigger T cells by themselves, can substantially boost T cell activation by a few cognate pMHC complexes (51, 52). Conceptually it was proposed that such mixed pMHC dimers, or by analogy vicinal pMHC complexes on antigen presenting cells, promote coreceptor/lck association with TCR/CD3 and/or promote initial TCR/coreceptor co-aggregation on which T cell activation relies (53).

pMHC TETRAMERS AND OCTAMERS

Conventional “tetramers” obtained by reacting biotinylated pMHC monomers with PE (or APC) labeled streptavidin are heterogeneous, and unable to dissociate completely from CD8⁺ T cells (54). True tetrameric pMHC complexes can be prepared based on tetra-grade PE-SA or SA labeled with low molecular weight fluorochromes, e.g., Cy dyes. Real tetramers typically exhibit substantially lower binding avidities and fluorescence intensities, even when PE is used as in the conventional multimers. Moreover, as noted for pMHC dimers, tetramers containing short flexible linkers connecting pMHC to SA avidly bound to cloned CTL and vigorously activated these, whereas those containing long (>80 Å) rigid linkers bound less avidly, barely activated CTL and were able to antagonize CTL activation (36) (Figures 2C,D).

Finally octameric complexes were prepared by reacting Cy5-SA with Y shaped dimers in which two pMHC complexes were connected via short flexible or long rigid linkers to a biotin containing stem (36). Octamers composed of dimers with short inter pMHC distances avidly bound to and activated cloned CTL, whereas those consisting of dimers containing long rigid linkers bound less avidly and barely activated CTL. Owing to their high valence even octamers containing long rigid linkers exhibited avid binding to CTL and were able to efficiently inhibit CTL killing *in vitro* and *in vivo* and hence constitute a novel type of CD8⁺ T cell antagonists (36) (Figures 2C–F). Moreover, comparative binding studies on Melan-A-specific CD8⁺ T cell clones derived from a melanoma patient with A2/Melan-A dimers, tetramers, octamers containing linkers of different length and flexibility revealed striking differences depending on the state of CD8⁺ T cell differentiation (55). In particular dimeric complexes containing a short linker avidly and selectively stained a population of incompletely differentiation, dysfunctional CD8⁺ T cells, that are typically observed in advanced melanoma patients, but never in healthy individuals (55).

SOLUBLE pMHC COMPLEXES BUILT ON Ni^{2+} -NTA-HIS-TAG CHELATE COMPLEXES

THE MAKE AND NATURE OF REVERSIBLE pMHC COMPLEXES

Conventional BSP multimers are prepared by using the biotin-streptavidin irreversible tandem. Although for some applications this irreversible binding is desirable, for T cell staining it is not as multimers can bind and strongly activate those cells, leading sometimes to their death (Figure 3A). Nitrilotriacetic acid (NTA) at neutral aqueous solutions chelates bivalent cations, e.g., Ni^{2+} , which in turn binds vicinal side chains of histidines. While the monomeric interaction is in the micro-molar range, picomolar dissociation constants were obtained for linear tetra-NTA compounds binding to pMHC molecules containing a C-terminal tandem His-tag (2xHis₆) (35). While these coordination complexes are stable, also at elevated temperatures, they decay within a few seconds upon addition of free imidazole.

SORTING OF ANTIGEN-SPECIFIC T CELLS BY REVERSIBLE SOLUBLE pMHC COMPLEXES

Although CTL are highly cytotoxic cells, they are remarkably susceptible to cell death, which can be induced by soluble pMHC complexes, e.g., directly by Fas/FasL mediated cytotoxicity, mitochondrial damages due to excessive CTL activation, or indirectly by fratricide caused by transfer of peptide from soluble to CTL-associated MHC molecules (37, 56). To prevent this and functional alterations, three types of reversible pMHC multimers have been described (Figure 3B) that allow sorting of “untouched” antigen-specific CD8⁺ (and CD4⁺) T cells (33–35, 57). Because T cell activation and signaling takes place at physiological temperatures, but not in the cold and pMHC monomers rapidly dissociate from T cells, they are stained with the multimers and sorted by FACS or MACS in the cold and cell-associated reagents are removed before culturing the sorted cells at 37°C. Two reversible multimers are built on altered biotin-SA scaffolds (33, 34, 57). The first ones, called streptamers, which are commercially available from IBA Life Science, consist of pMHC complexes containing C-terminal one or two streptags, i.e., a linear peptide sequence that mimics biotin and fluorescence labeled streptactin, a SA mutant that more avidly binds streptags than SA. Because the binding of one streptag to streptactin is weak (micromolar range), it is advantageous to use a tandem streptag for improved stability. While streptamers (containing two streptags) in the cold are stable, decay becomes appreciable at 20°C, which risks to affect their shelf life, especially when brief periods of warming up cannot be excluded. The other type of reversible multimer contains desthiobiotin (DTB) in place of biotin, a biotin derivative with greatly weakened binding to SA (33). As the streptamer, these multimers can be readily dissociated by addition of free biotin. These reagents are only stable in the cold and readily decay at elevated temperatures, which can be reduced when using two DTB per pMHC entity. A third type of reversible pMHC multimers are NTAmers which being built on Ni^{2+} -NTA-His-tag chelates complexes, rapidly decay upon addition of free imidazole (35). NTAmers built on NTA₄- Ni^{2+} -tandem His₆-tag are nearly as stable as biotin-SA multimers and therefore are by far more stable than the other reversible multimers, especially at elevated temperatures (Figure 3C). As described

below, multimer staining of antigen-specific CD4⁺ T cells is greatly improved at elevated temperatures (18°C) and therefore for their sorting NTAmers are recommended.

Sorting of antigen-specific CD8⁺ CTL with Streptamers or NTAmers yielded fully functional CTL, whereas when conventional tetramers were used their cytotoxicity and proliferation was impaired (35, 57, 58). The cloning efficiency of NTAmers sorted CTL was approximately twofold higher as compared to cloning with conventional, irreversible multimers. Analysis of CTL clones revealed that those preferentially lost by sorting with conventional multimers exhibited high physical and functional avidity, which is adverse in view of studies showing that high avidity CTL mediate superior tumor control (59) (Figures 3D,E). The use of reversible multimers thus is strongly recommended especially for sorting of antigen-specific T cells used in immunotherapy.

ADDITIONAL APPLICATIONS FOR NTA-BASED REVERSIBLE SCAFFOLDS

PE-NTAmers allow detection of low avidity antigen-specific T cells

Previous pMHC binding studies on CD8⁺ T cells have revealed that the binding avidity substantially increases at decreasing pMHC-pMHC distances. In SA (and avidin derivatives) pMHC built complexes the minimal inter distance is in excess of the distance of their biotin binding sites (about 30 Å). By means of site-specific alkylation, we conjugated PE with linear NTA peptides and reacted this derivative with tandem His-tag carrying pMHC complexes [(35) and unpublished data, Figures 4A–C]. Phycoerythrin, a saddle shaped molecule, about fourfold larger than SA and with an abundance of surface lysines that permit the preparation of PE-pMHC conjugates with smaller pMHC-pMHC distances than in conventional multimers. The maximal degree of conjugation is 12–16 pMHC complexes per PE, but the maximal binding affinities were observed with conjugates containing 8–10 pMHC complexes per PE. Additionally these PE-pMHC NTAmers are molecularly better defined than conventional multimers, as they contain only one central PE, whereas conventional multimers contain higher order conjugates. In accordance with this, PE-pMHC NTAmers binding exhibit clear saturation and greatly increased binding avidity compared to conventional pMHC multimers and therefore can detect low avidity T cells that are missed by conventional staining reagents (Figure 4D). Conclusive detection of low avidity T cells is one of the formidable challenges in T cell monitoring, namely in cancer research where tumor antigen-specific T cells tend to be low avidity due to deletion of high affinity specificities by negative selection (60) and for CD4⁺ T cells, on which multimer binding is not strengthened by the coreceptor.

NTAmers allow pMHC monomer dissociation kinetic measurements

NTAmers are not only substantially more stable than other reversible pMHC multimers, but their induced dissociation is also more rapid and therefore can be used to measure accurate pMHC monomer dissociation kinetics on living CD8⁺ T cells (35). To this end NTAmers are prepared with Cy5 labeled monomers. CD8⁺ T cells are stained in the cold and the cell-associated PE and Cy5 fluorescences are measured before and after addition of imidazole (Figure 4E). After very rapid disintegration of the NTAmers,

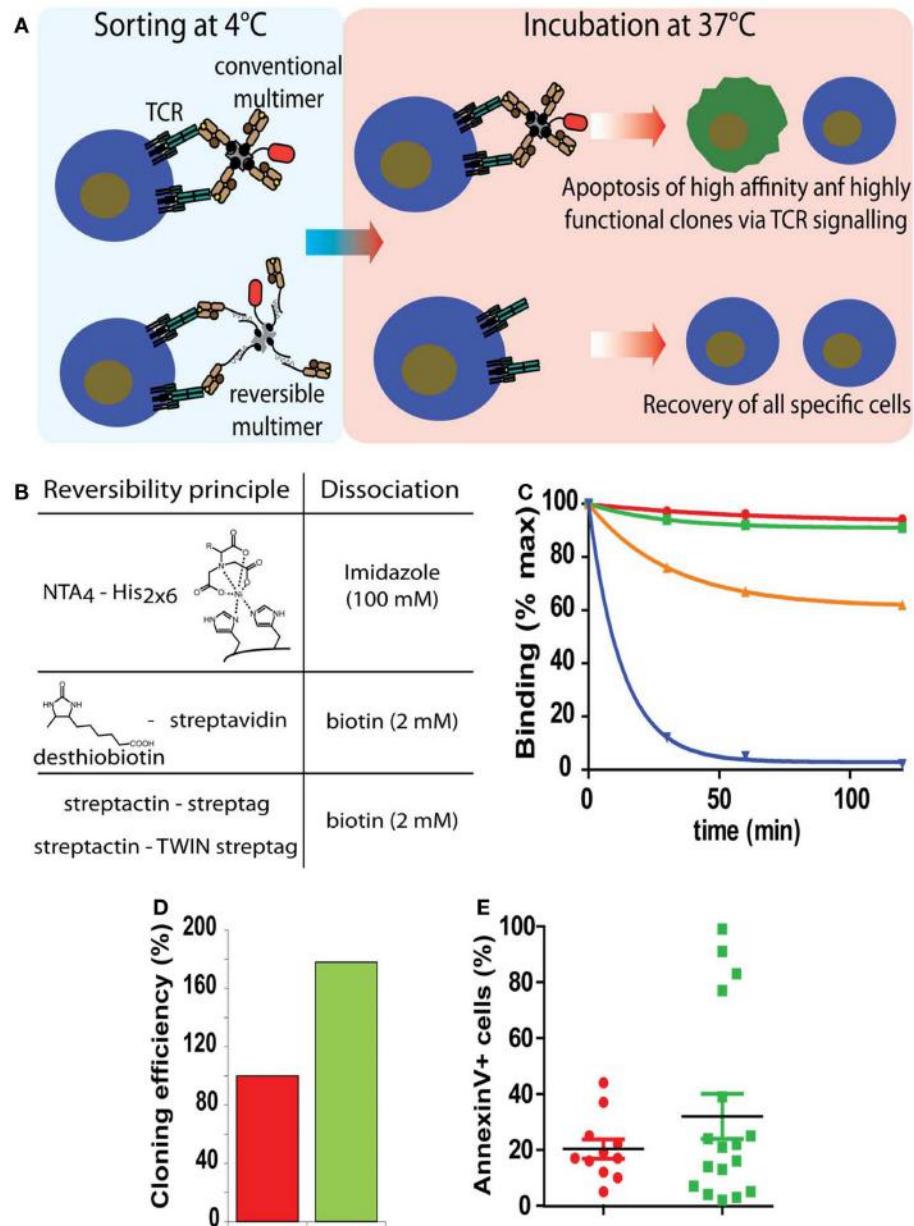


FIGURE 3 | Sorting of antigen-specific T cells with reversible pMHC multimers. **(A)** Cartoon illustrating the principle of sorting of “untouched” T cells. After cell staining and sorting, reversible, but not conventional multimers can be removed and the T cells cultured at 37°C in the absence of potentially harmful pMHC complexes. **(B)** Representation of the three types of reversible bonds used for the preparation of pMHC oligomers and agent used to provoke dissociation. **(C)** Flu matrix₅₈₋₆₆ peptide stimulated PBMC were stained at 4°C with A2/Flu multimers containing conventional

irreversible (red), NTA₄-His (green), streptactin-streptag (orange), or desthiobiotin-streptavidin (blue) scaffolds, washed and cell-associated fluorescence measured after different periods of incubation at 20°C. **(D)** Alternatively cells labeled with BSP (red) or NTAMers were FACS sorted and cloned by limiting dilution. Hundred percent refers to the number of clones obtained from BSP multimer sorted cells. **(E)** Randomly chosen clones were incubated with BSP multimer at 37°C, stained with AnnexinV, and analyzed by flow cytometry. Presented data were derived from (35).

reflected by rapid disappearance of PE fluorescence, pMHC monomer dissociation from cells can be determined by from the decrease of cell-associated Cy5 fluorescence. While dissociation kinetics with pMHC multimers tend to be inaccurate due to incomplete dissociation and heterogenous composition, pMHC monomer dissociation reaches baseline and is more accurate

(Figures 4F,G). This technique is less sensitive than SPR measurements, and inaccurate in the case of rapid kinetics, but provides information on pMHC-TCR interactions on cells, namely in the presence of CD8. Comparison of data obtained on a wide range of TCR-transduced cells, with NTAMers or directly by SPR showed a good correlation (unpublished data).

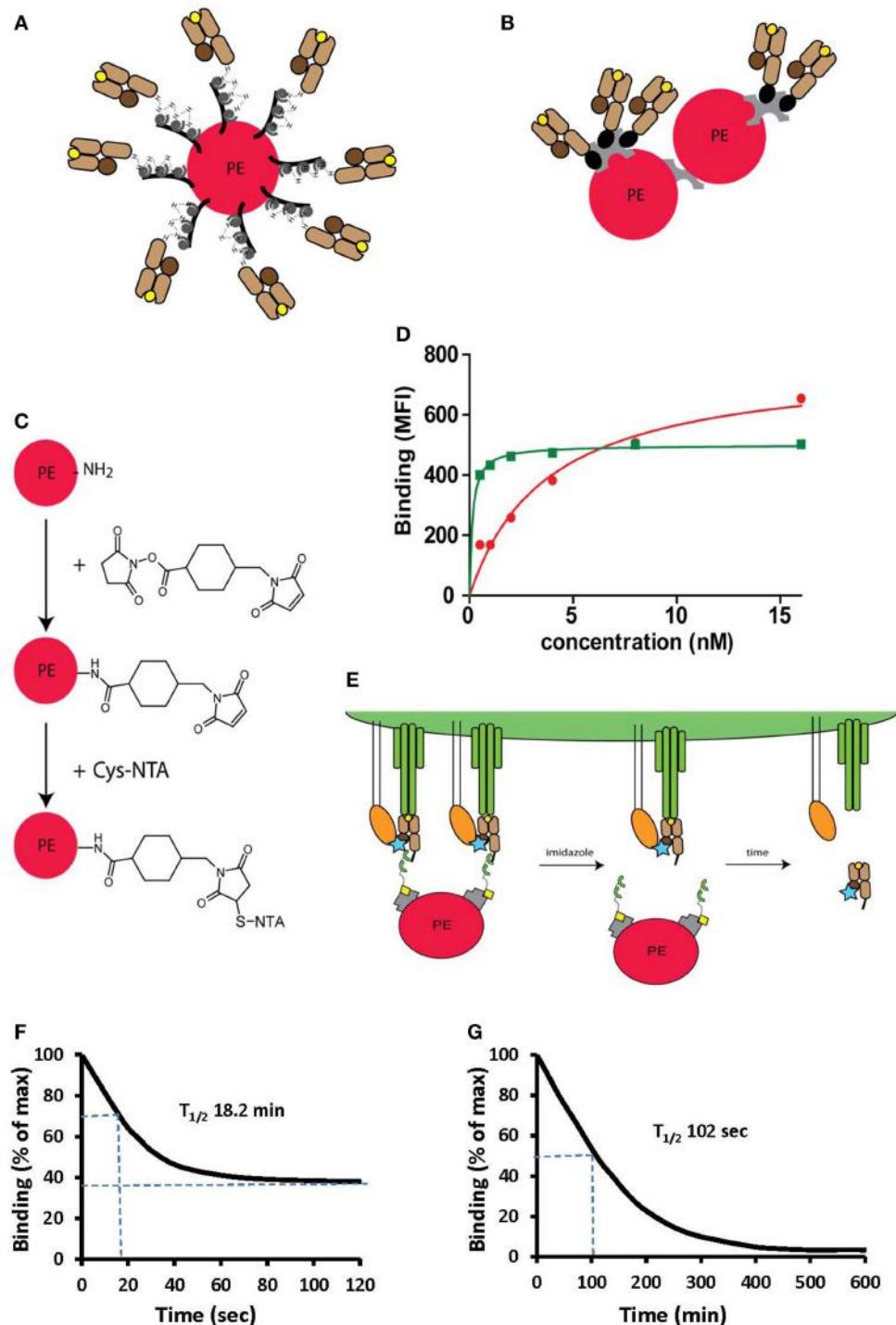


FIGURE 4 | Peptide-MHC oligomers built on switchable Ni^{2+} -NTA-His-tag chelate complexes. (A,B) Cartoons illustrating conventional BSP multimers pMHC **(B)** and reversible monomers directly coupled on PE via the NTA strategy **(A)**, where PE designates phycoerythrin. **(C)** The structure of a phycoerythrin molecule is shown in which lysine side chains reacted with a maleimido-*N*-hydroxysuccinimide ester and the resulting maleimido-PE will subsequently react with thio-NTA derivative. **(D)** Room temperature isotherms were shown for HLA-A2-Flu matrix₅₈₋₆₆ BSP multimers (red circles) or PE-NTAmer (green squares) as assessed on Flu peptide stimulated PBMC. **(E)** Cartoon illustrating the two-step dissociation of PE labeled pMHC NTamer

from T cells. Imidazole induced rapid decay of cell-associated NTAmers and disappearance of PE fluorescence, followed by slower dissociation of Cy5 labeled blue monomers from the cells. **(F)** Cloned Flu matrix-specific CD8⁺ T cells were stained in the cold with conventional A2/Flu₅₈₋₆₆ PE multimers, washed and cell-associated PE fluorescence measured by flow cytometry after the indicated periods of time. **(G)** Alternatively the cells were labeled with PE-NTAmers containing Cy5 conjugated A2/Flu₅₈₋₆₆ monomers and the monomer dissociation from the cells was assessed by flow cytometry as illustrated in **(E)**. The inserted numbers indicate the dissociation half-lives $T_{1/2}$. Presented data were derived from (33) and (35).

IMPROVED DETECTION AND ANALYSIS OF ANTIGEN-SPECIFIC CD4⁺ T CELLS BY MOLECULARLY DEFINED MHC CLASS II MULTIMERS

The performance of pMHC II trimers lags behind the one of pMHC I trimers, in particular they often fail to detect low avidity antigen-specific CD4⁺ T cells, which limits integral analysis of CD4⁺ T cell responses. Factors accounting for poor pMHC II trimer staining include (i) the coreceptor CD4, unlike CD8 fails to increase the pMHC binding avidity, (ii) certain types of glycosylation and sialylation of CD4⁺ T cell molecules, (iii) lower affinity of MHC class II-peptide restricted TCRs, (iv) conformational diversity of pMHC complexes, and (v) poor quality of pMHC II trimers (61). While refolding of pMHC I complexes provides highly pure monomers, production of pMHC II monomers by loading of “empty” MHC II molecules with peptides of interest does not and therefore the corresponding pMHC trimers contain a reduced fraction of active monomeric complexes. The fraction of correctly loaded pMHC II monomers depends on the peptide and the MHC class II allele and can be well below 50% (62, 63). Tethering the peptide on the MHC II beta chain does not circumvent this caveat. Different strategies were described that allow isolation of molecularly defined pMHC II monomers. Peptide-MHC II trimers containing defined fractions of cognate and non-cognate monomers were used to investigate the dependence of pMHC II trimer staining of CD4⁺ T cells on pMHC II monomer purity. The efficiency of trimer staining of antigen-specific CD4⁺ T cells depends on the trimer’s monomer purity; it decreases dramatically with the monomer purity for low avidity CD4⁺ T cells, but much less for high affinity cells. This explains why most successful pMHC II trimer staining reported thus far concerns pathogen-specific CD4⁺ T cells and not tumor antigen-specific ones, which typically are of low avidity. The use of molecularly defined pMHC II trimers permits conclusive analysis of low avidity CD4⁺ T cells (64).

PEPTIDE-MHC II PRODUCTION

Soluble recombinant MHC II molecules typically consist of alpha and beta chain where transmembrane and cytoplasmic domains of which have been replaced by acidic and basic leucine zippers (10). With very few exceptions soluble MHC II molecules are expressed in insect cells, either stably in *Drosophila* Schneider cells or transiently in sf9 cells using baculo virus (11, 61). Because in insect cells there is no MHC II-peptide loading apparatus, nor any retention mechanisms, they are typically secreted in “empty” form, i.e., without a nominal peptide cargo (12). These soluble MHC II molecules are purified usually by means of affinity chromatography using anti-MHC II antibody columns (65). Even though this provides highly pure MHC II proteins, the conditions used for elution, such as extreme pH, are prone to cause partial denaturation, which is an important factor for incomplete peptide loading of “empty” MHC molecules. In order to circumvent this, soluble pMHC molecules have been made containing a C-terminal His-tag, which allows gentle affinity purification at neutral pH on chelating columns, e.g., NTA or IDA columns. Loading of peptides of interest onto “empty” pMHC molecules is usually accomplished at reduced pH and elevated temperatures (e.g., pH 5.5–6, 30–37°C

for 24–72 h) and is on faith, i.e., without knowledge of the extent of loading.

The peptide loading efficiency can be assessed by means of a peptide tag, usually an N-terminal one, such as His-tag, dinitrophenyl (DNP), or biotin (31, 66, 67). Such studies indicated that the degree of peptide loading can vary over a wide range and get as low as 10–20%. In addition peptide tags allow isolation of correctly loaded pMHC complexes, henceforth referred to as immunopure pMHC complexes (31). While this is attractive it renders the synthesis of pMHC class II trimers more tedious; importantly, the addition peptide tags may affect CD4⁺ T cell antigen recognition as suggested by observations that peptide truncations can have significant effects on CD4⁺ T cell antigen recognition (68–70). Therefore the use of conditional peptide tags would be desirable and could be prepared similarly as conditional peptide ligands, namely by linking it to the peptide via a photocleavable residue such as 2-[2-nitro-phenyl]-propionic acid (71, 72).

An alternative strategy to prepare soluble pMHC monomers is by tethering a peptide of interest to the N-terminus of the beta chain via a long and flexible linker (10). This method allows the preparation of murine pMHC class II monomers and trimers, which are difficult to obtain via peptide loading, as “empty” mouse MHC II molecules are unstable. It is important to note that tethered on peptides may or may not be properly nested in the MHC II’s binding groove and that therefore the same concerns of denaturation, namely those related to immunoaffinity chromatography are applicable as mentioned above. Moreover, it has been reported that depending on peptide truncations and linker length the tethered on peptides may bind in different registers and hence be differently recognized by antigen-specific CD4⁺ T cells (30, 62).

A major shortcoming of pMHC II trimers is that they fail to stain a fraction of antigen-specific CD4⁺ T cells (29). This is especially a concern for tumor and self-antigen-specific CD4⁺ T cells, which tend to be of low avidity, due to deletion of high affinity cells by thymic negative selection and much less for pathogen-specific CD4⁺ T cells (29). This shortcoming curtails the usefulness of pMHC II trimers especially in research related to cancer or autoimmunity (63, 73), because these fields of research become increasingly more important, we review in the following sections studies related to improving pMHC class II staining performance especially on low avidity CD4⁺ T cells.

ON THE USE OF PEPTIDE TAGS TO ASSESS MHC II-PEPTIDE LOADING AND TO ISOLATE IMMUNOPURE MONOMERS

In order to be able to measure peptide loading and to purify correctly loaded pMHC II complexes, several peptide tags have been described; for example: (1) An N-terminal dinitrophenyl (DNP) residue, allowing ELISA detection and isolation of DNP-pMHC complexes by means of anti-DNP mAb. This technique is expensive and addition of DNP renders poorly (Figure 5A) soluble peptide completely water insoluble (74). (2) N-terminal His₆-peptide tags have been used that allow detection and isolation of specific pMHC complexes by means of chelators, such as Ni²⁺-NTA or IDA (31, 75). Advantages of this strategy include facile peptide synthesis and improved solubility of peptides and inexpensive availability of chelators for affinity chromatography and ELISA applications. Disadvantages include (i) losses due to inadequate binding affinity,

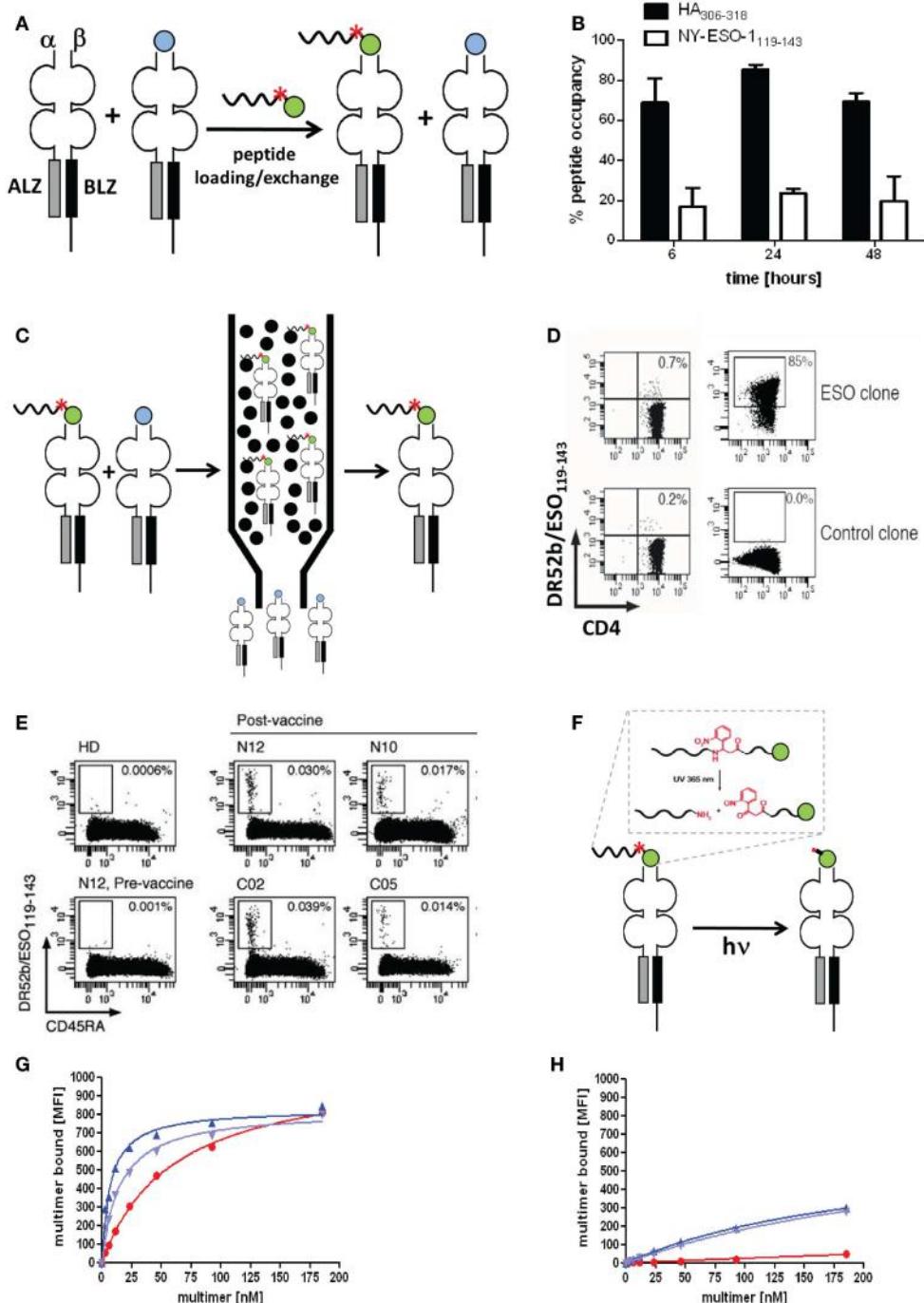


FIGURE 5 | Molecularly defined pMHC class II multimers detect low avidity CD4⁺ T cells. (A,C,F) Cartoons illustrating the use of photolabile (red asterisk) peptide tags to discriminate pMHC II complexes containing the peptide of interest (green ball) or irrelevant peptides (blue ball) (A), to affinity purify correctly loaded complexes (C) and to remove the peptide tag, either His₆ or desthiobiotin that is linked to the peptide via a β-nitrophenylglycine residue, which can be cleaved by UV irradiation. (B) “Empty” HLA-DR4 was incubated for 6, 24, and 48 h at 37°C at pH 6, 32°C for 4 h with His₆-HA₃₀₆₋₃₁₈ (black bars) or His₆-NY-ESO-1₁₁₉₋₁₄₃ (white bars). Peptide occupancy was calculated as (amount of His₆-peptide bound to the DR4)/(amount of total DR4). After gel-filtration the peptide occupancy was determined by ELISA using affinity-purified complexes as reference (100%).

(D) Staining of DR52b/NY-ESO-1₁₁₉₋₁₄₃-specific (top) or an irrelevant (SSX2-specific) CD4⁺ T cell clone (bottom) were stained with 5.6 nM of conventional (left) and “immunopure” DR52b/NY-ESO-1₁₁₉₋₁₄₃ multimers (right) at 37°C for 1 h and analyzed by flow cytometry. (E) Ex vivo CD4⁺ T cells from PBMC of a DR52b⁺ healthy donors (HD) or of DR52b melanoma patients either pre- or post-NY-ESO-1 vaccination were stained as in E plus anti-CD45RA antibody and analyzed by flow cytometry. (G,H) The 37°C binding isotherms of “immunopure” DR4/HA₃₀₆₋₃₁₈ (blue) and conventional multimers (red) on the DR4/HA₃₀₆₋₃₁₈-specific clones 9 (G) or 8 (H). The immunopure multimers contained [dark blue or not (light blue)] the N-terminal His₆-tag that was used for affinity purification. Presented data were derived from (31).

(ii) backgrounds due to non-specific co-ligation, and (iii) incompatibility with multimer formation based on His-tag-NTA chelate complexes (35). (3) Biotin or desthiobiotin tag can be used for the detection of specifically peptide loaded pMHC II-peptide complexes. Disadvantages of these strategies include: (i) incompatibility with tetramer formation by means of biotin and streptavidin; (ii) for affinity purification of specifically peptide loaded pMHC complexes, biotin binding tends to be too strong and DTB binding too weak (unpublished data); and (iii) aggravation of peptide solubility problems by addition of biotin.

Previous studies with His₆ or Cy5.5 tagged peptides revealed that peptide loading to “empty” HLA-DR4 and DR52b molecules is remarkably variable. As shown in **Figure 5B** the viral Flu HA peptide bound to over 80% of “empty” DR4 molecules, whereas the tumor antigen NY-ESO-1 peptide bound to only 20%. Similar striking differences were observed for other viral and tumor antigen derived epitopes on “empty” DR4, DR52b, and DP4 molecules, with the viral peptides typically being the better binders. It may be speculated that viruses that are older than adaptive immunity of mammals, like the influenza virus, actively participated in the evolutionary shaping of the MHC locus.

By means of an N-terminal His₆-peptide tag and IDA chromatography immunopure DR52b/NY-ESO-1_{119–143} monomers were produced and with these PE labeled multimers, which exhibited dramatically improved staining a cloned ESO-specific CD4⁺ T cells as compared to conventional DR52b/NY-ESO-1_{119–143} multimers (**Figures 5A,C–E**) (31). More importantly, these immunopure multimer allowed detection and detailed phenotypic and repertoire analysis of DR52b/NY-ESO-1_{119–143} specific CD4⁺ T cell in PBMC from melanoma patients post, but not pre vaccination with NY-ESO-1 protein. In view of numerous reports indicating that residues flanking the peptide binding core sequence can sensibly affect their interaction with MHC class II molecules, we established a procedure to remove the peptide tag after affinity purification. To this end we used photocleavable 2-[2-nitro-phenyl]-propionic acid to link the His₆-peptide tag to the HA_{306–318} peptide, which allowed removing this tag by UV irradiation at nanometers (**Figure 5F**). The photocleavage is rapid (~4 min using a 45 W mercury fluorescence lamp emitting at (365 ± 40 nm) and nearly quantitative (~90%) (data not shown). We then used this technique to prepare immunopure DR4/HA_{306–318} multimers and compared their staining of cloned, flu-specific CD4⁺ T cells before and after removal of the His₆-peptide tag. The multimer staining on one clone was modestly higher when the His₆-tag was present, but was unaffected on another clone, indicating that this tag may affect pMHC II binding and/or recognition on some, but not other CD4⁺ T cell clones. What this experiment also shows is that the immunopure multimers bind significantly more avidly than the conventional multimer and that this difference is especially notable on low avidity clones, which are not significantly detected by the normal multimers (**Figures 5G,H**).

DETECTION LIMITS OF pMHC II MULTIMERS AND HOW THEY CAN BE INCREASED

A major shortcoming of pMHC II multimers is their failure to detect some antigen-specific CD4⁺ T cells. Studies using 2D

binding assays it has been shown that a substantial fraction of antigen-specific CD4⁺ T cells responding in functional assays are not detected by tetramer staining, but are visible in a 2D binding assay (29). Interestingly comparison of CD4⁺ T cells specific for a self versus viral antigens showed that the proportion of tetramer undetectable CD4⁺ T cells is higher for the latter than the former. This is consistent with the view that for self-antigens CD4⁺ T cells expressing high affinity TCR are deleted by negative thymic selection (76). It is important to note that the vast majority of tumor antigens are self-antigens and that therefore tumor antigen-specific CD4⁺ (and CD8⁺) T cell responses tend to be in average of lower avidity than viral specific CD4⁺ T cells.

While on CD8⁺ T cells pMHC binding is substantially strengthened by coordinate binding of CD8 to TCR-associated pMHC complexes, CD4 does not significantly contribute to pMHC binding (10). In addition, pMHC I complexes obtained by refolding all contain the peptide of interest; because this is not the case for pMHC II complexes obtained by loading of empty MHC II molecules, CD4⁺ T cell tetramer staining can be compromised by incomplete peptide loading. To investigate this, tetramers were prepared that contained defined proportions of immunopure cognate and non-cognate pMHC monomers. Using binomial distribution the average contents of cognate pMHC II monomer, dimer, trimer, and tetramer were calculated for different ratios of cognate/non-cognate complexes used to make the tetramer (**Figure 6A**); from these four different binding curves can be calculated for the scenarios that a tetramer can stain a CD4⁺ T cell when it contains (1) one, two, three, or four or (2) two, three, or four or (3) three and four or (4) only four cognate monomers (**Figure 6B**). Staining of seven randomly chosen DR4-restricted Flu HA_{306–318} specific CD4⁺ T cell clones (**Figure 6C**) with tetramers containing graded fractions of cognate, immunopure DR4-HA_{306–318} monomer, showed that such binding curves are found, i.e., that clones can be grouped in different discrete avidity ranges (**Figures 6D–F**). Importantly, this shows that low avidity CD4⁺ T cells will not be detected by tetramer staining, unless these contain only cognate pMHC monomers (**Figure 6F**). In view of the fact that pMHC II tetramer staining risks to miss a substantial fraction of antigen-specific CD4⁺ T cells, the use of immunopure tetramers is highly recommended in order to detect low avidity cells (**Figures 6G,H**).

The tetramer staining efficiency also critically depends on CD4⁺ T cells surface glycosylation and pretreatment of cells with a broadly active neuraminidase (e.g., from *Vibrio cholerae*) can increase tetramer staining by nearly 100% (77, 78). Testing on 37 Flu-specific Th1 clones we found increases of tetramer staining upon desialylation in the range of 20–95% (unpublished data).

CONCLUSION

Wide ranges of soluble pMHC I-peptide oligomers have been prepared and used to analyze, activate, inactivate, and sort antigen-specific CD8⁺ T cells. Well-defined pMHC dimers, tetramers, and octamers containing linkers of defined length and flexibility were instrumental in dissecting mechanisms governing pMHC binding by CD8⁺ T cells and the ensuing cell activation and eventual cell death. Reversible multimeric pMHC complexes emerged as useful reagents to sort “untouched” CD8⁺ T cells; of particular

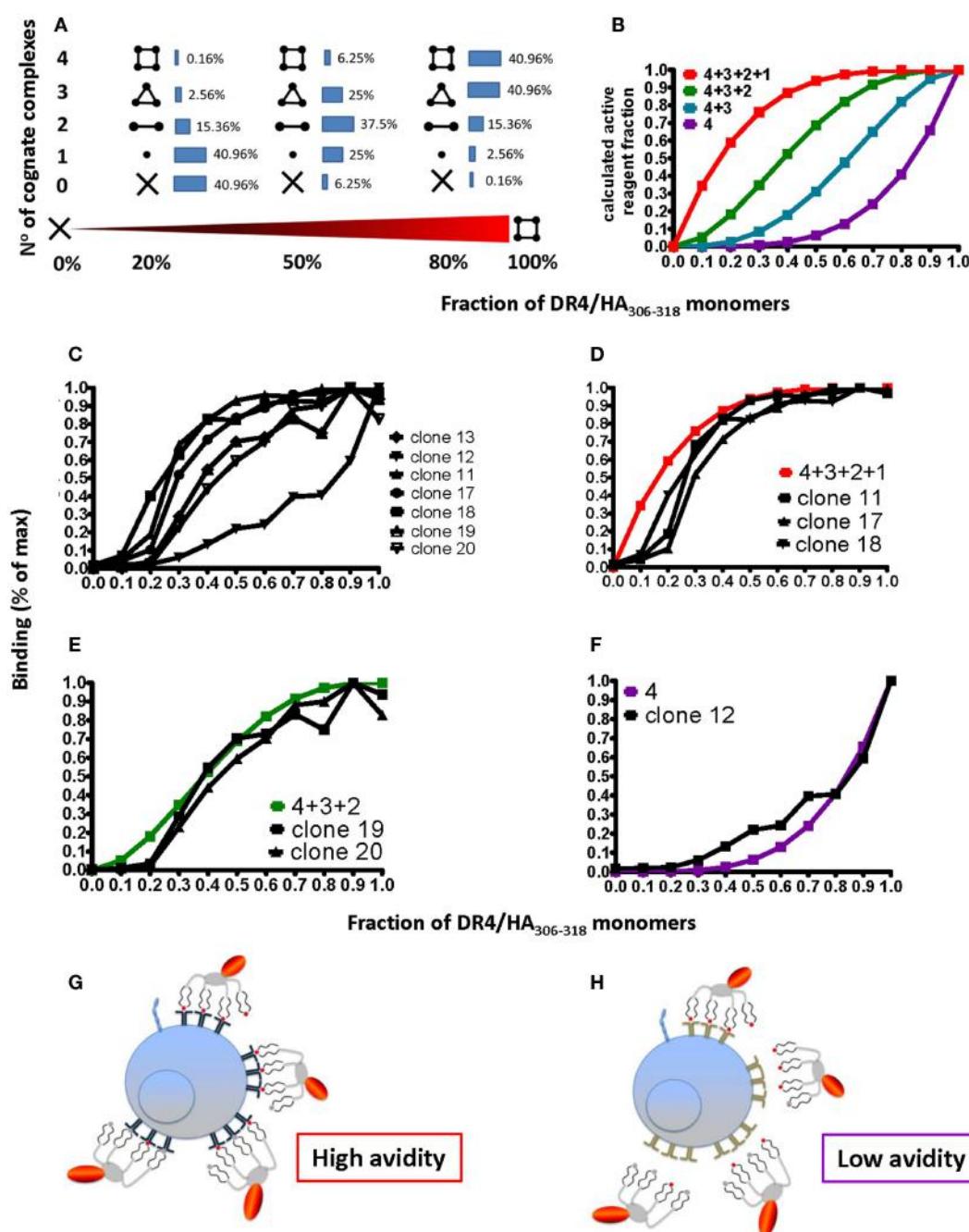


FIGURE 6 | Degree of peptide loading determines the avidity detection threshold on pMHC II multimer detection. **(A,B)** Cognate (DR4/HA₃₀₆₋₃₁₈) and irrelevant (DR4/CLIP) pMHC II monomers were mixed in different ratios (x-axis; fractions of one) and reacted with streptavidin-PE to make tetramers. For 0, 20, 50, 80, and 100% of cognate monomers the compositions of tetramers containing $X=0, 1, 2, 3$, or 4 cognate monomers were calculated according to the binomial distribution and expressed in %, indicated by the inserted numbers **(A)**, with 100% being the sum of each row; alternatively it was plotted on the y-axis as fraction of 1 against the fraction of cognate/non-cognate complexes (x-axis) for the indicated four avidity levels T cell binding (red: all

four complexes bind; green: three; blue: two; and purple: one). **(C)** Seven DR4/HA₃₀₆₋₃₁₈-specific CD4⁺ T cell clones were incubated with 18.5 nM DR4 tetramers with DR4 tetramers containing the indicated fractions of HA₃₀₆₋₃₁₈ and CLIP peptides (x-axis; fraction of one) for 1 h at 37°C and cell-associated fluorescence assessed by flow cytometry and expressed in % of maximal binding (y-axis). **(D–F)** From these binding isotherms those observed on the clones 11, 17, and 18 match the high avidity binding curve (in red) **(D)**, those recorded on the clones 19 and 20, match the green curve **(E)** and the one measured on the clone 12 the purple curve **(F)**. **(G,H)** The cartoon representation of high **(G)** and low avidity **(H)** CD4⁺ T cells as defined by their ability to bind mixed tetramers.

interest were “switchable” NTAMer that additionally allowed measurement of pMHC monomer dissociation kinetics on living cells and detection of low avidity T cells. Detection of antigen-specific CD4⁺ T cells by pMHC II multimers overall was more challenging and prone to miss significant fractions of cells, namely low avidity CD4⁺ T cells; detection of these cells was dramatically improved by using molecularly defined multimers.

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Monitoring the dynamics of T cell clonal diversity using recombinant peptide:MHC technology

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The capacity to probe antigen specific T cells within the polyclonal repertoire has been revolutionized by the advent of recombinant peptide:MHC (pMHC) technology. Monomers and multimers of pMHC molecules can enrich for and identify antigen specific T cells to elucidate the contributions of T cell frequency, localization, and T cell receptor (TCR) affinity during immune responses. Two-dimensional (2D) measurements of TCR-pMHC interactions are at the forefront of this field because the biological topography is replicated such that TCR and pMHC are membrane anchored on opposing cells, allowing for biologically pertinent measures of TCR antigen specificity and diversity. 2D measurements of TCR-pMHC kinetics have also demonstrated increased fidelity compared to three-dimensional surface plasmon resonance data and are capable of detecting T cell affinities that are below the detection level of most pMHC multimers. Importantly, 2D techniques provide a platform to evaluate T cell affinity and antigen specificity against multiple protein epitopes within the polyclonal repertoire directly *ex vivo* from sites of ongoing immune responses. This review will discuss how antigen specific pMHC molecules, with a focus on 2D technologies, can be used as effective tools to evaluate the range of TCR affinities that comprise an immune response and more importantly how the breadth of affinities determine functional outcome against a given exposure to antigen.

Keywords: kinetics, 2D assays, T cell activation, recombinant pMHC, T cell affinity

DETECTION OF ANTIGEN SPECIFIC T CELLS

The ability to mount an effective immune response is essential to the survival of a living organism. Adaptive immunity in particular allows vertebrates a defense mechanism against countless pathogens. Antigen receptors on lymphocyte surfaces allow for recognition of a specific target, leading to activation and subsequent expansion of effector cells. This process is heavily dependent on affinity and on/off rate binding kinetics of the receptor for antigen. Though it is generally accepted that the highest affinity and thus most fit lymphocytes are selectively expanded (1, 2), the exact affinities of lymphocytes needed for an optimal immune response are still unknown.

During the course of a B cell response, somatic hypermutation in the germinal center allows for editing of the B cell receptor (BCR) to improve the affinity of the responding cells. This process involves the introduction of random mutations in the antigen binding site that can result in enhanced recognition of the target antigen. B cell affinity maturation allows higher affinity cells to outcompete less fit, lower affinity clones. While T cells also selectively expand responders based on specificity for antigen, T cells do not undergo receptor editing to improve the specificity of the response. Of interest, some reports have shown that mature T cells can re-express V(D)J recombination machinery and facilitate rearrangement of the T cell receptor (TCR) (3, 4). As the concept of TCR editing in the periphery may require further investigation, this review will assume that the TCR is fixed once the T cell has matured and entered the periphery. From the predetermined TCR

repertoire, mature T cells are still able to generate diverse antigen specific polyclonal responses. This leads to the questions of what affinity range defines an optimal T cell response and what technology is best suited to evaluate this aspect of T cell diversity.

One way to detect diversity of the TCR repertoire is through the analysis of antigen driven changes in V β chain usage and complimentary determining region (CDR3) sequences during the course of an immune response. The $\alpha\beta$ chains of the conventional TCR are encoded by V, D, J genes. Recombination of these gene segments concomitant with nucleotide insertions and imprecise joining events yields highly diverse T cell receptors. The CDR3 region, formed from the joining of the $\alpha\beta$ TCR chains, directly contacts the antigen in the binding groove and thus reflects the antigen specificity of the clone (5). Studies show that during the course of an immune response, certain V β chains are preferentially expanded to create a unique signature of antigen specificity and clonal dominance of an immune response (6–8). Spectratyping or immunoscope analysis is a technique in which the sequence length of the CDR3 is derived from the DNA of bulk clonal populations typically identified by V β usage (9). CDR3 sequence length has been used to subset and characterize T cell clonal populations for specific antigens (10, 11). Tracking CDR3 lengths and V β profiles can also provide insight in monitoring disease progression and for diagnostic purposes (12–15). Though repertoire analyses via these methods have revealed useful information, they lack the fine resolution to assess the diversity of a T cell clonal response. For example, these methods are primarily done on bulk cellular

populations resulting in conclusions based on a population average rather than on individual clones. More recent inquiries have shown this critical limitation fails to identify the paired TCR α and β chains responsible for the antigen recognition (16) and as a result, attempts to modify the techniques for single-clone analysis are being pursued (17). Future research combining single-cell analyses of TCR $\alpha\beta$ chain usage along with functional readouts and kinetic measurements will greatly enhance our knowledge of the T cells that comprise the polyclonal repertoire.

The detection of antigen specific T cells concomitant with the characterization of their functional responsiveness has been key to providing insights into the factors that promote pathogenic specific and protective immunity. Historically, the tracking of antigen specific T cells in a polyclonal environment has been performed with functional assays assessing proliferation, production of cytokines, cytotoxic mediators, and protein markers of cell activation. These indirect markers are important for characterizing T cell phenotype but may poorly represent the entire polyclonal repertoire because detection depends on antigen dose utilized in the assay as well as the efficiency of the assay itself. Stimulation with high dose, cognate antigen may negatively bias T cell detection toward a low affinity profile by eliminating the higher affinity clones though activation induced cell death (18), while low dose cognate antigen may selectively detect cells with higher affinity TCR. Therefore, a direct means for quantifying antigen specific T cells utilizing recombinant cognate or variant peptide:MHC (pMHC) molecules could provide more sensitive analytical tools for assessing the complexity of the entire responding T cell population (19).

The development of recombinant pMHC molecules for detection of a myriad of MHC class I and II epitopes from both foreign (bacteria, viruses, and parasites) and self proteins (tumors and targets of autoimmune attack) provide a method for specific assessment or targeting of the T cell repertoire. Multimers of pMHC, especially the biotin:streptavidin-based pMHC tetramer technology, provide accessible tools to determine the frequency of antigen specific T cells via flow cytometry (19) and to deplete antigen specific T cells *in vivo* (20, 21). Importantly, tetramers are useful for enumerating and enriching antigen specific T cells. The fluorophore attached to tetramers can allow for the “pull down” or enrichment of antigen specific cells from a polyclonal population for downstream applications such as determining precursory frequency of tetramer positive cells (22, 23). The efficiency of detection by multimers is due to the aggregation of TCR:antigen interactions that increase avidity and circumvent the short half life of interactions between TCR and pMHC (19, 24). MHC class I and II tetramers are the most commonly utilized multimer largely because monomers and dimers exhibited insufficient binding kinetics for TCR to facilitate detection by flow cytometry and were less stimulatory than tetramers (25). Advancements on multimer technology have been focused on increasing avidity through creation of progressively higher order oligomers, most notably the commercially available 5-armed pentamers (26–28) or 10-armed dextramers (29, 30). Despite the increased avidity provided by these reagents, multimers of higher order magnitude beyond pentamers provide, at most, modest increases in sensitivity of T cell detection (29, 30), possibly due to the physical constraints needed

for multiple simultaneous TCR–pMHC interactions (31). Even in the case of pMHC tetramers, it is unlikely that all four monomers bind simultaneously due to steric hindrances (25, 32).

The efficiency of pMHC molecules to detect antigen specific T cells is also dependent on peptide orientation within the MHC groove. Peptide-MHC anchor residues, which typically lie at positions 1, 4, 6, and 9 of the core peptide for MHC class II, are key to the stability of the peptide within the MHC. Variations in the amino acid residues that contact MHC, termed MHC variant peptides, can weaken or stabilize the interaction between TCR and pMHC (33–36). Though MHC variant peptides have been used to stabilize interactions with MHC to enhance T cell detection, these modifications could confound downstream analyses. For instance, these changes could modify the secondary structure, altering the TCR contact residues (37, 38) and may impact accurate kinetic and functional analysis. Furthermore the non-covalent interactions between peptide and MHC class II are of particular concern because the binding groove is open at both ends and can allow for the peptide to slide into different binding registers and influence TCR detection of the pMHC complex (39). For example, we and others identified three to four peptide registers in the well described OVA_{323–339} 17-mer peptide (40, 41) that have made uniform recombinant pMHC monomer production and especially the creation of tetramers somewhat difficult (41). One method to improve the tetramer production is through the use of a limited set of linker amino acids used to covalently attach peptide to the N-terminus of the MHC class II molecule (42). In addition, multiple binding registers can be limited by creating a disulfide bond or “lock” engineered via a cysteine residue on the peptide and on the MHC as reported for insulin B_{9–23}, OVA_{323–339}, and HA_{126–138} peptides (41, 43). Despite the effective use of recombinant pMHC and tetramers for the identification of antigen reactive T cells, their use as direct measures of TCR frequency and affinity during an immune response can be problematic.

MEASURING TCR AFFINITY FOR pMHC

A critical determinant for an antigenic response is the strength of signal derived through the TCR (44, 45). Although many factors contribute to the translation of signals into a biological response (i.e., costimulation (46), duration of signal (47, 48), etc.), affinity is a major parameter that establishes and controls the contribution of all additional factors in this response. Characterization of T cell response dynamics requires methods to obtain biophysical measures of affinity and kinetic on/off rates between TCR and recombinant pMHC. Many of the models describing T cell activation have been postulated based on kinetic-binding data from three-dimensional (3D) and two-dimensional (2D) binding assays.

Purified TCR and pMHC proteins can be used to study binding kinetics in 3D using techniques such as surface plasmon resonance (SPR). In this case, TCR and pMHC protein interactions occur in a fluid filled 3D space and affinity is measured in terms of the molar concentration needed to generate binding; TCR-pMHC affinity can range from 1 to 100 μ M while the half life of the interaction can range from 10 to 100 s (49–52). SPR analysis provided the biophysical basis for models correlating TCR binding kinetics and T cell triggering in order to explain the functional

differences seen between agonists, weak agonists, and antagonists (53–55). The most popular models are the kinetic proofreading and kinetic discrimination models, which ascribe optimal T cell responses to binding kinetics that allow sufficient time for TCR triggering (53, 54, 56, 57). Ligands that stimulate outside of this optimal time range, i.e., too long or too short, will not lead to a productive response according to these models. Despite the accuracy of these models in predicting agonist responses, several instances were identified where the biophysical measures did not relate to T cell activation state, particularly in response to weaker ligands (51, 57–62). These exceptions raised questions regarding the accuracy of 3D kinetic measurements derived from purified molecules to reflect the kinetics of proteins within the membrane environment. 3D assays are also limited in their ability to assess the full scope of a response due to the difficulty in purifying TCR from all participating antigen specific T cells. Therefore, alternative technologies are needed to probe the breadth of a polyclonal T cell response.

Analysis of receptor/ligand interactions using 2D technologies provides a physiologically relevant context in which to assay TCR affinity and the scope of polyclonal T cell responses because the TCR and pMHC are bound within cell membranes. Therefore these assays, namely the fluorescent based assays of FRAP and FRET, as well as the mechanical based micropipette techniques, biomembrane force probe and flow cell, can potentially better interrogate T cell kinetics with pMHC (63–69). The interactions between pMHC and TCR were found to occur more rapidly when analyzed in 2D rather than 3D, lending support to the serial triggering model where high affinity interactions generate fast off rates and rapid on-rates amenable for sampling multiple pMHC (69, 70). For the most part, one cannot readily convert the 2D area based measurements to 3D volume based affinities and on rates. A conversion of affinity from 2D FRET data to 3D measurements was suggested based on approximations of the contact area and intercellular volume between the T cell and surrogate APC bilayer (66). The approximations of contact area and intercellular volume are difficult to attain for T cells, which possibly explains why there is a discrepancy between the converted 3D K_d and SPR values for the MCC agonist and T102S weak agonist peptides. In contrast, the 2D and 3D half-life measurements are comparable because they are reported in units of time, yet in 2D, the time of interaction is more rapid than found in 3D analyses (66, 70).

We have focused on the mechanical 2D micropipette adhesion frequency assay as it provides a novel platform for evaluating T cell antigen specificity, frequency, and cross reactivity between epitopes within a polyclonal repertoire. Importantly, small numbers of T cells can be individually analyzed directly *ex vivo* from the blood and sites of ongoing immune responses. This assay allows for the visualization of TCR binding events with pMHC on opposing cells via a modified inverted microscope (Figure 1). The T cell and pMHC coated red blood cell are placed on opposing micropipettes and moved in and out of contact by means of a piezoelectric actuator for a defined contact and retraction cycle that will facilitate a binding event at equilibrium (71). A binding event is seen as distension of the RBC membrane on the video monitor as the cells are moved out of contact. The concentration of pMHC monomer coating on the RBC is optimized to yield an average binding frequency between 10 and 90% for several repeated

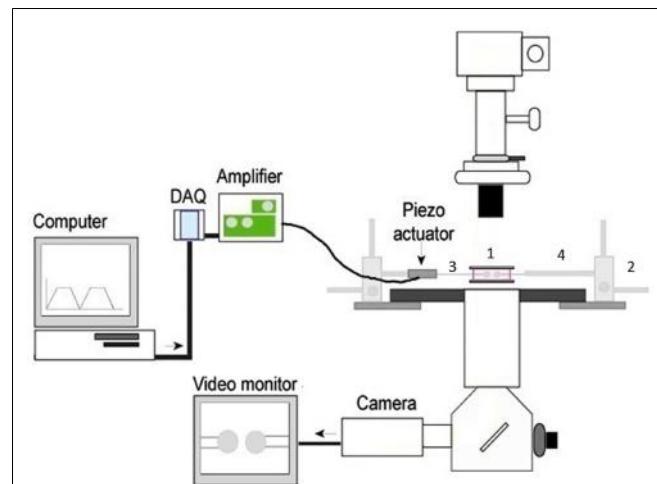


FIGURE 1 | The basic 2D micropipette adhesion frequency assay system. The foundation for this system is an inverted microscope. The stage has been modified with a metal adapter (1) to rest a media filled chamber containing the T cells and pMHC coated RBCs above the 100 \times oil immersion objective lens. The stage is also fitted with coarse micromanipulators (2) allowing for the movement of the micropipettes (3) within the chamber. The micropipettes are attached to a hydraulic pressure system (not shown) by means of a micropipette holder (4) allowing for individual cells to be aspirated and held within the chamber. A piezoelectric actuator is attached to one micropipette holder such that it can be moved in and out of contact with the opposing cell. The DAQ, digital acquisition board, converts the cyclical digital signal from a computer program to an analog voltage signal that drives the piezoelectric actuator. Cells can be visualized on the video monitor and adhesion events can be subsequently recorded.

contacts (usually 50 contacts). The micropipette assay is sufficiently sensitive to measure the binding of a single receptor-ligand bond, a feature that cannot be achieved with pMHC multimer technology. The higher sensitivity of the micropipette assay is not based on increased valency but likely due to the 2D orientation of the pMHC and TCR incorporated within the cell membranes. This closely replicates the interaction as it would occur between two cells and allows for measurement of TCR: pMHC kinetic parameters in a biologically relevant context. The effective 2D binding affinity, with a detection range from high to low, 10^{-2} to 10^{-7} μm^4 , is a composite term that incorporates the affinity (K_a) and the contact area (A_c) between the T cell and surrogate APC. Derivation of the effective 2D binding affinity ($A_c K_a$) requires quantification of the receptor density (m_r), the ligand density (m_l), and the frequency of adhesion (P_a) between the cells as represented by $A_c K_a = -\ln(1 - P_a)/m_r m_l$ (71, 72). The adhesion frequency assay is the fundamental model of 2D mechanical assays where binding frequencies can be used to derive affinities and on/off rate kinetic readouts (73). The biomembrane force probe is a modified adhesion frequency assay that can allow for detection of individual bonds with increased sensitivity for detecting faster on/off rates and it can be modified to readout the effects of force on the bond between TCR and pMHC (73). Furthermore, the 2D micropipette system can be altered to permit visualization of functional fluorescent readouts such as calcium signaling, which has already been integrated into the FRET based 2D assays (66). The capacity of

multiple readouts and increased sensitivity with the 2D mechanical tools is evidence for the power/significance of these techniques.

BREADTH OF AFFINITY IN THE POLYCLONAL REPERTOIRE

The affinity of TCRs for antigen can be discussed at both the single clone and population levels. A single TCR clone expands into multiple daughter cells that will possess a measurable but narrow range in affinity despite expressing an identical TCR. A polyclonal population of cells will possess a wider range or distribution of affinities comprised of all TCR clones activated to expand in response to any specific antigenic stimuli. Additionally, TCRs by their very nature are degenerate or crossreactive and can interact with many different peptide antigens. The estimated $\alpha:\beta$ TCR diversity is $\sim 10^{18}$, a seemingly large number that is significantly reduced to several hundred millions of T cells during thymocyte maturation (74, 75). Even with these reduced numbers, T cells still possess the ability to respond to most possible antigens. Therefore, the ability of a single TCR to recognize multiple antigens, albeit with varying degrees of affinity, is critical to increase the number of T cells that recognize each antigen.

The concept of TCR degeneracy is demonstrated by the capacity of monoclonal TCRs to recognize MHC variant peptides or altered peptide ligands, defined as epitopes with modified affinity for cognate TCR (76, 77). They also provide insight into the effective affinity range recognized by a single TCR. For instance, OT-1 CD8⁺ TCR transgenic T cells exhibit a V α 2/V β 5 rearranged TCR that recognizes the cognate SIINFEKL (OVA) peptide on H2-K b with a high 2D effective affinity ($\sim 10^{-3} \mu\text{m}^4$). Modifying this peptide sequence changes the affinity of OT-1 TCR for pMHC generating a breadth of affinities (70) that can alter downstream functional outcomes to yield agonist (A2, 2D affinity of $\sim 10^{-4} \mu\text{m}^4$), weak agonist (G4 and E1, 2D affinity of $\sim 10^{-5} \mu\text{m}^4$), or antagonist (V-OVA and R4, 2D affinity of $\sim 10^{-6} \mu\text{m}^4$) responses (70). Overall the OT-1 TCR exhibits an approximate, 1000-fold range in affinities depending on the peptide being presented by MHC class I. Additional T cells will have to be analyzed to determine whether this breadth of the 2D affinity range is characteristic of all CD8⁺ or CD4⁺ T cells.

The identification that a single TCR can exhibit a broad range of affinities to different peptide antigens led to the study of the array of affinities found within a polyclonal CD4⁺ T cell response against one peptide antigen. The breadth of 2D effective affinities for a single antigen within a polyclonal population exhibited a Gaussian distribution possessing a defined mean and standard deviation. For example, CD4⁺ T cells primed with the LCMV (lymphocytic choriomeningitis virus) GP₆₁₋₈₀ peptide epitope, showed between a 100- and 1000-fold range of affinities by the 2D micropipette assay with a mean of $4.21 \pm 1.48 \times 10^{-4} \mu\text{m}^4$ (78). A similar distribution and range with a lower mean affinity $1.63 \pm 0.48 \times 10^{-5} \mu\text{m}^4$ was also observed for the polyclonal response against the myelin oligodendrocyte glycoprotein self antigen MOG₃₅₋₅₅ (78). As one would expect, the analysis of a single TCR does not replicate the affinity range found within a polyclonal population. It is currently unclear how well conclusions made based on monoclonal models informs on the polyclonal response to the same antigen (79). This is affirmed by comparing the 2D affinities between the monoclonal CD4⁺ SMARTA

T cell clone and the polyclonal CD4⁺ T cell population. Both populations are specific for the same GP₆₆₋₇₇: IA b antigen, but the monoclonal SMARTA population only represents a fraction of the affinity breadth seen in the polyclonal response. In this case, the monoclonal cells have a mean affinity of $\sim 10^{-3} \mu\text{m}^4$ which is ~ 10 -fold higher than the mean polyclonal affinity of $\sim 10^{-4} \mu\text{m}^4$ [Figure 2A adapted from Ref. (48, 78)]. Although the SMARTA TCR is monoclonal, it is interesting that this TCR exhibits a range of affinities, albeit more narrow than the responding polyclonal T cells. An affinity range can even be detected

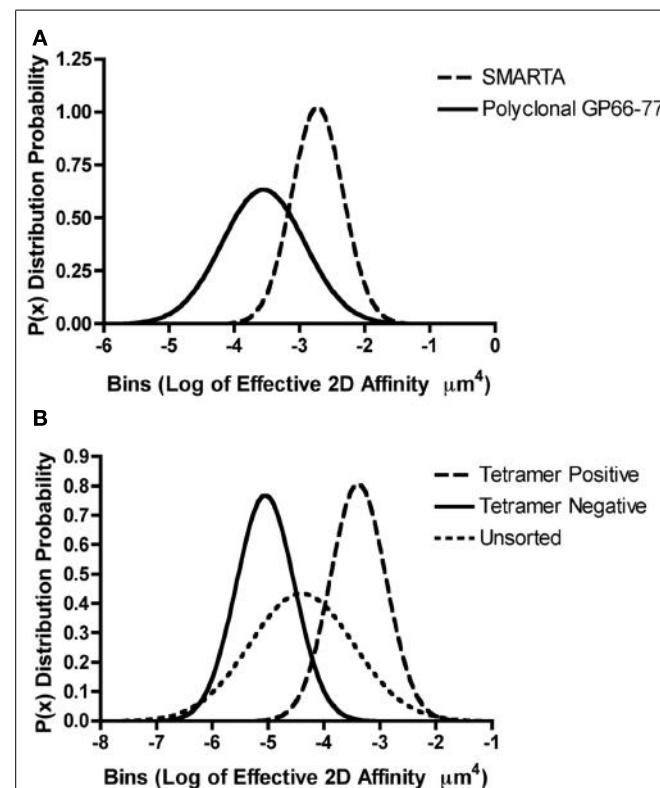


FIGURE 2 | Range of TCR affinities for an individual pMHC in a polyclonal repertoire. (A,B) Gaussian distributions were modeled for the described T cells by utilizing previously published effective 2D affinity means and standard deviations using the equation $P(x) = 1/\sigma \times \sqrt{2\pi} \times e^{-(X - \mu)^2/(2\sigma^2)}$ where $P(x)$ is the probability density function or distribution, σ is the standard deviation, X is the variate or bin interval, and μ is the mean log of the TCR affinities. (A) The monoclonal SMARTA T cells and the polyclonal GP₆₁₋₈₀ population both recognize GP₆₆₋₇₇: IA b . The 2D micropipette adhesion frequency assay was used to determine the mean effective 2D affinities and standard deviations as previously reported (48, 78). Gaussian distributions indicated that SMARTA T cells exhibit a higher log of affinity μm^4 (-2.7 ± 0.39) ~ 10 -fold higher than the polyclonal T cell populations (-3.5 ± 0.63), indicating that monoclonal population underrepresented the polyclonal affinity range. (B) The 2D micropipette adhesion frequency assay was used to ascertain the mean effective 2D affinities and standard deviations for the polyclonal GP₆₁₋₈₀ repertoire (unsorted) and FACS sorted GP₆₆₋₇₇: IA b tetramer positive and negative populations (78). Gaussian distributions indicated that both the tetramer positive (\sim peak at -3.0) and tetramer negative (\sim peak at -5.0) populations under represented the range of affinities exhibited by the polyclonal (unsorted) repertoire.

among TCRs expressed on a single T cell because FRET analyses with the 5C.C7 CD4⁺ TCR have shown a 250-fold 2D affinity range for MCC between microclusters of the same cell (66). Furthermore, monoclonal models are often thought to represent the highest affinity TCRs within a polyclonal response, which is not necessarily the case. Clones are often selected *in vitro* for optimal growth, effector function, and reagent availability for tracking the TCR *in vivo*. For example, we have found that the transgenic 2D2 TCR, widely used for the study of demyelinating autoimmune disease, is of low affinity for its antigenic ligand yet shows measurable reactivity through proliferation and cytokine production assays (48, 80).

While the ability to track antigen specific T cells within a polyclonal repertoire has been revolutionized by the use of pMHC tetramers recent investigations call into question the fidelity of these reagents to sufficiently capture all participating effectors in an immune response. Our laboratory and others have shown a discrepancy in the use of tetramers to determine the breadth of CD4⁺ T cell responses. Tetramers will not detect T cells where the affinity of the TCR for antigen is below the avidity threshold needed for binding. We have estimated this 2D effective affinity cutoff to be between 10⁻⁵ and 10⁻⁴ μm^4 for MHC class II restricted T cells; it is more difficult to define for MHC class I based tetramers as CD8 significantly contributes to the overall stability and binding while CD4 does not (48, 78, 81). Analysis of the tetramer positive subset of polyclonal GP_{66–77} reactive T cells showed enrichment for higher affinity T cells with a mean 2D affinity of $\sim 10^{-3} \mu\text{m}^4$ as compared to the affinity of the intact polyclonal T cell population of $\sim 10^{-4}$ or the tetramer negative T cells $\sim 10^{-5} \mu\text{m}^4$ (78). Of interest, the tetramer positive and negative cells are a subset of the overall polyclonal affinity repertoire [Figure 2B, adapted from Ref. (78)]. For both the self and pathogen specific CD4⁺ T cell response, the percentage of tetramer reactivity was lower and did not correlate to the percentage of cytokine responders or the frequency of antigen specific T cells measured by the 2D micropipette adhesion frequency assay (78). Therefore, the sole use of tetramers to monitor the antigen specificity, frequency, magnitude, and affinity of a polyclonal repertoire in order to predict the overall composition of an immune response appears somewhat limited, missing the contribution of the lower affinity T cells.

Underestimating the contribution of low affinity T cells is a significant issue for models of autoimmune disease where negative selection likely enriches for a low affinity repertoire reactive against self antigen. Our studies with MOG_{35–55} specific CD4⁺ T cells indicated that tetramer generally reacted with 7–10% of T cells within the target organ, while functional effector responses and 2D affinity analysis detected much higher levels of cells (78). In the 2D2 TCR transgenic model of EAE, the 2D2 T cells promoted spontaneous paralytic disease (4%) or spontaneous optic neuritis (35%) (80). This TCR has very low affinity for antigen (48) and does not interact with MOG-specific tetramers and therefore contrasts with data suggesting low affinity or low avidity T cells are less pathogenic. Furthermore, retrogenic derived monoclonal TCR models suggest that TCR of low avidity can support the development of spontaneous EAE in the absence of higher

avidity T cells (82). The challenge in studying the contributions of low affinity T cells has been the lack of reagents to do so. The 2D adhesion frequency assays gives us one such tool to characterize lower affinity T cells alongside the higher affinity contributors within a polyclonal population.

BENEFITS OF AN INCLUSIVE RESPONSE

To date, current models of T cell clonal expansion suggest that high affinity T cell clones are preferentially enriched over low affinity clones (83–85). As a result, many current T cell therapeutic initiatives seek to elicit or artificially create high affinity T cells to enhance pathogen specific and anti-tumor responses (86). However, recent investigations have shown that T cells manipulated to have supraphysiological affinity were unexpectedly less potent effectors than lower affinity counterparts due to triggering of inhibitory mechanisms (87, 88). It would therefore be plausible that an effective immune response may benefit from a balance of high, intermediate, and low affinity T cell responders.

Polyclonal TCR affinity composition can be shaped by TCR activation thresholds. For example, CD27 costimulation has been shown to support the emergence of lower affinity CD8⁺ T cells that mediate greater protection against reinfection with an influenza variant (89). Similarly, clones with low functional avidity have been shown to be important in the maintenance of an effective anti-tumor response (90). Although affinity was not the sole focus of this study, reduction of p56^{Lck} expression significantly decreased T cell sensitivity to activation which mimics a lower affinity response. These low affinity effectors were less susceptible to an exhausted phenotype and mediated better protection in subsequent rechallenge. Such investigations provide evidence for why low affinity clones may exist within the repertoire and how therapeutics to limit them may be shortsighted.

The role for low affinity T cell populations can be obscured by the nature of the assay used to analyze the response. In a study examining the therapeutic efficacy of tumor vaccines, high affinity clones (as determined by SPR) responded optimally in *in vitro* assays, but intermediate affinity clones mediated the best anti-tumor responses *in vivo* (91). Similarly, a study evaluating optimal T cell responses to peptide in the 5C.C7 model (85, 92) showed that intermediate affinity clones mediated the most optimal *in vivo* responses while high affinity clones demonstrated the strongest response *in vitro*. Future studies may benefit from understanding the interplay of individual T cell affinity subsets in the overall efficacy of tumor and pathogen specific responses. These findings underscore the potential role for lower affinity effectors in an immune response and therefore they should be an important consideration in the design of therapeutic interventions.

Understanding how T cell affinity mediates protective immunity also has important implications for vaccine design because recent studies have shown the priming agent and the adjuvant can alter the CD4⁺ TCR affinity composition. In one study, vaccinations using either cytochrome C peptide or whole protein were compared (93). Though both vaccination regimens generated diverse clonal responses, peptide vaccines elicited high

affinity dominated responses while protein vaccines generated a repertoire inclusive of both low and high affinity responses. The maintenance of low affinity effectors was found, at least in part, to require CD27-CD70 signaling. Another study demonstrated the ability of vaccine adjuvants to affect the affinity composition of T cells generated in response to pigeon cytochrome C, PCC (94). All the adjuvants tested were effective in enhancing a PCC-specific T cell response, but alum, IFA, and CFA induced lower avidity responses while CpG and monophosphoryl lipid A generated higher avidity responses as determined by pMHC tetramer and CDR3 spectotyping. This observation suggests that adjuvants could differentially influence recruitment into the polyclonal response. The effect was dependent on the dispersive ability of the adjuvant and activation of different TLRs that resulted in changes in CD4⁺ T cell recruitment and/or migration. It is worth noting that even adjuvant choice can affect the balance of low and high affinity clonotypes (94, 95) and should be furthered explored with 2D assays. The application of 2D based pMHC technologies to these questions will allow us to uniquely explore the breadth of TCR affinities and redefine our understanding of the dynamic interplay between TCR affinity subsets within the polyclonal repertoire.

CONCLUSION

The use of pMHC technology is at the forefront of monitoring antigen specific immune responses. We promote 2D mechanical based assays with purified pMHC for several reasons. First, they display increased sensitivity for detecting antigen specific T cells when compared to functional responses or pMHC tetramer based assays. Secondly, the polyclonal repertoire can be monitored without purification of individual TCRs because the analysis is carried out using intact T cells. Lastly, 2D assays provide a more accurate

representation of the relationship between T cell affinity and functional responsiveness. The findings to date have highlighted the presence of antigen specific CD4⁺ T cells exhibiting a range of affinities from low to high in both autoimmune and pathogen specific models. Low affinity, tetramer negative populations elicit effector functions and expand in response to antigen suggesting their capacity to contribute to adaptive immune responses. The idea that lower affinity T cells effectively compete within and contribute to the effector T cell repertoire at the very least modifies our view that high affinity T cell clones would dominate the lower affinity counterparts. Future work is needed to examine how affinity of the initial TCR:pMHC interaction contributes functionally to the initiation, maintenance, and/or resolution of a polyclonal immune response. In addition, we need sensitive techniques that allow for analysis of TCR crossreactivity and in the case of autoimmunity, epitope spread to new antigens. At this point in time, 2D based assays together with recombinant pMHC molecules are useful tools available to characterize individual T cell affinity contributions to the breadth of an immune response. Potential clinical outcomes for this research include the use of TCR affinity as a biomarker to monitor disease progression and to provide information for the development of high efficacy antigen specific therapies.

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“Model T” cells: a time-tested vehicle for gene therapy

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T lymphocytes first carried foreign genes safely into humans over two decades ago. Since these pioneering studies, scientific techniques to better understand the genomic landscape of cells has directly led to a more sophisticated appreciation of the diversity, functional complexity, and therapeutic potential of T cells. Through the use of mouse models, we now know the function of the many genes that are critical for T cells to recognize foreign, mutated, or self-antigens and the factors responsible for the lineage diversification of T cells that lead to inhibitory or stimulatory immune responses. This knowledge combined with well-established modalities to introduce genes into T cells allows for the design of effector and memory CD8 and CD4 T lymphocytes specific for viral, fungal, bacterial, parasitic, and tumor-antigens and to design regulatory lymphocytes specific for the self-antigens responsible for autoimmune and inflammatory diseases. Here, I review strategies for designing the ideal T cell by introducing genes controlling (1) the secretion of cytokines/chemokines and their receptors, (2) T-cell receptor specificity, (3) chimeric-antigen receptors that enable for the recognition of surface antigens in an MHC-independent fashion, (4) co-stimulatory/inhibitory surface molecules, and (5) disease defining single-gene factors.

Keywords: gene therapy, cancer, immunotherapy, T cells, inflammation, chimeric-antigen receptors, cytokines, severe combined immunodeficiency

INTRODUCTION

The Deoxyribonucleic acid (DNA) molecule, perhaps one of biology's greatest discoveries, helped unlock the secrets to the flow of genetic information that we now know forms the basis for the complexity of all life on earth. In the 1960s, the scientific community demonstrated for the first time that exogenous DNA could be taken up and ectopically expressed in mammalian cell lines (1). Shortly after, a growing understanding of viral reverse transcription processes and advances in recombinant DNA technologies paved the way for engineering viruses to carry therapeutic genes into cells (2).

Fast-forward 40 years and there now exists numerous viral and non-viral modalities to introduce therapeutic genes into cells. The most common viral vectors include retroviruses, adenovirus, and herpes simplex viral backbones with non-viral modalities centered on physical (DNA transfection/electroporation) or chemical (synthetic oligonucleotides, lipoplexes, nanoparticles) methods of delivery and transposon systems (3–6). Of these various modalities, gene therapy using retroviral based vectors is perhaps the most established methodology both in experimental models and in human clinical trials due to the ability to stably integrate genes into dividing cells (7–9).

In addition to the technologic advancements in gene therapy, a growing understanding of the genetic causes of human disease and the downstream function and network-like interactions between specific genes are enabling scientists to devise strategies to treat ailments once thought incurable (10, 11). While the *in vivo* delivery of genes targeting specific cell types remains a grand hope for the future, current methodologies readily enable for the stable

introduction of foreign genes into cells *ex vivo*, allowing for the transfer of these cells back into patients (6).

T lymphocytes represent the ideal vehicle for carrying therapeutic genes into humans. T cells are easily obtained through peripheral blood draws or apheresis procedures and can be induced to divide robustly *ex vivo*, a characteristic that allows them to be highly permissible to retroviral introduction of ectopic genes (12). The first clinical trial to safely infuse a foreign gene into humans consisted of transducing tumor-infiltrating lymphocytes with a neomycin resistant gene that enabled for the detection of the transgene within a tumor biopsy several days following transfer (13). Today, the adoptive transfer of tumor-infiltrating lymphocytes combined with total-body irradiation, lymphodepleting chemotherapy, and high-dose IL-2 achieve response rates as high as 70% in patients with metastatic melanoma (14). The rapid development of gene therapy in this field promises to vastly improve current cellular therapies and opens the door to treat cancers of various histologies and wider arrays of human disease. Here, I discuss potential therapeutic genes that may improve current gene therapies, although rigorous pre-clinical testing and careful phase 1 clinical trials will be required for many of the suggestions in this review.

CYTOKINES, CHEMOKINES, AND THEIR RECEPTORS

The theory of immune surveillance in cancer is controversial but there exists reproducible scientific data pointing to the importance of interferon-gamma as a critical mediator for the elimination of malignantly transformed cells (15). Furthermore, there is a clear

increase in the incidence of cancer in patients with HIV, Immunodeficiency syndromes triggered by mutations in genes such as GATA-2 (MonoMAC) and post-transplant patients receiving immune-suppressive drugs (16, 17).

Additional support for the importance of an immune response for cancer elimination can be garnered from clinical data with robust long-term follow up showing the ability of systemic IL-2, anti-CTLA-4 antibodies, and anti-PD-1/anti-PD-L1 antibodies, and the adoptive transfer of T lymphocytes to induce tumor regression in patients with metastatic melanoma and metastatic renal cell carcinoma (18, 19). Three major factors are important for an effective immune response against cancer: (1) overcoming suppressive factors induced by mutated cancer cells within the tumor microenvironment, (2) the quality of the T cells transferred, and (3) polymorphic factors of an individual's host immune response. Some of these factors can be readily modified by over-expressing cytokines, chemokines, and their receptors in transferred T cells, enabling lymphocytes to secrete supra-physiologic amounts of therapeutic immune-stimulatory molecules.

THE IL-12/IFN- γ /TH-1 AXIS

IL-12 is a hallmark inflammatory cytokine and is critical for driving an effective immunologic response against cancer and foreign pathogens (20). It is mainly produced by inflammatory cells such as dendritic cells, macrophages, and neutrophils and directly augments the functionality of multiple end effectors such as CD4 $^{+}$ T cells, CD8 $^{+}$ T cells, natural killer (NK) cells, and NKT cells (20). The anti-tumor effects of IL-12 are well documented (21). IL-12 enhances the ability of CD8 $^{+}$ T cells to cause the regression of large established tumors by potently stimulating the production of high-levels of IFN- γ , resulting in an increase in the cross-presentation of tumor-antigens and the reversal of suppressive functions of myeloid-derived suppressor cells, alternatively activated macrophages, and dendritic cells (22). These changes subsequently lead to the collapse of the tumor stroma and the regression of large established masses (22, 23).

Unlike activated lymph nodes stimulated by pathogen-activated molecular patterns, sterile conditions within tumors lead to low levels of IL-12 secretion by innate immune cells. This lack of a danger signal within the tumor microenvironment results in a skewing away from a Th-1 type effector immune response. One attractive approach is to increase the levels of IL-12 directly at the point of T-cell/Tumor cell and T-cell/Antigen-presenting contact within tumors (24) (Figure 1). Several studies show that over-expressing a single-chain, functionally active IL-12 gene in tumor-antigen-specific lymphocytes significantly increases the levels of IL-12 to supra-physiological levels within tumors, leading to the regression of large established masses (25–28). This modification enables for therapeutic anti-tumor immunity with smaller numbers of T cells and does not require the use of systemic gamma-chain cytokines to support the transfer of cells *in vivo*. Currently, clinical trials are determining if the benefits of IL-12 gene therapy outweigh the many risks associated with a systemic increase in IL-12 and IFN- γ .

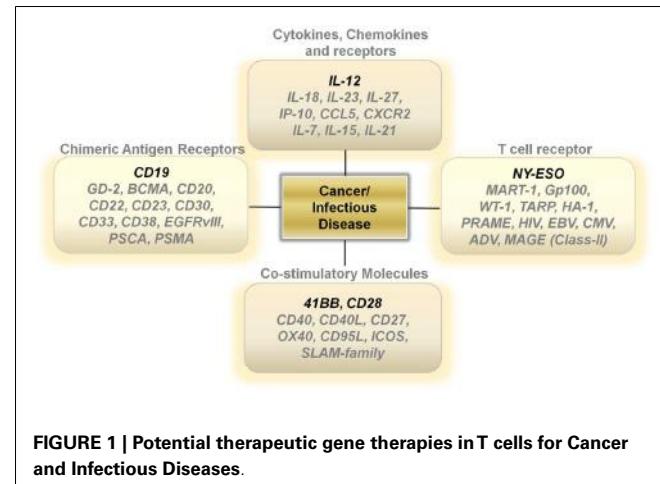


FIGURE 1 | Potential therapeutic gene therapies in T cells for Cancer and Infectious Diseases.

ADDITIONAL CYTOKINES, CHEMOKINES, AND CHEMOKINE RECEPTORS

The importance of gamma-chain cytokines in the proliferation and maintenance of memory T cells remains a critical and extremely important avenue of research for many investigators (29–32). However, clinical trials using TIL transduced with the IL-2 gene did not show a clinical benefit (33). Over-expressing the other gamma-chain cytokines such as IL-7, IL-15, or IL-21 in T cells may lead to better results. However, the constitutive expression of genes that drive T-cell proliferation carries the risk of causing an uncontrollable expansion of transferred T cells due to the stable integration of retrovirally transduced genes being expressed in every daughter cell (Figure 1). Designing viral vectors using a NFAT promoter for inducible gene expression upon T-cell receptor (TCR) ligation may provide an important degree of safety (26). Another strategy is to use adenoviral vectors or systems that only transiently express the genes that control T-cell memory formation.

Other potentially attractive cytokines include those within the IL-12 family, such as IL-23, and IL-27 (Figure 1). These cytokines may invoke beneficial downstream mechanisms for anti-tumor immunity without the heavy reliance on the induction of IFN- γ secretion. Furthermore, genetic polymorphisms within the human population may make certain individuals more likely to mount an anti-tumor response to one of the alternate members of the IL-12 family rather than IL-12 itself.

Another strategy that may turn out to be fruitful is the over-expression of chemokines and chemokine receptors in T cells (Figure 1). Melanomas can secrete chemokines such as CXCL1 and CXCL8 to aid in the recruitment of monocytes into local microenvironments and studies show that expressing the chemokine receptor CXCR2 on transferred T cells aids in the ability of T cells to infiltrate tumors and cause regression (34). This approach can be easily tailored to other tumor histologies depending on the chemokine secretion profile of the cancer. Additionally, over-expressing chemokines in T cells may also provide some benefit. Upon recognizing cognate antigens, T cells arrest their migration and accumulate at sites with productive antigen presentation. The over-expression of chemokines such as IP-10 or CCL5 in transferred T cells may enable antigen-specific T cells to secrete

products that attract activated T cells to the local microenvironment they inhabit. This in turn may provide a positive feedback loop that enables for an increase in infiltrating antigen-specific T cells and an improved therapeutic outcome. Thus, the possibility to genetically alter the cytokine or chemokine profile of adoptively transferred T cells may prove to enhance and simplify current treatments requiring lymphodepletion and high-dose IL-2.

CHIMERIC-ANTIGEN RECEPTORS

The ability to generate a single fusion molecule that can bind surface antigens and trigger T-cell function holds great promise for the future of cell therapy. Chimeric-antigen receptors (CAR) are the latest form of gene therapy, where a single vector is constructed with a binding moiety recognizing a surface antigen [usually designed from a single-chain variable fragment (scFv) derived from a tumor-antigen-specific monoclonal antibody] (35–37). The beauty of CAR generated T cells is the ability to generate lymphocyte specificity in an MHC-independent fashion due to the ability to design receptors that recognize surface antigens. This is accomplished by cloning the sequences from the variable region of antibodies (many of which already exist) and adding T-cell signaling and co-stimulatory domains to the vector construct.

Early phase trials for CARs recognizing the antigen CD19, expressed on many B cell lymphomas and leukemias are showing promising results in adult and pediatric patients at multiple institutions (38–41). One of the major advantages of using CARs as the main platform for gene therapy is the ability to rapidly and clearly define the expression of the target protein. Often, the antibodies whose variable region is cloned into the CAR vector can also be used diagnostically to look for the expression of the desired target.

Other antigen targets that may be worthwhile exploring for CAR development includes GD-2 for neuroblastomas (42), CD20 (43), and CD22 for B cell lymphomas (44), BCMA (B cell maturation antigen) (45), and CD38 for multiple myeloma (46), CD23 for chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) (47), CD30 for Hodgkin's lymphoma and anaplastic large cell lymphomas (48), CD33 for acute myeloid leukemias (49), EGFRvIII for glioblastomas (50) and PSCA (51) and PSMA (52) for prostate adenocarcinomas (Figure 1).

T-CELL RECEPTORS

Although CAR-directed gene therapy remains a promising modality for the future, many cancers, especially carcinomas and sarcomas, do not possess known surface expression of unique non-shared antigens. Targeting surface proteins that may be expressed on normal tissue with CARs may cause serious end organ damage and toxicity. Gene therapy using high avidity TCR enables for the design of lymphocytes targeting epitopes from differentially expressed or mutated intra-nuclear and/or intra-cytoplasmic proteins such as transcription factors (22, 53). Emerging data now shows that tumor-infiltrating lymphocytes possess the ability to recognize mutated melanoma antigens (54, 55). This exciting finding opens up a large window of opportunity to develop effective TCR gene therapies. It is possible that in the future we may perform whole exome sequencing for every tumor for

diagnostic purposes, enabling us to design TCR recognizing the most frequently mutated epitopes for different tumor histologies.

A great example of the success of TCR gene therapy was recently described with a clinical trial utilizing the NY-ESO TCR (56). This study led to significant tumor regression in four out of six patients with synovial sarcoma and five out of 11 patients with metastatic melanoma. Overall, the cancer-testis antigens represent an ideal set of target antigens due to their relatively low to negligible expression on normal tissue, except in the testis, where cells express low levels of MHC Class I. Identifying antigens with limited normal tissue distribution will be critical to extending TCR gene therapy to different types of cancer. Developing TCRs for breast, prostate, and thyroid cancer also seems reasonable since targeting of normal tissue in these organs might not be accompanied with serious life-threatening adverse events (Figure 1).

CO-STIMULATORY MOLECULES

Generating both a specific and a productive T-cell response requires not only appropriate signaling through the TCR but an additional secondary co-stimulatory signal. The most well studied co-stimulatory molecule is CD28, a disulfide linked homodimer that is constitutively expressed on naive T cells (57). CD28 engagement with CD80 and CD86 on antigen-presenting cells enables T cells to differentiate and become functionally activated (57). However, after initial antigen encounter and under altered cytokine conditions, T cells can lose or decrease their expression of CD28, leading to replicative senescence and functional anergy. The lack of CD28 signaling can also result in an impaired memory response and activation induced cell death (AICD) (58). One strategy to circumvent these physiological restraints is to constitutively over-express CD28 in T cells. Currently, second and third generation CAR constructs use the intracellular domain of CD28 to improve the persistence, function, and activity of CAR transduced T cells (59). Other important co-stimulatory molecules include 41BB, CD27, OX40, CD40, CD27, ICOS, Fas ligand, and the Slam family of proteins (60, 61). These molecules all have been implicated in tipping the balance in favor of generating a functional T-cell response and helping avoid AICD during antigen re-stimulation. The intracellular domains of 41BB, OX40, and CD27 are currently being incorporated into various CAR constructs that are being developed. Thus, it is possible that the constitutive over-expression of these various co-stimulatory molecules may aid in designing long lived, functionally active T cells that are resistant to cellular senescence (Figure 1).

SEVERE COMBINED IMMUNODEFICIENCY SYNDROMES

The first successful therapeutic gene therapy in humans in the early 1990s involved treating two children with severe combined immunodeficiency syndrome (SCID) caused by a genetic defect in the enzyme adenosine deaminase (SCID-ADA) (62). This syndrome resulted in defective T and B cells, leading to debilitating recurrent opportunistic infections. A normal/wild type ADA gene, enabling for the production of a functional enzyme, was introduced into T cells and infused back into the patient. The results were striking, and for the first time in these patients, there was evidence for IgM antibody production and the detection of tetanus antibody in the serum following immunization (62). In

one patient, approximately 20% of the circulating lymphocytes still expressed the retrovirally inserted gene 10 years following transfer (63).

Although these initial results led to heightened optimism, attempts to develop gene therapies for SCID-X1, a disease characterized by a defective common gamma-c cytokine receptor subunit, by retroviral transfer of the corrected gene into CD34+ hematopoietic stem cells, led to the development of leukemias in some patients (64, 65). These setbacks sent shock waves through the scientific and medical communities. We now know that retroviral vectors can result in insertional mutagenesis, although this phenomenon still remains poorly understood (66). Five out of 20 patients treated in trials carried out in London and Paris developed leukemias secondary to the expansion of clones containing vector integration near proto-oncogenes such as *CCND* and *LMO2* (65).

Despite the clear dangers of gamma-retroviral gene transfer into hematopoietic stem cells, transferring genes into T cells *ex vivo* appears to be much more resistant to oncogenic transformation. There now exists robust long-term follow up for over a 100 patients treated on various gene therapy trials utilizing *ex vivo* retroviral insertion of genes into T cells with no evidence of malignant transformation (9). The mechanisms for the differences in oncogenesis between transducing hematopoietic stem cells versus T cells is not well understood. Perhaps introducing genes into more differentiated cells that contain a vastly different genetic and epigenetic landscape from stem cells leads to retroviral integration away from oncogenes.

Currently, gene therapists are continuing to try to improve safety through vector design. One strategy gaining support includes creating self inactivating gamma (SIN) retroviral vectors and lentiviral vectors by deleting the U3 region in the 3' LTR (67). This modification generates a pro-virus with defective transcriptional activity at both the 5' and 3' LTR end regions, preventing the possibility of transcriptionally activating cellular oncogenes near the site of viral integration. Importantly, an internal promoter will need to be designed to drive the expression of the desired transgene within the SIN vector construct. Additionally safety measures include the genetic modification of shorter lived cell populations or the use of suicide genes (68).

The current progress in improving the safety of gene therapies is helping the field move forward. In regard to SCID, although mutations in the common gamma-c chain receptor is the most common cause of the disease, a broad range of single-gene mutations can result in a similar disease pattern of recurrent opportunistic infections (Figure 2). Theoretically, for all the various defects that may occur, introducing a correctly functioning gene *ex vivo* into T cells or hematopoietic stem cells/monocyte/dendritic cell populations may re-capitulate the early excitement seen in the SCID-ADA trials and build on the recent successes of gene therapy for cancer.

AUTOIMMUNE AND INFLAMMATORY DISEASES

Although genetic modifications to stimulate the immune system is beneficial for battling infectious organisms and cancer, there also exists a set of devastating diseases that are caused by an over-zealous and unchecked immune response. The targeting of self-antigens under normal physiologic conditions can cause a range of serious ailments including type 1 diabetes, multiple

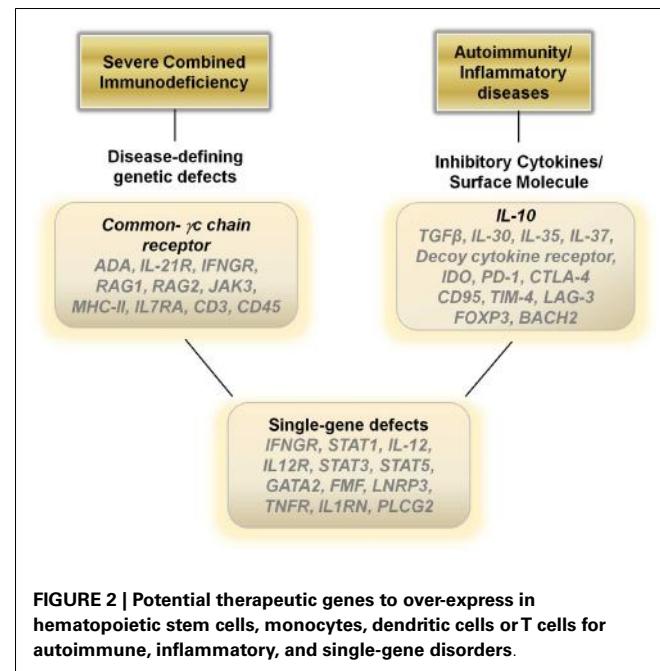


FIGURE 2 | Potential therapeutic genes to over-express in hematopoietic stem cells, monocytes, dendritic cells or T cells for autoimmune, inflammatory, and single-gene disorders.

sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and autoimmune encephalomyelitis. Recently, a relatively new set of autoimmune diseases categorized as autoinflammatory diseases are beginning to be characterized such as familial Mediterranean fever (FMF), neonatal onset multisystem inflammatory disease (NOMID), tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), deficiency of the Interleukin-1 receptor antagonist (DIRA) and Behcet's disease (69, 70). Additional inflammatory diseases that cause morbidity and mortality in a large number of patients include inflammatory bowel disease (Crohn's disease and ulcerative colitis), chronic granulomatous disease (CGD), and the various forms of vasculitis (71, 72).

In general, dampening the immune response is the ideal treatment for autoimmune and inflammatory diseases and current therapies revolve around the use of steroids, cytokine antagonists, or directly down regulating the immune system utilizing various modalities. Gene therapies may provide a viable biological alternative to directly blunt an over-active immune response. Over-expressing anti-inflammatory cytokines such as IL-10, TGF-β, IL-30, IL-35, or IL-37 in T cells or monocyte/dendritic cell populations *ex vivo* with a re-infusion of the modified cells may aid in decreasing inflammatory driven symptoms (Figure 2). Another alternative may be to construct a decoy cytokine receptor that contains the correct receptor sequence to enable for binding of pro-inflammatory cytokines such as IL-12 combined with a non-functioning cytoplasmic signal transducing sequence. Over-expressing these "dominant-negative" receptors would enable re-infused immune cells to function as sinks for the inflammatory cytokines responsible for the pathophysiology of the disease. Other genes that may aid in dampening the immune response include over-expressing indoleamine 2,3-dioxygenase in monocytes/dendritic cells or CTLA-4, PD-1, CD95, LAG-3, FOXP3, and BACH2 in T cells (73) (Figure 2).

SINGLE-GENE DEFECTS

Although many human diseases are caused by complex genetic polymorphisms, perhaps the greatest potential for gene therapy is in the ability to treat diseases caused by single Mendelian gene defects. Mutations in genes such as *IFNGR1*, *STAT1*, *IL-12*, and *IL-12R* can lead to immune dysfunction and recurrent mycobacterial infections (74). Genetic disruptions also cause many of the autoinflammatory diseases, such as mutations in the *FMF* gene in FMF, the *LRN3* gene in NOMID, the *TNFR* gene in TRAPS, and *IL1RN* gene in DIRA. Inserting the corrected sequence for these genes into hematopoietic cells or more safely into differentiated immune cells may result in dramatic improvements in the health of these patients (Figure 2).

CONCLUSION

T lymphocytes represent one of the first vehicles to carry therapeutic genes into humans, and its current use, centered on the adoptive transfer of T cells, is proving to be a promising cancer therapeutic modality. However, logistic hurdles still exist for the wider use of this technology due to costs associated with GMP quality viral production and the requirement of significant

technologic infrastructure and expertise. Increased collaboration between industry and academia for developing gene therapies may help overcome current financial limitations by developing viable business models.

There exist over 4000 known single-gene disease causing disorders in addition to the innumerable genetic polymorphisms that increase susceptibilities for diseases. Gene therapy in T cells is paving the way for a broader application of this therapeutic modality in human disease. The ability to stably introduce functional genes into hematopoietic stem cells or differentiated cells *ex vivo* provides hope for the thousands of patients diagnosed with a wide range of devastating genetic diseases, highlighted by recent successes in childhood cerebral adrenoleukodystrophy (75) and hemophilia B (76). Gene therapy represents the ultimate form of personalized medicine, and in the future, it is conceivable to imagine that diseases that were once considered untreatable will be readily controlled or eradicated with a single specialized treatment.

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TCR-engineered T cells meet new challenges to treat solid tumors: choice of antigen, T cell fitness, and sensitization of tumor milieu

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Adoptive transfer of T cells gene-engineered with antigen-specific T cell receptors (TCRs) has proven its feasibility and therapeutic potential in the treatment of malignant tumors. To ensure further clinical development of TCR gene therapy, it is necessary to target immunogenic epitopes that are related to oncogenesis and selectively expressed by tumor tissue, and implement strategies that result in optimal T cell fitness. In addition, in particular for the treatment of solid tumors, it is equally necessary to include strategies that counteract the immune-suppressive nature of the tumor micro-environment. Here, we will provide an overview of the current status of TCR gene therapy, and redefine the following three challenges of improvement: "choice of target antigen"; "fitness of T cells"; and "sensitization of tumor milieu." We will categorize and discuss potential strategies to address each of these challenges, and argue that advancement of clinical TCR gene therapy critically depends on developments toward each of the three challenges.

Keywords: antigens, inhibitory micro-milieu, solid tumors, T cell avidity, T cell co-stimulation, T cells, TCR affinity, TCR transgenes

TCR GENE THERAPY: CLINICAL POTENCY AND TOXICITIES

T cells possess distinct properties such as the ability to specifically recognize tumor antigens, serially kill tumor cells, self-replicate, form memory and induce a complete tumor response. It is because of these properties that the therapeutic use of T cells in certain types of cancer may be advantageous when compared to drugs, antibodies, or small molecule inhibitors.

T cell therapy intends to treat cancer by transferring autologous and *ex vivo* expanded T cells to patients. Therapy with tumor-infiltrating T lymphocytes (TILs) preceded by non-myeloablative lymphodepletion resulted in objective responses in about 50% of metastatic melanoma patients in two different medical centers (1, 2). Equally notable were the durable complete responses observed in these trials that ranged between 10 and 22% (ongoing for more than 3 years) (1, 2). Likewise, adoptive transfer of tumor-specific T cell clones generated from autologous peripheral T cells resulted in regression of individual metastases, and responses in 8 out of 10 melanoma patients (3). In addition, co-culture of peripheral T cells with artificial antigen-presenting cells (APC) loaded with tumor antigens resulted in T cells that were clinically effective in four out of seven evaluable melanoma patients (4). Response rates observed with T cell therapy are generally higher than those observed for other treatments of melanoma, such as chemotherapeutic drugs, high-dose cytokines, inhibitors of kinases, or antibodies against T cell co-inhibitory molecules. See Table 1 for an overview of clinical outcomes of T cell therapies and other treatments of melanoma.

Despite its clinical successes, T cell therapy has its limitations in availability and generation of therapeutic T cells for a larger

group of patients. Genetic introduction of T cell receptors (TCRs) or chimeric antigen receptors (CARs) into autologous T cells, termed gene-engineering of T cells, can provide an alternative that is more widely applicable and can potentially be extended to multiple types of cancer (5). Key preclinical achievements and clinical tests with TCR-engineered T cells, the focus of the current review, are depicted in Figures 1A,B, respectively. Therapeutic advances with CAR-engineered T cells is reviewed elsewhere (6). The principle of clinical TCR gene therapy is straightforward: transferral of TCR $\alpha\beta$ genes into T cells; *ex vivo* expansion of T cells; and infusion of T cells into the patient. In this way, TCR α and β genes are used as "off the shelf" reagents to confer tumor reactivity to patients whose tumor expresses the appropriate antigen and HLA restriction element. At the moment of writing this review, eight clinical trials using TCR-engineered T cells have reported their results (see Figure 1B and Table 2 for details), and at least another 10 trials using TCR-engineered T cells are open and actively recruiting patients or will recruit patients soon¹.

Most clinical TCRs tested so far were HLA-A2-restricted and directed against either melanoma-associated antigen recognized by T cells 1 (MART-1), glycoprotein (gp) 100, carcinoembryonic antigen (CEA), p53, melanoma-associated antigen (MAGE-)A3, or New York esophageal squamous cell carcinoma antigen (NY-ESO)1. Another TCR tested clinically was HLA-A1-restricted and

¹www.clinicaltrials.gov

Table 1 | Overview of standard and experimental none-gene-based therapies for metastatic melanoma.

Therapy	Function	Type of trial	OR (%) ^a	CR (%) ^a	Reference
T CELL THERAPY					
Tumor-infiltrating lymphocytes (TILs)	Adoptive transfer of tumor-specific T cells	n.c.	52/93 (56)	20/93 (22)	(1)
		n.c.	15/31 (48)	3/31 (10)	(2) ^b
T cell clones		n.c.	8/10 (80)	n.r.	(3)
“Educated T cells”		n.c.	4/9 (44)	1/9 (11)	(4)
STANDARD THERAPY					
High-dose IL-2	Cytokine that induces T cell growth	n.c.	43/270 (16)	16/270 (6)	(178)
Dacarbazine (DTIC)	Drug that alkylates DNA	Phase III trial	18/149 (12)	4/149 (3)	(179)
Vemurafenib (PLX-4032)	Small molecule that inhibits BRAF kinase activity	Phase III trial	106/219 (48)	2/219 (1)	(180)
EXPERIMENTAL THERAPY					
Dabrafenib	Small molecule that blocks BRAF kinase activity	Phase III trial	29/54 (54)	n.r.	(181)
Dabrafenib + Trametinib	Small molecules that block BRAF and MEK kinase activities	Phase III trial	41/54 (76)	n.r.	(181)
Ipilimumab (MDX-010) + vaccination	Antibody that blocks T cell CTLA4	Phase III trial	39/137 (28)	3/137 (2)	(182)
Ipilimumab + DTIC		Phase III trial	34/252 (14)	26/252 (10)	(183)
Nivolumab (MDX-1106) ^c	Antibody that blocks T cell PD1	Phase I trial	5/39 (13)	1/39 (3)	(184)
		Phase I trial	26/94 (28)	n.r.	(185)
Nivolumab + Ipilimumab		Phase I trial	21/53 (40)	n.r.	(186)
Lambrolizumab (MK-3475)	Antibody that blocks T cell PD1	Phase I trial	51/135 (38)	n.r.	(187)
Anti-PD-L1 (MDX-1105)	Antibody that blocks tumor cell PDL1	Phase I trial	17/135 (13)	n.r.	(188)

^aOR, objective responses; CR, complete responses; both according to Response Evaluation Criteria for Solid Tumors (RECIST). Number of patients with responses = before dash; total number of patients treated = after dash; percentage of responses = between brackets.

^bDr. Jacob Schachter, *Cellular Therapy of Cancer Symposium, September 24–27th, Montpellier, France, 2010*.

^cThis study included patients with metastatic melanoma, but also patients with renal cell carcinoma, colorectal cancer, prostate cancer, and non-small-cell lung cancer. BRAF, gene responsible for production of B-Raf-kinase; CTLA4, cytotoxic T-lymphocyte antigen 4; IL-2, Interleukin 2; n.c., not classified; n.r., none reported; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; PD1, programmed cell death 1 receptor; PDL1, programmed cell death 1 ligand.

directed against MAGE-A3. Collectively, these trials have not only demonstrated feasibility but also demonstrated significant clinical responses in patients with metastatic melanoma, colorectal carcinoma, and synovial sarcoma (Table 2). Responses, although variable and tested in a cumulative number of about 80 patients (based on trials listed in Table 2), ranged from 12 to 67%. Notably, the finding that TCR gene-engineered T cells were able to traffic to the central nervous system and cause complete responses of brain metastasis in patients with melanoma was not only encouraging but also underscored the strength of T cell therapy toward metastasized and poorly accessible tumors (7). Clinical testing, however, also clearly demonstrated that therapy is currently hampered by treatment-related toxicity and a transient nature of tumor regression. Treatment-related toxicity became evident from studies with TCRs, in particular those of high-affinity, directed against antigens that are over-expressed on tumors but also expressed on healthy cells. Toxicities included severe but treatable inflammation of skin, eyes, ears (MART-1/HLA-A2; gp100/HLA-A2), and colon (CEA/HLA-A2). In addition, lethal neurological toxicities were observed in two patients when targeting MAGE-A3/HLA-A2, and lethal cardiac toxicities were observed in three patients when targeting MART-1/HLA-A2 (another epitope as above) or MAGE-A3/HLA-A1. The transient nature of tumor regression became evident from observations that anti-tumor responses are initially significant but not sustainable and ultimately incomplete in 80–90% of patients. Table 2 offers an up-to-date and detailed overview

of toxicities as well as clinical responses reported for TCR gene therapy trials.

Strategies that aim at preventing or limiting toxicities as well as tumor recurrences have already been developed, some of which need further preclinical testing and some of which have already been implemented in clinical trials. In this review, we have categorized these strategies along three renewed challenges, i.e., “choice of target antigen”; “fitness of T cells,” and “sensitization of micro-milieu for T cell therapy,” as illustrated in Figure 2. We propose and will argue that optimizations along each or combinations of these challenges will contribute most significantly to the advancement of clinical TCR gene therapy.

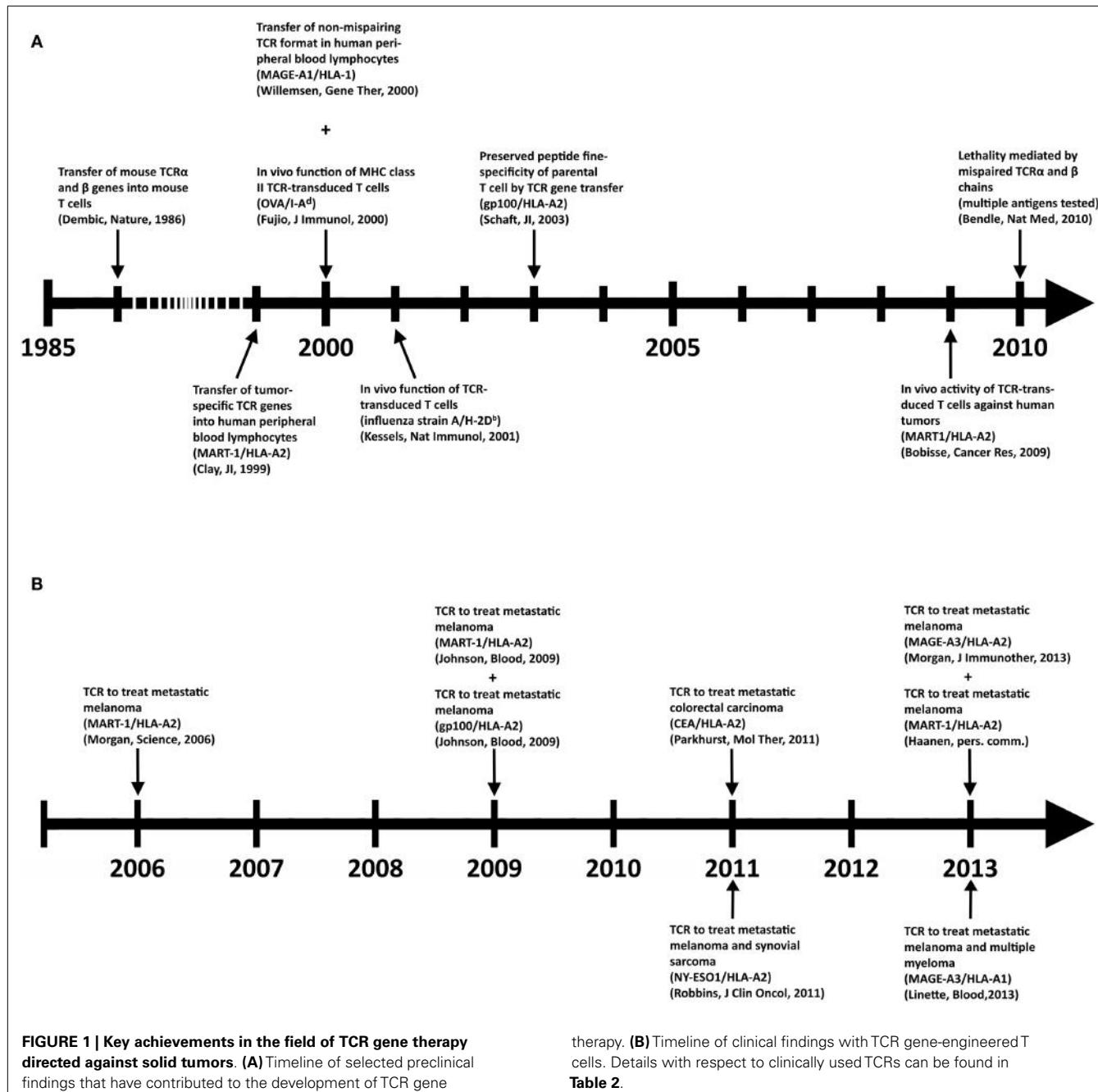
CHOICE OF TARGET ANTIGEN

Ideally, target antigens are selectively expressed by tumor tissue and not healthy tissue, and hence not expected to evoke a response against self. At the same time, target antigens should have proficient immunogenicity to initiate an effective anti-tumor response.

SELECTIVE EXPRESSION

Tumor-associated antigens (TAAs) can generally be divided into four groups (8).

- Differentiation antigens: cell surface proteins that are expressed at different stages of tissue development or cell activation.



Expression of these antigens may discriminate tumor cells from surrounding healthy cells, but expression by healthy cells is not absent. Examples include MART-1, gp100, CEA, and tyrosinase related protein (TRP)1 and 2.

- Over-expressed antigens: cell surface proteins that are highly, but not selectively, expressed by tumor cells when compared to healthy cells. Examples include the epidermal growth factor receptor (HER)2 or survivin.
- Cancer Testis Antigens (CTAs): proteins that are expressed by tumors and a limited number of healthy and adult cell types. A defined number of CTAs may not be expressed by healthy

adult cell types. Examples include MAGE-A1, MAGE-C2, and NY-ESO1.

- Neo-antigens: proteins that result from gene mutations or aberrations in tumor cells. These proteins are uniquely expressed by tumor cells but not healthy cells. Examples include mutated protein (p)53, B-Raf kinase, and cyclin-dependent kinase 4 (CDK4).

Looking at these four groups of TAAs, CTAs, and neo-antigens may represent the best available choices for therapy with TCR-engineered T cells. With respect to CTAs, over several hundreds

Table 2 | T cell receptor gene therapy trials – an update on efficacy and safety.

Target antigen (epitope)	Original T cell clone/lines	Tumor type	OR (%)	CR (%)	Toxicity (%) ^a	Type of toxicity	Reference
MART-1(AAG)/HLA-A2	TIL clone DMF4 from responding patient	Metastatic melanoma	2/17 (12)	n.r.	0/17 (0)	n.r.	(189)
MART-1(AAG)/HLA-A2	TIL clone DMF5 from responding patient with high <i>in vitro</i> avidity	Metastatic melanoma	6/20 (30)	n.r.	9/36 (25)	Severe melanocyte destruction in skin, eye, and ear (in some cases leading to uveitis and hearing loss)	(190)
gp100(KTW)/HLA-A2	Splenocytes from immunized mouse	Metastatic melanoma	3/16 (19)	n.r.			
CEA(IMI)/HLA-A2	Splenocytes from immunized mouse; TCR is affinity-enhanced	Metastatic colorectal carcinoma	1/3 (33)	n.r.	(3/3) (100)	Severe inflammation of colon	(191)
NY-ESO1(SLL)/HLA-A2	T cell clone 1G4 from human subject; TCR is affinity-enhanced	Metastatic melanoma	5/11 (45)	2/11 (18)	0/11 (0)	n.r.	(192)
		Metastatic synovial sarcoma	4/6 (67)	0/6 (0)	0/6 (0)		
MAGE-A3(KVA)/HLA-A2	Splenocytes from immunized mouse; TCR is affinity-enhanced	Metastatic melanoma	5/9 (55)	2/9 (22)	3/9 (33)	Changes in mental status, two patients fell into coma and subsequently died, one patient recovered	(29)
MART-1(ELA)/HLA-A2	T cell clone 1D3 from human subject; TCR is codon-optimized and murinized	Metastatic melanoma	n.r.	n.r.	1/1 (100)	Lethal cardiac toxicity in one patient	^b
MAGE-A3(EVD)/HLA-A1	T cell clone a3a from human subject; TCR is affinity-enhanced	Metastatic melanoma and multiple myeloma	n.r.	n.r.	2/2 (100)	Lethal cardiac toxicity in two patients	(30)

OR, objective responses; CR, complete responses, both according to Response Evaluation Criteria for Solid Tumors (RECIST). Number of patients with responses = before dash; total number of patients = after dash; percentage of responses = between brackets.

^aNumber of patients with Serious Adverse Events (toxicity grading ≥ 3 according to National Cancer Institute common toxicity criteria) and total number of patients treated are put before and after dash, respectively.

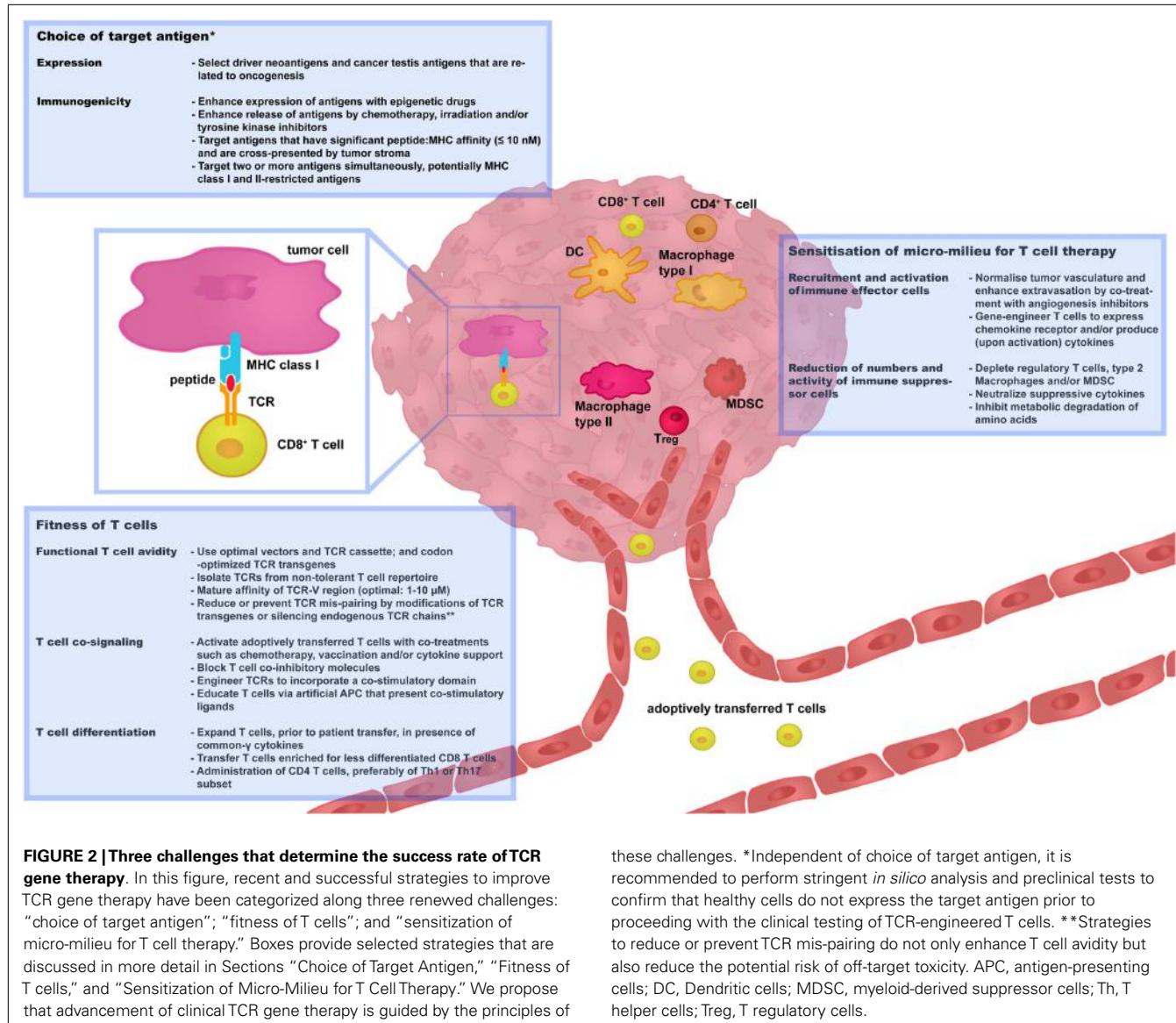
^bDr. John Haanen, Cellular Therapy of Cancer Symposium, London, UK, February 27th–March 2nd, 2013.

CEA, carcinoembryonic antigen; gp, glycoprotein; HLA, human leukocyte antigen; MAGE, melanoma-associated antigen; MART, melanoma antigen recognized by T cells; n.r., none reported; NY-ESO1, New York esophageal squamous cell carcinoma 1.

of genes have been identified (see for a full description of CTAs²). Approximately 40 of these genes belong to multigene families that are located on the X-chromosome. A selected number of mostly X-chromosome-located CTAs may be of interest for T cell therapy. *First*, these antigens are not expressed by healthy tissues except testes and placentas (determined using RT-PCR), and these latter tissues do not express Major Histocompatibility (MHC) molecules and cannot be targeted by T cells (9). *Second*, CTAs are expressed by tumor tissues of various histological origins as a result of aberrant epigenetic regulation (9), and expression of CTAs has been associated with advanced stages of disease and unfavorable patient prognosis (10). Along these lines, there is evidence that MAGE

proteins are related to oncogenesis as they suppress p53-dependent apoptosis and cause fibronectin-controlled increase in tumor cell proliferation and metastasis (11–15). *Third*, CTAs are immuno-genic proteins that have been reported to induce both humoral and cell-mediated immune responses in patients without the concomitant induction of toxicities (10, 16, 17). Undeniably, current patient studies emphasize the need for careful identification of target CTAs. In one study, Robbins and colleagues demonstrated that a TCR directed against NY-ESO1/HLA-A2 showed significant anti-tumor responses in patients with metastatic melanoma and synovial sarcoma without detectable toxicities (Table 2). Unexpectedly, in another study using a TCR directed against MAGE-A3/HLA-A2, two patients with metastatic melanoma lapsed into coma and died. These adverse events were most likely caused by T cell recognition of rare neurons that were positive for MAGE-A12

²<http://www.cta.lncc.br>



and possibly MAGE-A9 antigens, which contain shared or highly similar epitopes compared to MAGE-A3 antigen (Table 2). In a third study, in which a TCR was used directed against MAGE-A3/HLA-A1, one patient with melanoma and one patient with myeloma suffered from cardiovascular toxicity and died. This toxicity was possibly caused by T cell recognition of a similar but not identical peptide from the muscle protein titin (so-called "off-target" toxicity, Table 2).

With respect to neo-antigens, the expression of these antigens may vary significantly among different patients, but their expression is unique to tumor tissues. In case a neo-antigen is the result of "driver mutations," the antigen may constitute an ideal target for T cell therapy. Driver mutations are related to oncogenesis, may be linked to known genes (~400), and may provide tumors with a selective growth advantage (18, 19). Nevertheless, it is important to realize that only 15% of up to 100,000 mutations that are encountered in tumor genomes are considered "driver" mutations

these challenges. *Independent of choice of target antigen, it is recommended to perform stringent *in silico* analysis and preclinical tests to confirm that healthy cells do not express the target antigen prior to proceeding with the clinical testing of TCR-engineered T cells. **Strategies to reduce or prevent TCR mis-pairing do not only enhance T cell avidity but also reduce the potential risk of off-target toxicity. APC, antigen-presenting cells; DC, Dendritic cells; MDSC, myeloid-derived suppressor cells; Th, T helper cells; Treg, T regulatory cells.

(18, 20). Moreover, not all driver mutations may result in new immunogenic antigens. A quest for neo-antigen targets does not only require next-generation sequencing techniques to identify tumor-specific mutations (21), but also techniques to determine whether a neo-epitope can be presented by MHC and recognized by T cells (22, 23).

In short, we consider epitopes from selected (non-shared) CTA and neo-antigens as potentially safe T cell target antigens. However, no matter what the antigen, it is recommended to perform stringent *in silico* analysis and preclinical testing to confirm the antigen's absence from vital organs. Strategies used to identify titin as a cross-recognized peptide, such as amino acid scanning, gene database searches, and use of three-dimensional cell cultures, are potentially helpful in this respect (24). In addition, one could consider using suicide systems to deplete self-reactive T cells prior to proceeding with clinical testing (25–28). Although suicide genes provide the option to delete TCR-transduced T cells,

it is questionable whether such a switch could counteract the fast kinetics of toxicity reported in the above-mentioned trials (29, 30).

IMMUNOGENICITY

The immunogenicity of an antigen, i.e., its ability to initiate immune responses, is determined by the level of its expression, how it is processed and presented, and how well it is recognized by T cells.

Level of expression and processing of antigens

Ideally, target antigens should be expressed at high levels by most if not all tumor cells. Such a property is generally restricted to those antigens that are related to oncogenesis and that tumors cannot easily do without (see Selective Expression). It is noteworthy that the production of antigens, such as those of MAGE-A family members and NY-ESO1, is enhanced and becomes more homogeneous within tumors by treatment with demethylation agents and/or histone deacetylases (31–34). In a phase II clinical study, in which hematological malignancies were targeted and which included treatments with epigenetic drugs, it was observed that T cell responses directed against CTA were enhanced with no evidence of adverse events (35). In addition, the production of antigens may depend on immune or intermediate proteasomes, rather than standard proteasomes, and on unconventional post-translational events such as reverse splicing and deamidation of proteins (36–38). Such processing of antigens, in particular when mediated by immune proteasomes, may benefit from local production of interferon (IFN) γ . Finally, the release and hence the availability of antigens may be enhanced via treatment-induced cell death following (co-treatments with) chemotherapy, irradiation, and/or therapy with tyrosine kinase inhibitors (39, 40).

Cross-presentation of antigens

Antigen cross-presentation may take part in the infiltration of antigen-specific CD8 T cells (41) and cause activation of T cells and subsequent stroma destruction, thereby preventing outgrowth of antigen-negative tumor cells. Recently, Engels and colleagues revealed that peptide:MHC affinities of 10 nM or less allowed for cross-presentation of antigens by stromal cells (42). Notably, using an experimental model in which mice transgenic for TCRs with different antigen specificities were used either as donors or recipients of T cells, they showed that the use of peptide targets that can be cross-presented result in complete anti-tumor responses. Destruction of tumor stroma, a bystander response that may put an advantage to T cells over drugs (43, 44), may require optimal T cell fitness (as measured by production of IFN γ) and IFN γ -mediated preservation of Fas expression by stromal cells (45).

Robustness of antigenicity

Loss of tumor antigen expression after infusion of T cells, and its impact on the recurrence of tumors, is an important yet controversial aspect. Decreased antigen expression has been proposed to be a consequence of molecular alterations in tumor cells, such as genetic and epigenetic changes in antigen genes, MHC genes, and genes related to antigen processing and presentation (46–48). Indeed, selective loss of antigen or HLA-A2 expression has been

reported in primary and metastatic melanoma lesions in non-treated patients (49, 50) as well as patients treated with T cells (51, 52). Also, Landsberg and colleagues, using a gene-engineered model of melanoma, have eloquently demonstrated that a therapy-resistant phenotype may be directed by an inflammatory milieu and tumor necrosis factor (TNF) α 's ability to lead to epithelial dedifferentiation and decreased expression of melanoma antigens (53). In contrast to these findings, there is increasing evidence to support the view that tumors progress without loss of T cell antigens. In various preclinical models, in which either skin, lung, or ovary tumors were studied, it was observed that tumors progressed despite continued antigen expression (54–56). In these models, tumor progression was rather a consequence of reduced T cell infiltration and reduced T cell responsiveness. We postulate that in the setting of T cell therapy, loss of target antigen, whether by T cell-dependent selection or epigenetic silencing (57, 58), is *not* necessarily a driving mechanism in tumor recurrence (Straetemans et al., manuscript submitted).

Target multiple antigens simultaneously

In current TCR gene therapy trials, single MHC class I-restricted antigens are targeted. Preclinical studies have suggested that the targeting of two or more antigens enhances the therapeutic potential of T cells. For example, adoptive transfer of two CD8 T cell populations to simultaneously target ovalbumin and gp100, rather than either one antigen, resulted in delayed recurrence of tumors (59). Interestingly, treatment with viruses positive for three MHC class II-restricted antigens, i.e., neuroblastoma RAS, TRP1, and cytochrome c1, resulted in complete anti-tumor responses that were accompanied by significant CD4 T helper cell type 17 (Th17) responses (60). Since cooperation of CD4 and CD8 T cells appears important in the effector phase of an anti-tumor response and may contribute to the bystander elimination of tumor stroma (61), it may be worthwhile to simultaneously target MHC class I and II targets. With respect to human antigens, it is interesting to note that X-chromosome linked CTAs are coordinately expressed in tumor tissues (62), which may allow the simultaneous targeting of multiple CTAs.

FITNESS OF T CELLS

The responsiveness of T cells toward tumor antigen is generally tuned down, most likely at various levels. First, reactive T cells may be deleted during T cell development in the thymus; second, peripheral T cells may be susceptibility to anergy; and third, intra-tumoral T cells may require enhanced co-stimulation (63). To overcome such T cell tolerizing mechanisms one can optimize T cell fitness. Here, we define T cell fitness according to the following three T cell properties: functional T cell avidity, T cell co-signaling, and T cell differentiation.

FUNCTIONAL T CELL AVIDITY

Functional T cell avidity is considered as the ability of T cells to respond to a given concentration of cognate peptide antigen, and can be enhanced via strategies, often involving gene-engineering of TCR $\alpha\beta$ transgenes, that either increase the level of cell surface expression of TCR chains or the TCR's affinity for peptide-MHC.

Expression level of TCR transgenes

One angle to enhance the surface expression of TCR transgenes is through optimization of the TCR gene transfer methodology, including choice of gene delivery method, use of optimal vector elements, and use of transgene cassettes [reviewed in Ref. (6, 64)]. Another angle to enhance the surface expression of TCR transgenes is through limitation or abolishment of TCR mis-pairing. TCR mis-pairing is the formation of TCR heterodimers that comprise one transgenic TCR chain and one endogenous TCR chain, and represents a phenomenon that is inherent to the generation of TCR-engineered T cells. Importantly, TCR mis-pairing dilutes the surface expression of the transgenic TCR $\alpha\beta$ chains, and mis-paired TCRs are of unknown specificity and can yield self-reactive T cells. Although in clinical trials performed so far, no formal observations of toxicities mediated by TCR mis-pairing have been made, pre-clinical studies have clearly demonstrated that TCR mis-pairing has the potential to induce harmful recognition of self-antigens (65, 66). Strategies to promote preferential pairing between transgenic TCR α and TCR β chains (and consequently prevent or reduce TCR mis-pairing) can be grouped according to those that depend on gene-engineering of TCR transgenes and those that do not. The first group of strategies are reviewed in Ref. (67). In short, these strategies include murinization of TCR (68), addition of cysteine amino acids to TCR (69, 70), mutations in TCR transmembrane and constant domains (71, 72), and equipment of TCR with a signaling cassette that replaces TCR transmembrane and intracellular domains with the CD3 ζ accessory molecule (73, 74). More recently, a limited number of murine amino acids have been identified that are responsible for enhanced expression and preferential pairing of murinized TCRs (75, 76). Similar efforts to minimize the number of amino acids in a CD3 ζ signaling cassette failed, and it was observed that properties of TCRs equipped with CD3 ζ signaling cassettes are best preserved when incorporating a complete CD3 ζ molecule (77). The other group of strategies includes technologies that enhance expression levels of CD3 molecules in T cells and those that interrupt expression of endogenous TCR chains. Co-transfer of CD3 and TCR genes into T cells resulted in higher levels of TCR expression and allowed T cells to respond to lower concentrations of antigen, and to infiltrate and eliminate tumors with faster kinetics (78). RNA interference techniques have been shown to specifically down-regulate the expression of endogenous but not transgenic TCR chains (79, 80). An alternative method encompasses the use of zinc finger nucleases and a sequential knock-out of endogenous TCR α and β chains, followed by introduction and sorting of TCR α and β transgenes (81). The latter method is relatively new and not yet widely or clinically applied, but holds promise to effectively address TCR mis-pairing.

Affinity-enhancement of TCR $\alpha\beta$ transgenes

Affinity-enhancement of tumor-specific TCRs, and its exploitation, relies on the existence of a window for optimal TCR affinities. The existence of such a window is based on observations that TCRs specific for HLA-A2-restricted pathogens have K_D values that are generally about 10-fold lower when compared to TCRs specific for HLA-A2-restricted tumor-associated self-antigens (82). In support of this notion are the observations that a high-affinity MART-1/HLA-A2 TCR mediated improved objective response

rates compared to a lower affinity MART-1/HLA-A2 TCR, and that an affinity-enhanced NY-ESO1 TCR mediated significant clinical responses (Table 2). Affinity-enhanced TCRs can be obtained through various routes. First, allo-reactive settings can be used to circumvent self-tolerance and yield T cells with a higher avidity when compared to T cells derived from autologous settings (=patients). Examples of such settings include *in vitro* generation of allo-HLA reactive, peptide-specific T cells (83–85), and immunization of mice transgenic for human-MHC or human TCR (86, 87). Second, TCR affinities can be enhanced by rationally designed mutations of the TCR's complementarity-determining regions (CDRs) (88, 89). Third, high-affinity TCR variants can be selected from a library of CDR mutants by yeast, phage, or T cell display (90–92). Although the affinity of TCRs significantly contributes to the functional avidity of T cells, recent studies warrant caution when therapeutically implementing this strategy. Clinical reports suggest that CDR mutations in TCRs directed against CEA/HLA-A2, MAGE-A3/HLA-A2, and MAGE-A3/HLA-A1, but not NY-ESO/HLA-A2, were possibly related to patient toxicities (Table 2). Investigations whether defined locations and types of mutations are more prone to lead to toxicities than others would most likely benefit further development of CDR-mutated TCRs. Also, preclinical reports suggest the existence of a functional ceiling with respect to TCR affinity (93, 94). In fact, studies with primary human T cells transduced with affinity-enhanced TCRs directed against NY-ESO1/HLA-A2 (93) or gp100/HLA-A2 (Govers et al., manuscript submitted) pointed to the existence of a K_D threshold of 1–5 μ M, below which T cell function became compromised. The functional impairment of high avidity T cells in the presence of high levels of antigen, as is often the case in tumors, may be related to enhanced expression of the exhaustion marker programmed cell death (PD1) and enhanced activity of its downstream sarcoma homology domain 2 phosphatase (SHP)1 (95, 96).

T CELL CO-SIGNALING

T cell co-signaling is directed by interactions between co-stimulatory or co-inhibitory molecules and their ligands and determines, in addition to interactions between TCR and peptide-MHC, the functional outcome of T cells [reviewed by Chen and Flies (97)]. The best characterized co-stimulatory and co-inhibitory molecules expressed by T cells are CD28 and cytotoxic T-lymphocyte associated protein (CTLA)4, respectively, which both interact with CD80 and CD86 ligands expressed by APCs. More recent examples of co-stimulatory and co-inhibitory molecules include inducible T cell co-stimulation (ICOS), 4-1BB, OX40, CD40, B and T-lymphocyte attenuator (BTLA), and PD1.

Tumors provide continuous stimulation with antigen often in the absence of co-stimulatory ligands, which may result in exhausted T cells with reduced proliferative capacity, reduced effector function (such as IFN γ production) (98), and up-regulated expression of T cell co-inhibitory molecules (99). Immunotherapy with monoclonal antibodies to block the T cell co-inhibitory molecules CTLA4, PD1, PDL1, or the combination of CTLA4 and PD1 showed clear clinical successes in the treatment of advanced melanoma (see Table 1). These clinical activities have provided an impetus for the development of blocking other co-inhibitory molecules and/or stimulation of co-stimulatory

molecules (100–104). The beneficial outcome of targeting T cell co-signaling most likely relies on enhancement of infiltration of T effector cells (Teff) into tumor tissue and activation of Teff, as well as depletion of intra-tumoral T regulatory cells (Treg) (103–105). We would advocate explorative studies to test the combination of blocking T cell co-inhibitory molecules and adoptive transfer of Teff. In addition to this combination of immune therapies, two other approaches to implement T cell co-signaling in protocols of T cell therapy have already been clinically tested. First, TCR transgenes can be equipped with a signaling cassette that harbors a co-stimulatory molecule. Such a signaling cassette, designed in analogy to those used in co-stimulatory CARs (6), typically introduces accessory and co-stimulatory molecules to enhance the function of T cells expressing the TCR transgene. It is noteworthy that clinical trials using CARs containing CD28 or CD137 demonstrated significant objective responses in patients with B cell leukemia (106–108), and while CARs may evoke immune responses, these were directed against murine idiotypes, but never against boundaries between genetically introduced human molecules (109). According to this rationale, single and two-chain TCR genes have been coupled to a combination of CD28 and CD3 molecules and were shown to provide T cells with improved function *in vitro* (110, 111) (Govers et al., manuscript submitted). Second, T cells can be stimulated *ex vivo* with human artificial APC (aAPCs) that express co-stimulatory ligands (4, 112). In addition to co-stimulatory ligands, these aAPCs are mostly engineered to express HLA-A × 0201 and used to stimulate T cells in the presence of common- γ cytokines other than interleukin (IL)-2. These combined activations allow for the generation of HLA-A2-restricted, antigen-specific T cells with a less differentiated phenotype (CD45RA⁺ CD62L⁺) and superior T cell functions *in vivo* (112). In a clinical study, T cells educated with aAPC presenting CD80, CD83, and a MART-1 peptide, and cultured in the presence of IL-2 and IL-15, resulted in objective responses in patients with metastatic melanoma (Table 1). Notably, inclusion of T cell co-stimulation by either one of the two above-mentioned approaches relieved the requirement for patient preconditioning with chemotherapy and/or *in vivo* IL-2 administration (4, 106).

T CELL DIFFERENTIATION

The differentiation of naïve T cells into mature CD8 Teff or CD4 Th1 or Th17 cells is required for T cells to make full use of their functional attributes directed against tumor cells, such as cytotoxicity and production of IFN γ and TNF α . The differentiation of T cells is largely driven by environmental stimuli, with cytokines being well-studied examples of such stimuli (113, 114). Progression of T cells into a differentiated subset is not necessarily permanent, and in particular T helper cell subsets have shown plasticity and may change into another T helper cell subset (114). Differentiation of CD8 and CD4 T cells, although occurring according to similar principles, follow different routes and show different outcomes. Strategies to manipulate T cell differentiation to advance T cell therapy are discussed separately for both T cell subsets.

CD8 T cells

Naïve CD8 T cells can differentiate, depending on the quantity and quality of the initial antigenic and co-stimulatory stimuli, into

stem-cell memory T cells, central memory T cells, effector memory T cells, or T effector cells (115). An important observation that came from preclinical studies was the inverse relationship between CD8 T cell differentiation and proliferation, and hence the inverse relationship between CD8 T cell differentiation and *in vivo* persistence and therapeutic activity (113). Two strategies have been reported to exploit this inverse relationship and improve adoptive T cell therapy. In one such strategy, as shortly mentioned in Section “T Cell Co-Signaling,” T cells are exposed to common- γ cytokines other than IL-2 prior to adoptive T cell transfer. For example, treatments with either IL-7 + IL-15 or IL-15 + IL-21 generated gene-engineered T cells with a less differentiated CD8 T cell phenotype (i.e., central memory phenotype), prolonged peripheral persistence, and potent antigen reactivity (116, 117). In addition to soluble cytokines, Singh and colleagues reported on aAPC that express membrane-bound IL-15 and IL-21 and facilitate the generation of “young” T cells (112). In other reports, the anti-tumor efficacy of T cells was enhanced either via *in vivo* administration of IL-15 + IL-21 (118) or conjugation of nanoparticles, encapsulating these cytokines, to the surface of therapeutic T cells (119). In a second strategy, T cells are enriched for less differentiated T cell populations, i.e., based on CD62L expression, and subsequently used as recipient cells for gene transfer (120, 121). A recently identified population of “stem-cell memory” CD8 T cells, expressing high levels of CD95, IL2R β and demonstrating increased proliferative potential and ability to mediate anti-tumor responses, may represent a promising subset of T cells for gene-engineering and therapeutic application (122). In fact, Cieri and colleagues have set up a protocol to obtain and gene-modify stem-cell memory CD8 T cells, which includes the use of CD3/CD28 mAbs and IL-7 and IL-15 and could potentially be translated to a clinical setting (123).

CD4 T cells

Naïve CD4 T cells can differentiate into multiple subsets, including Th1, 2, 9, 17, 22, follicular helper and various Tregs, often defined by the expression of “signature cytokines” or typical functions, such as B cell activation or the down-modulation of T cell responses (124). With respect to anti-tumor responses, it appears that upon cell transfer Th1 and Th17 are the most potent CD4 T cell subsets (125, 126). Administration of CD4 T cells, and in particular Th1 cells, has been shown to prevent exhaustion of CD8 T cells, enhance tumor infiltration of CD8 T cells and result in effective tumor eradication (125, 127–130). More recently, it was discovered that adoptive transfer of Th17 cells effectively mediate rejection of TRP1-positive tumors in a TCR-transgenic mouse model (126). Furthermore, Th17 cells appear to be long-lived and their molecular signature resembles that of stem-cell memory CD8 T cells (131). Interestingly, the anti-tumor activity of Th17 cells depended on its (incomplete) differentiation and conversion into Th1 cells, resulting in a co-existence of Th17 and Th1 cells, and it may very well be this multi-potent aspect that provides a therapeutic advantage.

Collectively, these data argue in favor of a combined therapeutic use of CD8 T cells and Th1 or Th17 cells. To this end, CD4 T cells can be functionally endowed with MHC I-restricted TCR and/or CD8 via gene transfer (132–135). Alternatively, one could opt for strategies that induce *in vivo* conversion of CD4 T cells

into Th1 cells, such as IL-12, IFN α , IFN γ , or blocking PD1 ligation (136–139). Also, metabolic signals, such as activation of T cell mammalian target of rapamycin (mTOR) and aerobic glycolysis can enhance differentiation toward IFN γ -producing T cells and may be exploited therapeutically (140, 141).

SENSITIZATION OF MICRO-MILIEU FOR T CELL THERAPY

Tumors, following initial regression upon treatment with T cells, most often become resistant to T cell therapy and recur. Recent understanding suggests that, at least in some tumors, therapy resistance may be part of a negative feedback loop that is initiated once an anti-tumor CD8 T cell has occurred (142). Therapy resistance is often characterized by a dis-balance between numbers and activation state of immune effector cells versus those of suppressor cells. Strategies to manipulate numbers and activation state of immune cells are discussed separately for effector and suppressor cells.

RECRUITMENT AND ACTIVATION OF IMMUNE EFFECTOR CELLS

Immune effector cells that have been recognized for their contribution to an anti-tumor response are numerous and, in addition to CD4 and CD8 T cells, include natural killer (NK), natural killer T cells (NKT), macrophages, and neutrophils. Here we will focus on Teff and macrophages and how manipulation of the micro-milieu may enhance their recruitment and activation.

Enhance recruitment of T effector cells

Clinical studies have demonstrated an unfavorable prognostic value of a limited CD8 T cell infiltration in melanoma, colorectal and ovary carcinomas (143–145). Vascular changes have been reported to contribute to arrested T cell infiltration and include insufficient vascular maturation and enhanced expression of endothelin B receptor, regulator of G-protein signaling 5 (Rgs5) and/or extracellular matrix components [reviewed in Ref. (146)]. Such changes may be targeted, as evidenced by angiostatic therapy in which antibodies directed against vascular endothelial growth factor (VEGF) or angiopoietin 2, or in which T cells gene-engineered with a CAR directed against VEGF receptor (VEGFR)2 resulted in enhanced T cell infiltration (147–149). In addition, drugs that inhibit angiogenesis or endothelin receptor B were able to enhance the expression of intercellular adhesion molecule (ICAM)1 on endothelial cells and to normalize T cell infiltration (150, 151). In various solid tumors, T cell infiltration appears to be facilitated by vessels that closely mimic high endothelial venules (HEV) and which may be part of ectopic lymphoid structures in tumor stroma (152, 153). A better understanding of the development of such HEV in tumor stroma may provide novel targets to improve T cell infiltration in tumors.

In addition to vascular changes, spontaneous cutaneous melanoma tumors in mice demonstrated a decreased mRNA expression of chemoattractants that contribute to recruitment of CD8 T cells, such as chemokine (CC motif) ligand (CCL)5 and chemokine (CXC motif) ligands (CXCL)9 and 10 (146). In a subset of patients with melanoma metastases, lack of chemoattractants coincides with limited migration of CD8 T cells and limited presence of lymphoid structures (154). Current findings from our laboratory suggest that a decreased expression of selected chemoattractants and adhesion molecules are related to a decreased infiltration of CD8 T cells and tumor relapse following T cell therapy

(Straetemans et. al., manuscript submitted). Interestingly, Hong and colleagues have shown that the chemotherapeutic drugs dacarbazine, temozolomide, and cisplatin enhanced the expression of CCL5, CXCL9, and CXCL10 in patient melanoma, which in turn correlated with improved immune control of tumors (155). Vice versa, T cells when gene-engineered to express chemokine (CXC motif) receptor (CXCR)2 displayed enhanced trafficking toward tumor cells secreting the corresponding chemokine ligand CXCL1 (156). Also, in xenograft tumor models of mesothelioma and neuroblastoma, the genetic introduction of chemokine (CC motif) receptor (CCR)2 in T cells resulted in increased T cell infiltration in tumors secreting CCL2 and was associated with significantly increased anti-tumor activity (157, 158). Other molecules often present in the micro-milieu that, when targeted, resulted in enhanced T cell accumulation at the tumor site are indoleamine 2,3-dioxygenase (IDO) and reactive nitrogen species. Inhibition of IDO by a small molecule blocks tryptophan depletion, enhances T cell infiltration, and delays tumor growth (159). Reactive nitrogen species induce TIL unresponsiveness (160), nitration of the TCR complex (161), and modification of the chemokine CCL2 (162). Drugs affecting the local production of reactive nitrogen species restore TIL function and improve intra-tumoral T cell migration and an anti-tumor T cell response (160, 162). Taken together, the above studies show the drug-ability of molecules that are involved in T cell extravasation and T cell migration into tumor tissues, and advocate studies to combine such drugs with adoptive T cell therapy.

Enhance T cell effector functions

Early protocols of adoptive T cell therapy already demonstrated the beneficial effects of co-treatments such as chemotherapy, vaccination, and/or cytokine support on T cell activation [reviewed in Ref. (64)]. More recently, additional strategies that enhance anti-tumor functions of Teff have been reported. A first strategy became apparent from clinical success with additional T cell co-stimulation or blocking of T cell co-inhibition (see T Cell Co-Signaling and Table 1). A second strategy relates to the inhibition of T cell suppressive cytokines, such as transforming growth factor (TGF) β . For example, genetic introduction of a dominant-negative TGF β receptor II in TCR-engineered T cells resulted in increased anti-tumor T cell responses in a spontaneous tumor model of prostate cancer (163). Another study tested the safety of mouse T cells engineered with this dominant-negative receptor, and could not detect spontaneous proliferation of these T cells *in vivo* (164). Genetic knockdown of negative regulators of T cell activation represents yet another strategy to enhance T cell activation. T cells with siRNA-mediated knockdown of casitas B-lineage lymphoma b (Cbl-b) displayed a lower threshold for T cell activation and, when adoptively transferred in mice with disseminated leukemia, resulted in enhanced anti-tumor effects (165). These latter findings warrant further testing of T cells with enhanced T cell activation, including tests that assess the safe use of these T cells.

Enhance recruitment and activation of macrophages

High numbers of macrophages with a tumor-promoting (M2) phenotype, but not those with a tumor-inhibiting (M1) phenotype, correlate with poor prognosis for patients with various

cancers (166). When conjugated to a vascular homing peptide and targeted to tumors, TNF α resulted in a switch from M2 to M1 macrophages, which was accompanied by normalization of tumor vasculature and enhanced infiltration of CD8 T cells (167). Interestingly, T cells gene-engineered to release the cytokine IL-12 were shown to improve the therapeutic efficacy of T cells, an effect that is likely mediated by cells of the innate immune system (168, 169). T cells that express IL-12 under the control of the Nuclear Factor of Activated T cell (NFAT) promoter, and deliver IL-12 locally in the tumor environment upon encounter of cognate antigen, induce destruction of antigen-negative cancer cells with a prominent role for monocytes and monocyte-derived TNF α (168). Such findings are not necessarily restricted to IL-12 since IL-15, when provided locally into tumors, also enhanced the responsiveness of adoptively transferred T cells and facilitated the removal of antigen-negative tumor cells (170).

REDUCE NUMBERS AND ACTIVITY OF IMMUNE SUPPRESSOR CELLS

T regulatory cells, M2 macrophages, and myeloid-derived suppressor cells (MDSC) are among the major immune-suppressive cell types in the tumor micro-milieu. Immune suppressor cells can reduce T cell infiltration into the tumor and suppress local T cell responses by: release of reactive nitrogen and oxygen species (171); expression of IDO and arginase (159, 172); and production of cytokines such as TGF β , IL-4, and IL-13 (173). Despite initial removal of these cells by administration of chemotherapeutic agents, the populations of MDSCs and Tregs may recover at a faster rate than CD4 and CD8 Teff (174). Furthermore, Jensen and colleagues demonstrated that therapeutic CD4 $^+$ T eff can convert into a Foxp3 $^+$ CD4 $^+$ Treg population (175). Various strategies have been reported to deplete or inactivate Tregs. These strategies include administration of anti-CD25 antibodies, combined intra-tumoral injection of anti-CTLA4 and OX40 mAbs, or blocking IDO (104, 176). Interestingly, blocking IDO may induce conversion from Treg to Th17 helper cells, which can further contribute to anti-tumor T cell responses (176). With respect to MDSCs, it is of interest to note that classical chemotherapeutic agents, such as docetaxel, are able to deplete these cells. Docetaxel-mediated depletion of MDSC, when combined with adoptive T cell therapy and dendritic cell vaccination, was shown to enhance anti-tumor responses (174). Alternatively, differentiation of MDSC into mature myeloid cells, which can be established upon administration of β -glucans (glucose monomers from cell walls), may also provide an angle to relieve immune suppression (177).

FUTURE PERSPECTIVES

By now, the feasibility of TCR gene therapy studies has been well established by the pioneering trials listed in **Figure 1B**, and is further enhanced by current optimizations and standardizations of protocols. TCR gene therapy, alike any cell-based therapy, requires specialized good manufacturing practice (GMP) and patient treatment facilities. Such facilities allow the generation and testing of virus batches and the gene processing and expansion of T cells, and are already integrated in multiple academic and private centers. Notably, parameters, such as time-lines and costs to manufacture a therapeutic T cell product, are considered competitive when compared to other clinical-grade products, such as antibodies. An

ongoing EU project to treat metastatic esophagus-gastric cancer and melanoma with NY-ESO1 TCR-engineered T cells, in which we participate, shows that time-lines and costs to obtain a T cell product are about 2 weeks and 36 k€ per patient (13.5 k€ for production, quality testing, and test runs of virus batch; and 22.5 k€ for T cell processing), respectively. For comparison: estimated per patients costs of Ipilimumab (3 mg/kg every 3 weeks, 4 times) and Vemurafenib (0.96 g twice daily for 6 months), both registered treatments for metastasized melanoma in The Netherlands since 2012, are 84 and 57 k€ [Association of Health Insurances (CVZ), The Netherlands]. The next step, and allowing a more valid comparison, would be the testing of T cell therapy versus standard treatment of care in a randomized trial.

Clinical testing of TCR-engineered T cells, when looking at single trials, demonstrated impressive and unprecedented efficacy but at the same time is hampered by treatment-related toxicity and a transient nature of tumor regression (**Table 2**). There exists a multitude of strategies that are developed and tested toward advanced safety and efficacy of TCR gene therapy. Here, we have defined three challenges and have categorized recent and successful strategies along these three challenges, which have been schematically depicted in **Figure 2**. With respect to the first challenge, i.e., choice for target antigen, an important criterion is minimal or no expression of such an antigen by healthy tissues. In this respect, non-shared and tumor-restricted CTAs as well as neo-antigens should be considered as potentially safe target antigens. Advances in the isolation and characterization of anti-tumor T cells from individual patient samples may increase the number of CTAs and neo-antigens that may qualify as target antigens. T cell-based recognition of similar, but unrelated peptides should be excluded, and to this end it is strongly recommended to perform stringent *in silico* analysis and preclinical tests to confirm that cross-reactive antigens are absent in healthy tissue. In order to improve patient safety further, measures to allow directed killing of engineered T cells have been tested and should be considered, at least for novel TCRs tested in the near future. In addition to tumor-restricted expression, another criterion to choose target antigens is maximal immunogenicity. Peptide epitopes that are cross-presented or the targeting of a more than a single peptide have been reported to induce complete anti-tumor responses, and may represent examples to consider when selecting target antigens.

With respect to the second and third challenges, i.e., fitness of T cells and sensitization of tumor micro-milieu, we would like to propose a two-step treatment protocol. The first step represents the transfer of fit T cells. T cell fitness involves optimal T cell avidity, additional T cell co-signaling, and using T cells with a preferred differentiation stage. T cell avidity can be optimized by enhancement of TCR affinity, yet reported treatment-related toxicities warrant caution when using affinity-enhanced TCRs (**Table 2**) and recommend further studies to define rules of TCR binding of cognate versus non-cognate peptides. With respect to T cell co-signaling, antibodies that block T cell co-inhibitory molecules and T cells gene-engineered with co-stimulatory receptors have demonstrated clinical successes. The implementation of such strategies in T cell therapy protocols holds promise for future trials. Also, developments to obtain and gene-modify early differentiation stages of CD8 T cells, including stem-cell memory CD8 T cells, are at the

brim of being translated to a clinical setting. Whatever the chosen route, an important measure for T cell fitness *in vivo* is the ability of these cells, whether it be CD8 T cells or certain subsets of CD4 T cells, to produce IFN γ and TNF α . The production of these cytokines not only determines T cell responsiveness, but also to what extent innate immune cells are recruited into the tumor and become activated to further improve an anti-tumor response and potentially avoid tumor relapse. The second step represents antagonism of an immune-suppressed milieu. Various strategies, such as antibodies or drugs to mediate angiostasis, chemotherapeutic agents to enhance intra-tumoral T cell infiltration, and local (T cell-mediated) delivery of cytokines, have proven beneficial to enhance the local ratio between effector and suppressor immune cells. Development of such a two-step protocol, together with the targeting of a selected antigen, is the way forward and expected to further enhance the success rate of TCR gene therapy to treat solid tumors.

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Role of T cell receptor affinity in the efficacy and specificity of adoptive T cell therapies

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Over the last several years, there has been considerable progress in the treatment of cancer using gene modified adoptive T cell therapies. Two approaches have been used, one involving the introduction of a conventional $\alpha\beta$ T cell receptor (TCR) against a pepMHC cancer antigen, and the second involving introduction of a chimeric antigen receptor (CAR) consisting of a single-chain antibody as an Fv fragment linked to transmembrane and signaling domains. In this review, we focus on one aspect of TCR-mediated adoptive T cell therapies, the impact of the affinity of the $\alpha\beta$ TCR for the pepMHC cancer antigen on both efficacy and specificity. We discuss the advantages of higher-affinity TCRs in mediating potent activity of CD4 T cells. This is balanced with the potential disadvantage of higher-affinity TCRs in mediating greater self-reactivity against a wider range of structurally similar antigenic peptides, especially in synergy with the CD8 co-receptor. Both TCR affinity and target selection will influence potential safety issues. We suggest pre-clinical strategies that might be used to examine each TCR for possible on-target and off-target side effects due to self-reactivities, and to adjust TCR affinities accordingly.

Keywords: adoptive T cell therapy, TCR affinity, T cell sensitivity, T cell cross-reactivity, tumor-associated epitopes

INTRODUCTION

Immunotherapies of cancer use either passive or active approaches to recruit immune cells against tumor cells. Although most passive strategies to date have involved monoclonal antibodies, a growing body of work shows that T cells may provide more immediate and potent anti-tumor cell activity. In the most common adoptive T cell approaches under investigation, genes that encode a T cell receptor (TCR) or a chimeric antibody-based receptor (chimeric antigen receptor, CAR) are introduced into *ex vivo* activated T cells from a patient. Both receptors have shown significant promise, but the properties of these receptors that yield the most effective responses continue to be explored. In addition, because of their potency and sensitivity, adoptive T cells can present safety issues that have not generally been seen with antibodies. Aspects of TCR-mediated adoptive T cell approaches are reviewed here.

TCR-MEDIATED ADOPTIVE T CELL THERAPIES

It has been a reasonable tenet that the potency of TCR-mediated adoptive T cell therapies could be improved by using class I-restricted TCRs that are able to function both in their normal context, CD8 T cells, and in CD4 T cells. While CD8 T cell activities against cancer are important, recruitment of CD4 T cells to the site of a tumor can result in direct tumor control (1) and provide a cytokine milieu that promotes the function and survival of CTLs and NK cells (2–9), and CTL proliferation within tumors (10). CD4 T cells can also take on a cytotoxic phenotype, killing tumor cells directly (11, 12). Finally, CD4 T cells contribute to IFN γ -dependent mechanisms of angiogenesis inhibition (13, 14) and enhanced innate and adaptive responses (15, 16).

The recruitment of CD4 T cells with class I MHC-restricted TCRs is, however, confounded by the fact that most TCRs with

class I specificity require co-expression of CD8 for full activity. Nevertheless, some TCRs have been shown to mediate activity without CD8 suggesting that they have higher “functional avidity” (7, 17–23). Experimental studies using CD8 binding-impaired MHCs (24) or T cells that do or do not express co-receptor (25, 26) have defined affinity thresholds above which TCRs can respond to class I MHC without a requirement for CD8. There are now many approaches available to isolate or engineer TCRs that exhibit higher affinities and thus, act independent of CD8 (27–32).

ROLE OF CD8 IN ENHANCING T CELL SENSITIVITY

The dual roles of the CD8 co-receptor in binding to the class I MHC ligand and in signaling have been the topic of many investigations. The synergy between the TCR and CD8 allows just a few class I complexes on a target cell to stimulate cytotoxicity (33, 34). This exquisite sensitivity has evolved to allow our immune system to identify a potential target cell as “foreign” under conditions where the processed antigen levels are extremely low.

It has been argued that CD8 functions primarily by bringing the intracellular kinase Lck together with the TCR/CD3 complex (35). It should also be noted that CD8 binding to non-cognate pepMHC has a profound impact on increasing T cell sensitivity, and that the overall surface density of pepMHC is important in the contribution of CD8 (36, 37). Accordingly, MHC density on tumor cells can play a role in the function of both CD8 and the antigen-specific TCR.

Regardless of the exact mechanism, CD8 synergy with the TCR is so effective that cytolytic activity of CTLs can be induced even with very low TCR affinities [e.g., 300 μ M (38, 39)]. This might be particularly important in the case of CD8 T cell responses against self-cancer antigens, where the TCR affinities appear to be lower

than TCR affinities against foreign antigens (40, 41), most likely due to negative selection in the thymus. The TCR affinity threshold in the thymus that promotes negative selection is thought to be set very low in order to reduce the risk of peripheral autoimmune reactions (42–46). However, the well-known ability of CD8 to synergize with very low affinity TCRs also presents issues of undesirable autoreactivities against structurally similar self-peptides, when the affinity of the TCR against the cognate tumor antigen is increased (see below).

TCR:pepMHC AFFINITIES

Given the central role of TCR affinity in both driving T cell activity and in conferring the specificity of the reaction, we summarize concepts of affinity and its measurement here. More thorough reviews have been published elsewhere [e.g., (40, 47, 48)]. One straightforward way to describe TCR binding to pepMHC is as a simple, one-to-one interaction involving a bimolecular binding reaction:



where k_{on} indicates the association rate of the interaction, and k_{off} describes the dissociation rate of the interaction. Additional parameters describing the binding can be determined from these association and dissociation rates, including the half-life [$t_{1/2} = \ln(2)/k_{\text{off}}$] and the equilibrium binding constant ($K_d = 1/K_a = [\text{TCR}][\text{pepMHC}]/[\text{TCR:pepMHC}] = k_{\text{off}}/k_{\text{on}}$). The equilibrium binding constant may also be measured with equilibrium (or estimated from quasi-equilibrium) binding experiments, using techniques such as Scatchard plots or other fitting of the bound vs. free equation for K_d . In this review, we do not describe the key role of peptide affinity for the MHC product, but this parameter is also critical in the assessment of which peptide(s) to target (49–52).

The bimolecular binding equation above is used to describe the interaction between two free molecules in solution, with 3D mobility. Using soluble versions of pepMHC and/or TCR and measurement techniques such as binding to cell surfaces or surface plasmon resonance, a variety of models relating TCR binding parameters to T cell triggering have been developed (40). These included models based on the dissociation rate (k_d) such as “kinetic proofreading” (53), which suggested that a critical $t_{1/2}$ threshold must be exceeded for T cell activation to occur. An extension of this model proposed an “optimal dwell time” (54), incorporating the concept that exceptionally long $t_{1/2}$ values would result in reduced activity at low antigen density as a consequence of reduced serial triggering of multiple TCRs by each cognate pepMHC molecule (55). This model, which predicts reduced sensitivity of TCRs with long half-lives seems to be contradicted by very high-affinity TCRs engineered via directed evolution that can mediate sensitive T cell responses to low amounts of antigen (56).

Because the TCR, CD8, and pepMHC all exist as integral cell surface proteins on opposing cells, each present in various numbers, the corresponding multivalent interactions have been difficult to deconvolute from cell-free affinity measurements. Initial exceptions to the correlation between k_{off} and

activity among TCR:pepMHC pairs led to consideration of the value of k_{on} in the overall interaction (57–59). In the 2C system, which benefits from a large repertoire of reagents, measurements of pepMHC affinities by competition with a TCR clonotypic antibody on the live T cell surface gave good correlation with sensitivity and activity of 2C T cells against those targets (38). This approach allows direct measurement of the cell surface affinities, but unfortunately due to the lack of appropriate antibody reagents, most TCRs can not be probed in this manner. More recently, using careful statistical analyses and experimentation, a confinement time model of TCR triggering highlighted the contribution of k_{on} and potential re-binding of the same TCR:pepMHC (60). *In situ* measurements of TCR:pepMHC binding to opposing 2D surfaces were also performed, using single-molecule fluorescence resonance energy transfer (61) or mechanical force and contact surface area measurements (62). These studies revealed that binding parameters were altered/accelerated under the more physiological geometries, showing high correlation between faster on-rates, lower 2D- K_d values, and more potent agonist activity.

Regardless of the type of K_d measurement, 2D or 3D, or the involvement of kinetics, it is reasonable to conclude that TCR:pepMHC systems exhibit a: (1) minimum affinity threshold required to be stimulated by cognate pepMHC, (2) a maximum affinity threshold above which there is no longer improvement in sensitivity (or even a reduction in sensitivity), and (3) that these affinity-minima and -maxima will have different ranges, depending on whether the cognate co-receptors (CD8 for a class I pepMHC and CD4 for a class II pepMHC) are present.

ROLE OF TCR AFFINITY IN MEDIATING ACTIVITY OF CD4 AND CD8 T CELLS AGAINST A CLASS I MHC ANTIGEN

Class I MHC is engaged by the CD8 co-receptor with relatively low affinity ($K_d \sim 10$ –200 μM), that varies by allele (35, 63–67). Nevertheless, CD8 participation can increase sensitivity of a T cell to its cognate class I pepMHC complex by one-million fold (56), reviewed in (67). Accordingly, in the targeting of class I pepMHC, normal wild-type affinity TCRs in the range of 10–300 μM [reviewed in (40)] are sufficient to provide very sensitive responses (Figure 1). Indeed, normal CD8 T cells have been shown to respond to as few as one to three agonist pepMHC complexes on the surface of a cell (33, 34) due to the synergy with CD8. The ability of CD8 to synergize with even very low affinity TCRs [$K_d > 300 \mu\text{M}$ (25, 67, 68)] can be advantageous in the normal anti-tumor setting, as most anti-self (and, hence, anti-tumor) pepMHC reactive T cells would have been deleted in the thymus if they exhibited even modest affinities. Based on studies with various TCRs against class I pepMHC, the minimal affinity required for CD8 T cell activity appears to be in the range of 300 μM , whereas the optimal affinity above which there is no additional *in vitro* or *in vivo* improvement is about 10 μM (24, 26, 69, 70). However, there has been some evidence that higher-affinity TCRs yield faster T cell reactions, but reduced sensitivity at lower pepMHC densities (71, 72).

As indicated, it has been shown that CD4 T cell responses against tumors are very beneficial, a process that can be achieved by transducing CD4 T cells with TCRs that have higher affinities

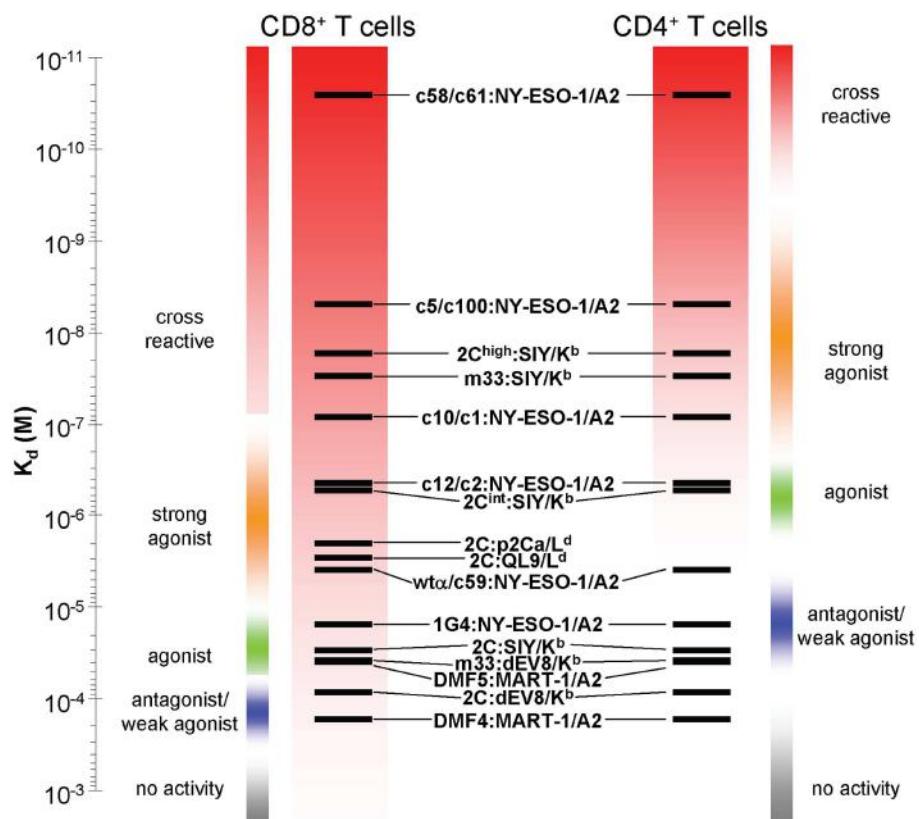


FIGURE 1 | Relationship between T cell activities and TCR affinities for a class I pepMHC antigen, in either CD8 or CD4 T cells. Various TCRs whose affinity for their target class I pepMHC complexes have been measured are depicted on an affinity scale (K_d). The relative activity ranges for those receptors are listed for those TCRs expressed in CD8 (left) and

CD4 (right) T cells. The activity boundaries are approximated from the best-known systems. Sensitivity at low TCR affinities is achieved due to TCR synergy with the CD8 co-receptor. This same principle can yield CD8-dependent, undesirable cross reactivities with structurally similar self-peptides.

($K_d < 10 \mu\text{M}$) against a class I MHC tumor antigen (25, 29, 73) (Figure 1). Even for CD4 T cells, however, there seems to exist an affinity threshold for class I pepMHC above which T cell activation occurs in the absence of the cognate peptide, as was seen for a picomolar-affinity TCR against HLA-A2/NY-ESO-1 (157–165) (73). This CD4 T cell activation appears to be due to the interaction of the affinity-engineered TCR with one or more self-pepMHC complexes with affinities above the CD8-independent threshold (i.e., $K_d < 10 \mu\text{M}$).

Raising the affinity of a TCR in order to achieve optimal CD4 T cell activity (i.e., CD8 independence) also increases the risk that the same TCR, in a CD8 T cell, will mediate activity against structurally related self-peptides. In this scenario, TCR affinities for such a self-peptide-MHC that were below the threshold (e.g., $K_d > 300 \mu\text{M}$, in the presence of CD8) for the wild-type TCR may now be elevated to $<300 \mu\text{M}$ with the affinity-enhanced TCR. In summary, in CD4 T cells a high-affinity TCR against a cognate pepMHC would need to cross-react with a structurally related self pepMHC at an affinity of at least $10 \mu\text{M}$ to stimulate autoreactivity, whereas in CD8 T cells a high-affinity TCR against a cognate pepMHC would need to cross-react with a structurally related self pepMHC

at an affinity of only $300 \mu\text{M}$ to stimulate autoreactivity, due to the synergy of CD8.

The consequences of these self-peptide cross-reactions can be varied. In one case (see 2C system below), a higher-affinity TCR introduced into CD8 T cells resulted in self-peptide reactivity and rapid deletion of the transduced CD8 T cells. While increased cross-reactivity by the mouse high-affinity TCR m33 in CD8 T cells resulted in deletion (74–76), several clinical trials in humans resulted in dangerous pathologies caused by the introduced T cells. The reasons for the difference in outcome are not entirely clear. One possibility is that the expression pattern of the cross-reactive epitope influences the outcome; for example, one cross-reactive epitope with the high-affinity m33 TCR, dEV8, is expressed ubiquitously, possibly overwhelming the introduced CD8 T cells and leading to AICD or even fratricide. By contrast, for cross-reactive epitopes that are tissue restricted (see below), the T cells may be able to persist and ultimately to mediate localized tissue destruction. Using appropriate animal models with tissue-restricted antigens, and adoptively transferred T cell with higher-affinity TCRs, it should be possible to investigate systematically the cause for different outcomes.

AFFINITY OF THE TCR CORRELATES WITH REACTIVITY FOR STRUCTURALLY RELATED PEPTIDES

Given the central role of the TCR:pepMHC interaction in activity and specificity it is not surprising that significant efforts have gone into dissecting the interface, often residue by residue. Of particular relevance is the role that TCR affinity plays in the recognition of structurally similar peptides, as such peptides could represent potential off-target safety issues. In order to consider this issue, we provide below a non-exhaustive review of several systems: the mouse class I pepMHC-specific TCR (2C), a mouse class II pepMHC-specific TCR (3.L2), and human TCRs against the cancer antigens MART-1, NY-ESO, MAGE-A3, and WT1. We focus on the activities mediated against the cognate peptides and, where available, structurally related peptides.

MOUSE 2C TCR AGAINST CLASS I ANTIGENS

The murine 2C T cell system (77, 78) has been studied extensively, from the level of central tolerance (79), to the level of structure/function (80–82), to its use in many tumor models (76, 83, 84). The CD8 T cell clone 2C was induced in a BALB.B mouse (H-2^b) by an alloresponse to the H-2^d tumor P815 (85). The 2C TCR was shown to mediate positive-selection by K^b (79), and a potential self-peptide, called dEV8, involved in this selection has been identified (86, 87). A synthetic peptide, called SIY, that acts as a strong agonist in the context of K^b was also identified (88).

The known reactions of 2C with a variety of ligands (K^b, L^d, and K^bm³) have provided a model system to study TCR degeneracy (89). Affinities for the allogeneic ligands [p2Ca/L^d and QL9/L^d $K_d \sim 1 \mu\text{M}$ (90, 91)], the putative positive-selection ligand [dEV8/K^b, $K_d \sim 80 \mu\text{M}$ (90)], and the strong agonist ligand [SIY/K^b, $K_d \sim 30 \mu\text{M}$ (26, 90, 91)] have been measured by various methods. The structure of this receptor in complex with dEV8/K^b was the first mouse TCR:pepMHC to be determined (80). Since then, the structures of the 2C TCR in complex with L^d ligands (81, 82) and K^b ligands (80, 92) have been solved, showing how the complementarity determining regions (CDR) accommodate the various ligands. CD8 2C T cells, have also been used to probe the exquisite sensitivity of T cells, suggesting that only a few agonist pepMHC molecules (or even one) on a target cell can mediate activity (33, 34). Finally, the 2C system and the strong agonist peptide SIY was used by Schreiber and colleagues to reveal the process of tumor antigen cross-presentation on stroma (83, 93, 94), and more recently the system has been exploited by Jacks and colleagues to reveal aspects of peripheral tumor tolerance (84) and the importance of mutated peptide antigens in immunoediting (95).

In the context of the present review, the 2C TCR ($K_d = 1 \mu\text{M}$ for QL9/L^d, and $30 \mu\text{M}$ for SIY/K^b) was also the first to be engineered for higher affinity by directed evolution, first against QL9/L^d (96) and then against SIY/K^b (71). A yeast display library of CDR3 α mutants in the 2C single-chain TCR (scTCR) were selected with QL9/L^d to yield various mutants, including m6 with a K_d value of 10 nM (91, 96, 97). The same 2C scTCR library, selected with SIY/K^b, yielded various mutants including m33 with a K_d value of 30 nM (26, 71, 91). Stimulation of a T cell hybridoma expressing the higher-affinity TCR variants showed that they exhibited increased sensitivity to agonist peptide presentation (71, 97). In addition to sensitive agonist responses, binding of high-affinity

TCR variants to structurally related pepMHC complexes were also increased (Figure 2A) (39, 71, 96, 97).

In addition to a broader range of reactivity with single-amino acid substitutions in the agonist peptide, the higher-affinity TCR m33 (isolated against the ligand SIY, with 1000-fold higher affinity) also showed CD8-dependent activity against the structurally similar self-peptide dEV8 (71). Although the m33 TCR only exhibited about a twofold increase in affinity for the self-pepMHC dEV8/K^b, this increase was sufficient for CD8 T cells expressing m33 to be stimulated by both exogenous dEV8 and endogenous peptides presented by H-2^b cells such as C57BL/6 splenocytes (71). While the sequence of dEV8 only contains two amino acids in common with the strong agonist SIY (SIY: SIYRYYGL; dEV8: EQYKFYSV), they are very similar structurally (Figure 2B), and can be considered to be analogous to single-amino acid substitutions of agonist peptides. This notion forms the basis of the more detailed discussion below concerning the examination of structurally similar self-peptides.

It is important to point out that in contrast to an increase in affinity for structurally similar pepMHC complexes (i.e., m6 TCR with QL9 and its variants, or m33 TCR with SIY and dEV8), the affinities of engineered 2C variant TCRs were not increased toward structurally dissimilar ligands. For example, the high-affinity TCRs m6 and m13 selected against the allogeneic ligand QL9/L^d, had reduced affinities for the syngeneic ligand SIY/K^b (91).

MOUSE TCR 3L2 AND ITS LIGANDS

Similar effects of increased affinity were observed for the class II-restricted TCR system called 3.L2 (98, 99). The 3.L2 TCR was derived from a CD4 T cell clone against a peptide from the minor d allele of the b chain of mouse hemoglobin, presented in complex with I-E^k. The 3.L2 TCR was engineered by yeast surface display for increased affinity to the Hb/class II pepMHC complex. A panel of TCRs with an affinity range from the wild-type 3.L2 [$K_d 20 \mu\text{M}$ (99, 100)] to the highest affinity variant, m15 ($K_d 25 \text{ nM}$) were isolated (99). In the case of these higher-affinity TCR variants, there were no apparent increases in CD4 T cell activity for the agonist pepMHC. This may be a result of a wild-type affinity already above the optimal activation threshold for this complex. However, the ability to respond to single-amino acid substitutions of the Hb peptide was much broader for the TCRs with increased affinity (99, 101). A recent study showed that even a TCR (m2) with a modest improvement in affinity (twofold) for Hb/I-E^k mediated broader peptide reactivity, and enhanced thymic negative selection (102). Thus, like the 2C system, the 3.L2 system also showed that structurally similar peptides have a higher probability of stimulating T cells that express affinity-enhanced TCRs.

HUMAN TCRs

A prioritized list of cancer-associated peptide antigens has been compiled, setting quantitative values on various properties, including antigenicity, relationship to oncogenicity, and specificity (103). Among the panel of peptides, some have been the antigenic peptides targeted by TCRs in adoptive T cell therapies. These include, most prominently, MART-1 (29), NY-ESO-1 (104, 105), MAGE-A3 (106), and WT1 (107, 108). Various strategies to improve the affinity, and it is hoped thus the efficacy, of TCRs for the adoptive

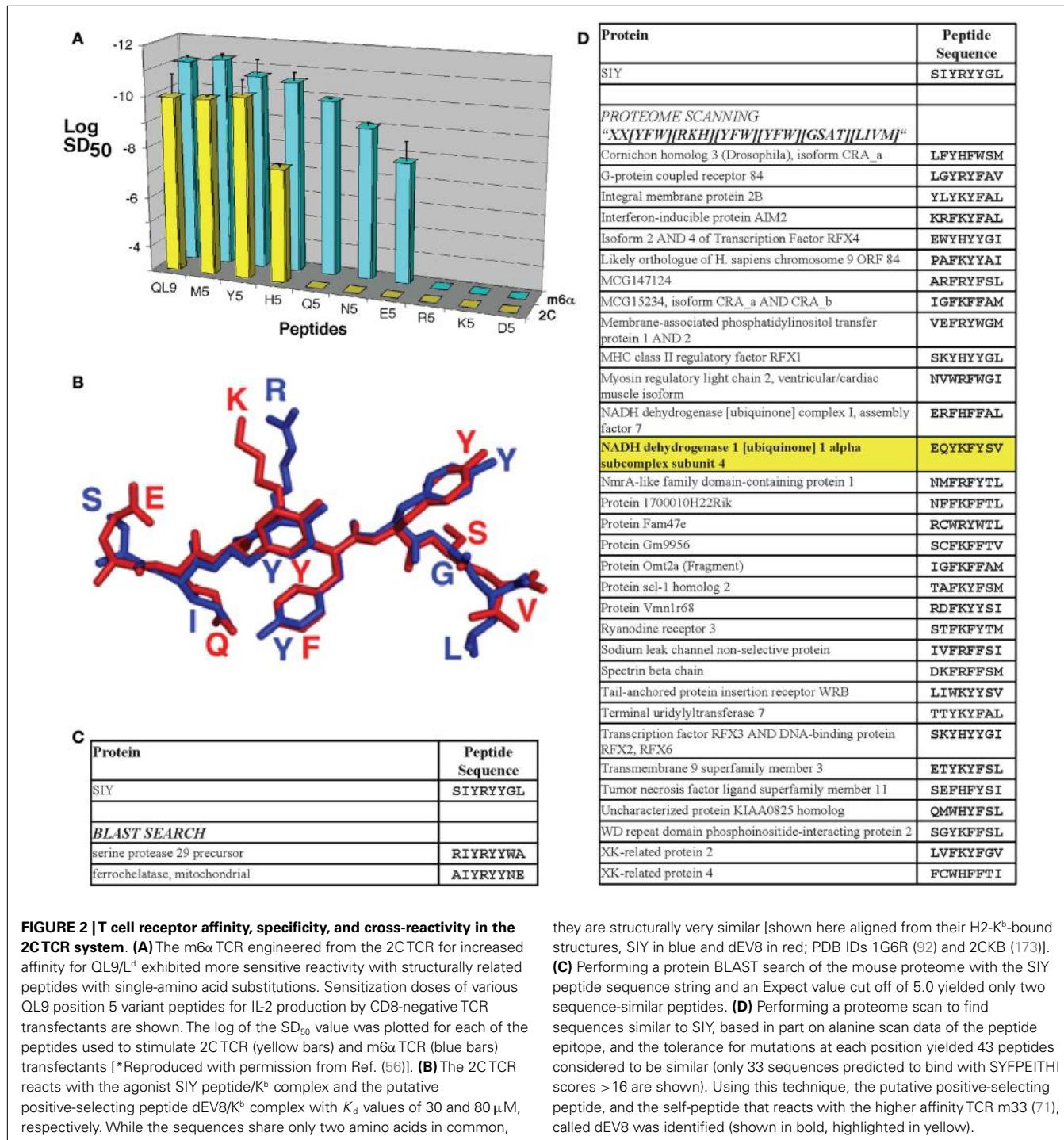


FIGURE 2 | T cell receptor affinity, specificity, and cross-reactivity in the 2CTCR system. (A) The m6 α TCR engineered from the 2CTCR for increased affinity for QL9/L α exhibited more sensitive reactivity with structurally related peptides with single-amino acid substitutions. Sensitization doses of various QL9 position 5 variant peptides for IL-2 production by CD8-negative TCR transfectants are shown. The log of the SD $_{50}$ value was plotted for each of the peptides used to stimulate 2CTCR (yellow bars) and m6 α TCR (blue bars) transfectants [*Reproduced with permission from Ref. (56)]. **(B)** The 2CTCR reacts with the agonist SIY peptide/K α complex and the putative positive-selecting peptide dEV8/K α complex with K_d values of 30 and 80 μ M, respectively. While the sequences share only two amino acids in common,

they are structurally very similar [shown here aligned from their H2-K α -bound structures, SIY in blue and dEV8 in red; PDB IDs 1G6R (92) and 2CKB (173)]. **(C)** Performing a protein BLAST search of the mouse proteome with the SIY peptide sequence string and an Expect value cut off of 5.0 yielded only two sequence-similar peptides. **(D)** Performing a proteome scan to find sequences similar to SIY, based in part on alanine scan data of the peptide epitope, and the tolerance for mutations at each position yielded 43 peptides considered to be similar (only 33 sequences predicted to bind with SYFPEITHI scores >16 are shown). Using this technique, the putative positive-selecting peptide, and the self-peptide that reacts with the higher affinity TCR m33 (71), called dEV8 was identified (shown in bold, highlighted in yellow).

T cell therapy trials have been taken. While anti-tumor responses have been observed, there have been serious adverse events with MART-1 TCRs due to on-target/off-tumor activity (109), and lethal events with MAGE-3 TCRs due apparently to off-target cross-reactivity with structurally similar epitopes (110, 111). For these reasons, we summarize below various aspects of reactivities mediated by TCRs against four of the candidates for adoptive T cell therapies (MART-1, MAGE-A3, NY-ESO-1, and WT1).

MART-1

MART-1, a differentiation antigen upregulated on the surface of melanoma cells, contains the well-studied HLA-A2-restricted peptide epitope AAGIGILTV [27–35] (112) and its N-terminal extended variant EAAGIGILTV [26–35] (113). CD8 T cell clone M1F12 (now called DMF4) against this peptide was isolated from a patient with an anti-tumor response (114). The DMF4 TCR has a relatively low affinity (K_d 170 μ M) for the predicted endogenous

epitope, AAGIGILTV/HLA-A2 (115). The DMF4 TCR was used in one of the first trials of gene modified adoptive T cell transfer in humans (116). While relatively low overall response rates were reported [4/31, or 13%, with 17 patients reported in the original publication (109, 116)], the study represented an important step toward proof of concept for TCR gene therapies.

In an attempt to improve the efficacy of MART-1-directed TCR gene therapy, a second generation T cell clone called DMF5, with higher functional avidity and detectable activity in CD4 T cells, was isolated (117). The affinity of DMF5 (K_d 40 μM) (115) was higher than DMF4, but interestingly still lower than the murine wild-type receptor 2C (K_d 30 μM) [Note: like DMF5, the 2C TCR exhibited some activity in CD4 T cells *in vitro*, although *in vivo* anti-tumor activity of CD4 T cells with the 2C TCR was less effective than the higher-affinity TCR m33, with a K_d of 30 nM (76)]. Similarly, because DMF5 showed greater *in vitro* activity than DMF4 in CD4 T cells, it was hypothesized that DMF5-transduced T cells might mediate improved anti-melanoma responses (109). Indeed, objective response rates were higher in the DMF5 trial (30 vs. 13%). However, unlike patients treated with DMF4, patients treated with DMF5 experienced a marked cytokine (IFN- γ) spike and serious skin rashes 3–5 days after T cell transfer. The cytokine spike induced was \sim 9-fold higher for patients treated with the affinity-enhanced DMF5 TCR when compared with previous patients who received cells with the DMF4 TCR, suggesting that the TCR reactivity was related to these results. Furthermore, since IFN- γ is produced by activated T cells, and patients were lymphodepleted prior to transduced T cell infusion (and still showed signs of lymphodepletion at the 3- to 5-day time point), it is likely that the cytokines were derived from the transferred cells. Importantly, DMF5 also mediated high rates of anterior uveitis, hearing loss, and dizziness, presumably due to reactions to MART-1 expressed in the normal eye and ear (109). Accordingly, these responses were characterized as on target/off tumor, and were only revealed by the potency of T cells transduced with the higher-affinity DMF5 TCR.

MAGE-A3

MAGE-A3 is a cancer-testis antigen and a member of a larger MAGE family. A related family member, MAGE-A1, was the first immunogenic gene found to elicit a natural CTL response in a melanoma patient (118). MAGE-A3 was identified several years later (119) and is one of the most commonly expressed MAGE family genes in cancers of different epithelial origins [reviewed in (120)]. Several peptide epitopes from MAGE-A3 have been identified, restricted by various MHC alleles. Here, we focus on the HLA-A2-restricted epitope, MAGE-A3 [112–120]: KVAELVHFL, which was the epitope targeted in a recent trial that resulted in the deaths of two patients (111), although a recent clinical trial with a MAGE-A3 epitope (EVDPIGHLY [161–169]) restricted by HLA-A1 also showed cross-reactivity, cardiovascular toxicity, and lethality in a clinical trial (110).

A high-avidity TCR was generated by vaccinating an HLA-A2 transgenic mouse with the MAGE-A3 [112–120] peptide (106). As murine CD8 does not bind efficiently to HLA-A2, T cells generated against peptide/HLA-A2 complexes in these mice presumably have affinities above the CD8 independence threshold, and would be sufficient to recruit CD4 as well as CD8 T cells. Human CD8,

but not CD4, T cells expressing the MAGE-A3 [112–120]-specific TCR stained with soluble pepMHC tetramers, and were activated *in vitro* by MAGE-expressing tumor cells. To identify a TCR with even higher avidity, various point mutations in the CDR3 α were examined for improved T cell activity (29), revealing an A118T variant that raised the functional avidity of the TCR, and mediated improved CD4 T cell activity (106). T cells transduced with these MAGE-A3/HLA-A2 TCRs were also screened against structurally similar peptides from other MAGE family members. An epitope from MAGE-A12 (differing only by a Val to Met substitution at position 2) was recognized indistinguishably from MAGE-A3, and detectable responses were seen with similar peptides from MAGE-A2 and MAGE-A6.

The MAGE-A3 A118T TCR was recently used in adoptive T cell therapy in nine melanoma patients (111). Five patients experienced objective regression of their tumors, including one complete response and one durable partial response that persisted for over 12 months. However, unexpected neurological toxicity was observed in three MAGE-A3 patients, resulting in two patient deaths. High levels of CD4 T cells with the murine TCR were detected in the cerebrospinal fluid of the patients that experienced toxicity, although brain infiltrating T cells were predominantly CD8 (CD4 T cells were rare). Cells expanded from the cerebrospinal fluid of one of the patients who succumbed showed specific IFN- γ release when stimulated with MAGE-A3 $^+$ /HLA-A2 $^+$ tumor cells.

To identify potential cross-reactive epitopes that might have accounted for these toxicities, a BLAST search of the MAGE-A3 peptide, KVAELVHFL, was conducted with the human genome, revealing various candidates (111). The peptides were synthesized and tested for their ability to stimulate CD8 T cells transduced with the MAGE-A3 A118T TCR. One peptide (SAAELVHFL from EPS8L2, for epidermal growth factor receptor kinase substrate 8-like protein 2) was reactive, but transfection of the full EPS8L2 gene into HLA-A2-positive cells did not stimulate activity. However, staining of brain sections with anti-MAGE family antibodies, as well as testing with Q-RT-PCR, revealed a subset of neurons that expressed MAGE genes, including MAGE-A12 (111). Thus, it was suggested that T cell recognition of the structurally similar peptide from MAGE-A12 likely accounted for the neuronal toxicity.

NY-ESO-1

NY-ESO-1 (or LAGE-1) is also a cancer-testis antigen that is expressed on a variety of tumors from different origins [reviewed in (121)]. An NY-ESO-1 peptide (NY-ESO-1 [157–165], SLLMWITQC) restricted by HLA-A2 was identified using CTL lines from a melanoma patient (122). A CD8-dependent TCR called 1G4 that is specific for this epitope was shown to have K_d value of 15 μM for the NY-ESO/A2 complex (104, 105).

As the native NY-ESO peptide bound poorly to HLA-A2, and was less active in solution due to reactions of the C-terminal cysteine (123), there have been efforts to design improved peptide analogs. Toward this effort, a positional alanine scan (124) indicated that P3-Leu, P4-Met, P5-Trp, P7-Thr, and P8-Gln were important for T cell recognition, while a crystal structure of the HLA-A2-bound peptide (125) showed that P2-Leu, P3-Leu, P6-Ile, and P9-Cys were unlikely to contact the TCR directly. To eliminate

the problems with the cysteine at P9, and to improve HLA-A2 binding, various P9 substitutions have been tested (104, 123, 125). A peptide with a valine substitution (SLLMWITQV) bound better to A2, was more stable in solution (123), and stimulated 1G4 T cells more effectively than the wild-type peptide *in vitro* (104). However, vaccination strategies with the C165V peptide did not lead to efficient cross-reactivity with the wild-type peptide (126), likely due to a repositioning of the peptide main chain with the different anchor residue at P9 (127).

While vaccination for NY-ESO-1 remains challenging, adoptive T cell therapy for this epitope has been shown to be effective and safe, even with a higher-affinity TCR variant of 1G4. Several single-site CDR mutants of the 1G4 TCR increased affinity and mediated improved activity of CD4 T cells (29). The 1G4- α 95LY TCR has been tested clinically in melanoma and synovial cell sarcoma with a significant benefit (overall response rate of 45 and 67%, respectively), and a good safety profile has been reported for the 17 treated patients (128).

In a separate strategy, the 1G4 TCR has been modified for higher affinity by phage display yielding affinities as high as 26 pM (129, 130). The highest affinity TCRs yielded self-reactivity in both CD8 and CD4 T cells (73) (Figure 1). A high-affinity (50 pM) variant generated by phage display has also been produced as a soluble, bispecific fusion with anti-CD3 to redirect T cells to NY-ESO *in vitro* and in a human xenograft model in mice (131, 132).

WT1

Wilms' tumor antigen (WT1) is a zinc-finger transcription factor that plays a significant role in embryogenesis but is minimally expressed in normal adult tissues. It is overexpressed in most leukemias, and in several other tumor types [reviewed in (133, 134)]. The recent prioritization of tumor-associated peptides (103) ranked WT1 as the top target due to its immunogenicity, restricted expression in normal tissues, and a strong correlation with tumorigenesis. An immunogenic HLA-A2-restricted epitope, WT1 [126–134]: RMFPNAPYL has been characterized (107, 135). Interestingly, the identical peptide sequence is present in the mouse WT1 homolog, and has been shown to be an immunogenic epitope in the context of H2-D^b (136, 137). [Note: a TCR targeting WT1 [235–243], restricted by HLA-A*2402 (138), is also being explored for adoptive immunotherapy, with reported efficacy and safety in pre-clinical systems (139); this peptide and TCR are not discussed further here.]

To date, several WT1 vaccination trials in mice and humans have been undertaken, showing excellent safety profiles but low response rates [reviewed in (140)]. A recent study (141) showed that only clones of low functional avidity for HLA-A2:WT1 [126–134] could be isolated from HLA-A2-positive individuals, while clones of higher functional avidity could be obtained from HLA-A2-negative individuals through allogeneic stimulation *in vitro*. However, the allogeneic clones showed promiscuous reactivity to different HLA-A2-bound peptides (141), highlighting that caution should be taken when taking advantage of allogeneic stimulation to isolate tumor-specific TCRs of improved affinity. A limited trial where anti-WT1 CTL clones were elicited *ex vivo* from patients, in the presence of IL-21, and re-introduced showed substantial persistence of the WT1-specific T cells (108). The

results also suggested an improved response over WT1 vaccines, while maintaining favorable safety. Looking toward adoptive T cell therapy, a WT1 [126–134]/A2-specific TCR isolated from peptide-specific, allo-induced CTLs (107, 142), exhibited good anti-tumor responses in a mouse xenograft model with TCR-transduced T cells (143, 144). A more recent study targeting WT1 for adoptive T cell therapies described a novel strategy to reduce endogenous TCR levels by using a targeted zinc-finger nuclease, followed by introduction of their WT1-specific TCR. This approach resulted in enhancement of overall functional avidity due to the higher T cell surface levels of the exogenous WT1-specific TCR (145).

With the possibility for improvement of anti-WT1 CD4 T cell responses with higher-affinity TCRs, our lab, working with Greenberg and colleagues has previously engineered an enhanced affinity (CD8-independent) TCR against the murine WT1/D^b complex (137, 146), and we have recently engineered a higher-affinity human TCR against WT1/HLA-A2 (unpublished). The mouse and human TCRs are being tested in mouse models with analysis of potential on-target/off-tumor responses, or cross-reactivity with structurally related pepMHCs (see below). Adoptive transfer studies with CD8 T cells and the mouse TCR against WT1/D^b have shown no signs of toxicity in the mouse models (146).

DOES THE ADVANTAGE OF HIGHER-AFFINITY TCRs IN CD4 T CELLS OUTWEIGH THE POTENTIAL DISADVANTAGE WITH SELF-REACTIVITY?

Given the connection between sensitivity and cross-reactivity with TCRs in CD8 T cells, it is reasonable to ask if the recruitment of CD4 T cells with higher-affinity TCRs is worth the risk of self-reactivity (by transducing all peripheral T cells including CD8 T cells). As described above, redirected CD4 T cells provide an opportunity for direct destruction of the tumor by the effector CD4 T cells. Our recent findings (76) and results from others (75) suggest that nanomolar affinity TCRs are more potent in CD4 T cells than wild-type TCRs. In fact, the only treatment which resulted in long-term control of established tumors, with no outgrowth, was CD4 T cells transduced with the 30-nM affinity TCR m33 (76).

We suggest that the major importance of CD4 T cell recruitment will be that they provide a cytokine milieu that facilitates the generation of endogenous responses against multiple class I MHC-restricted cancer antigens. These antigens might include individual unique peptides with tumor-specific, patient-specific mutations. Such mutated peptides have recently been shown to represent the dominant epitopes of an effective immune response that drives immunoediting (95, 147). Accordingly, it will be important to identify strategies that allow TCRs to mediate CD4 T cell activity, ultimately enabling a broad anti-cancer immune response. Since adoptive T cell therapies currently are configured to introduce the same TCR into both CD4 and CD8, an important issue is whether it is possible to improve current pre-clinical approaches to assess potential self-reactivity and consequent toxicity.

One possible strategy to take advantage of high-affinity TCRs in immunotherapy would be to separate CD4 and CD8 T cells *ex vivo* for transduction with separate TCR variants, as has been done in mouse studies (74–76). The CD4 T cells could be transduced with nanomolar affinity TCRs, while the CD8 T cells could

be transduced with a reduced affinity version of the receptor. The method for creating a lowered affinity version of a TCR is fairly straightforward, as conserved residues in the CDR2 loops may be substituted, reducing the overall binding affinity of the TCR while maintaining the peptide specificity. Using a library of receptors with different residues at a single CDR2 β position in the m33 and 2C TCRs, we recently showed that a range of binding affinities were achieved by the resulting receptor variants, and certain variants were sufficiently lowered in affinity to minimize cross-reactivity in CD8 T cells, but retain CD4 T cell activity (74). Several conserved positions in TCRs have been characterized [reviewed in (148)] which could be mutated to achieve lower-affinity variants of an anti-tumor TCR.

It is of course possible that adoptive T cell therapy could be combined with checkpoint blockade treatment to interfere with negative signals transmitted to T cells, for example from interactions of molecules such as CTLA-4 or PD-1 and their ligands, B7 and PD-L1 or PD-L2, respectively [reviewed in (149)]. With the advent of checkpoint blockade treatments, including antibodies that inhibit CTLA-4 (FDA-approved Ipilimumab) and PD-1 (or its ligand PD-L1), it is possible that lower-affinity TCRs will have improved efficacy in adoptive T cell therapies. Clinically, checkpoint blockade [reviewed in (150)] has been used to enhance endogenous T cell responses against a tumor. In melanoma patients, ipilimumab treatment showed a survival benefit, either alone or with a gp100 peptide-based vaccine, over the peptide vaccine alone (151). Patients treated with ipilimumab often exhibited tissue restricted, immune-related adverse autoimmune effects. Recently, there has been considerable excitement about blocking PD-1 signaling. As the PD-1 ligands, PD-L1 and PD-L2, are specifically upregulated at sites of inflammation and on many tumors (149), PD-1 blockade may more directly target immunosuppression in the tumor with fewer side effects than with CTLA-4. PD-1 blockade, currently in clinical trials in the form of several different antibodies (152–155), has shown promising response rates [up to 52% objective response rate in advanced melanoma patients treated with the MK-3475 (lambrizumab) PD-1 blocking antibody (155)], but these treatments were also associated with immune-related adverse effects, although at lower rates than CTLA-4 blockade. In combination with checkpoint blockade, it is possible that a lower-affinity TCR could act with higher potency in an adoptive T cell therapy setting, as has been seen in a mouse model (156). It remains to be seen if this may have similar safety concerns as with higher-affinity TCRs, in terms of cross-reactivity, or on-target/off-tumor responses.

POTENTIAL STRATEGIES TO EVALUATE SELF-REACTIVITY RISKS ASSOCIATED WITH HIGHER-AFFINITY TCRs

Along with the promise of adoptive therapy with engineered TCR-transduced T cells has come the very real dangers of on-target/off-tumor toxicity (as seen in the MART-1 trial) and cross-reactivity with similar epitopes in normal tissues (as seen in the MAGE-A3 trial). Several important techniques are already in use to check for cross-reactivity, including *in vitro* screening of CD4 and CD8 T cells transduced with tumor-specific TCRs, using as antigen-presenting cells various lines derived from normal tissues.

However, to safely take advantage of this therapeutic strategy and avoid serious adverse effects, it will be imperative to develop expanded strategies to screen candidate TCRs for safety and potential cross-reactivity prior to delivery into human patients. We propose below a combination of *in silico*, *in vitro*, and *in vivo* (murine) strategies to enhance current screens prior to clinical trials. In each case, we argue that having an engineered, high-affinity TCR would be of significant value in revealing potential safety concerns, even if a lower-affinity TCR may be desirable in a clinical setting, especially in CD8 T cells (75, 76). It is relatively easy to introduce mutations at one of several, well-characterized locations in the TCR [reviewed in (148)] that can reliably reduce affinity while maintaining specificity and anti-tumor activity (74).

One way to attempt to detect possible cross reactivities for a given TCR will be to take advantage of the vast amount of information available through genomic and proteomic databases. A standard protein BLAST (Basic Logical Alignment Search Tool, blastp algorithm)¹ search can be conveniently performed using the NCBI web interface, revealing similar sequences to a given peptide ranked with an Expect (E) value. The E value is a measure of the statistical significance of a particular match compared to random chance in the entire proteome, with lower E values being more significantly similar to the search string. As a model, the mouse proteome was searched by BLAST for sequences similar to the SIY peptide, which acts as an H2-K β -restricted agonist for the 2C TCR, but is not actually contained within the mouse proteome. This search revealed two peptides with Expect values <5.0 (Figure 2C). However, the previously identified positive-selecting antigen, dEV8, was not identified in the BLAST search, even extending the accepted E value up to 10,000. Thus, BLAST searches alone do not capture the criteria that would be best used to search for MHC-binding peptides with potentially similar TCR-contact residues.

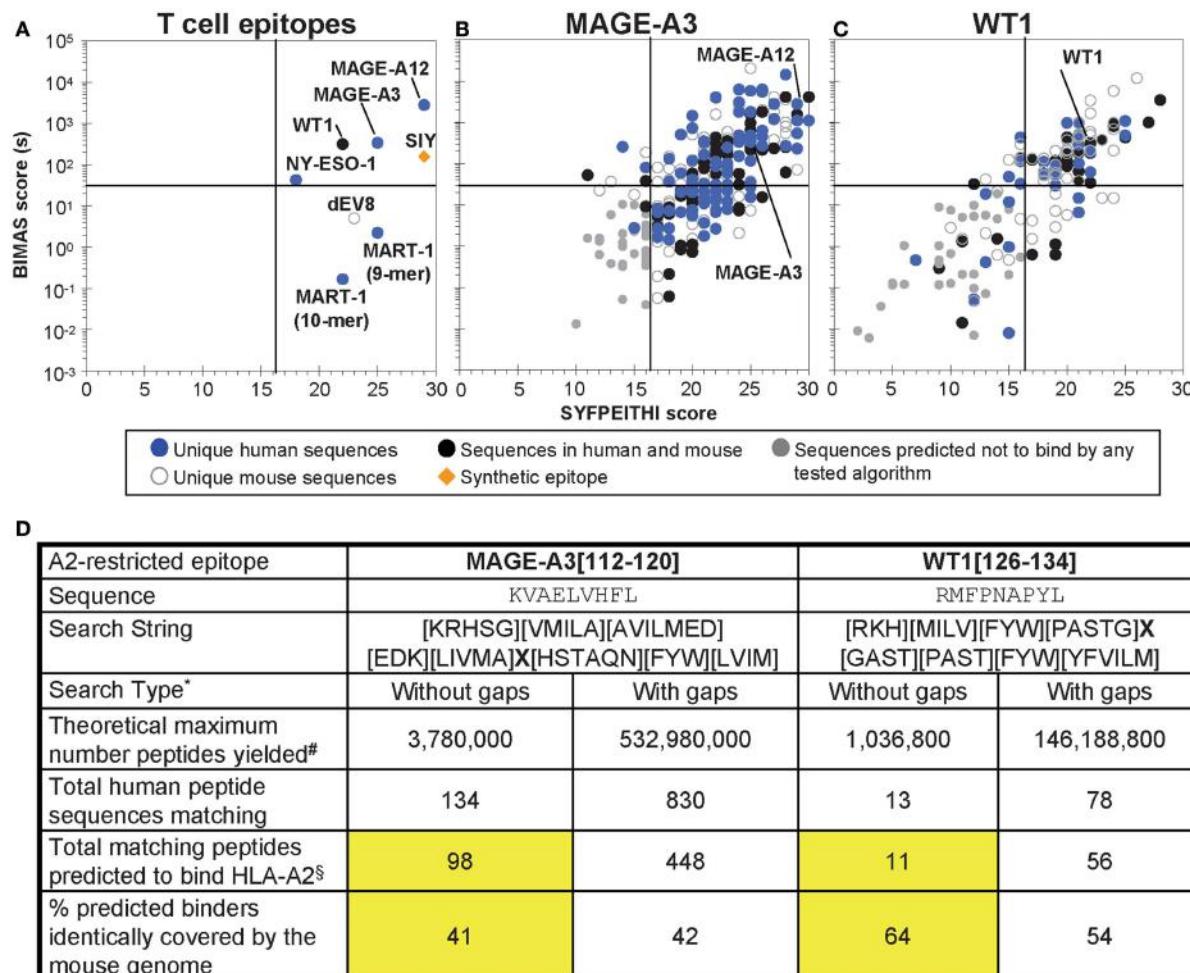
All peptides identified through *in silico* screens were tested in MHC-binding prediction algorithms with arbitrary cut-off values used previously for distinguishing qualitative binders vs. non-binders. Algorithms examined here included SYFPEITHI with a cutoff of >16 for binders (157)²; BIMAS with a cutoff of estimated $t^{1/2} > 30$ s³; Artificial Neural Network [ANN; (158)], and Stabilized Matrix Method [SMM (159)]. ANN and SMM were both applied with a cut-off value of $IC_{50} < 500$ nM, and both were accessed through the Immune Epitope Database (IEDB) Analysis Resource⁴. A plot of predicted MHC-binding values for epitopes discussed in this review are shown in Figure 3A. It has been estimated that an IC_{50} cutoff of 500 nM by ANN or SMM yields 80% or higher (up to 97%) accuracy in predicting MHC binders, depending on allele (160). However, it is important to keep in mind that some MHC-binding peptides may be missed using a threshold such as this; for example, using 500 nM as a binding threshold for netMHCpan H2-K β binding predictions would omit p2Ca, a peptide which is known to form a complex with H2-K β and stimulate 2C T cells (161).

¹<http://blast.ncbi.nlm.nih.gov/>

²<http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm>

³http://bimas.dcrt.nih.gov/molbio/hla_bind

⁴http://tools.iedb.org/analyze/html/mhc_binding.html#



* Without gaps indicates that the search was conducted for the string as stated only; With gaps indicates that the search also allowed a single, 1 residue gap (any amino acid) in the sequence at any position

if search were applied to a set containing every possible combination of amino acids

\$ Using a 500nM IC₅₀ cut-off value for binding predictions to HLA-A2 with the ANN and SMM prediction algorithms

FIGURE 3 | Analysis of selected tumor epitopes for homologous sequences in the human and mouse proteomes. (A) MHC-binding prediction scores for a set of characterized T cell epitopes, including six HLA-A2-restricted human tumor-associated epitopes. **(B)** MHC-binding predictions for peptides identified in a MAGE-A3 [112–120] homology scan (no gaps) of the human and mouse proteomes. **(C)** MHC-binding predictions for peptides identified in a WT1 [126–134] homology scan of the human and mouse proteomes (including an allowed, single-amino acid gap).

For **(B,C)**, peptides were subjected to ANN and SMM prediction algorithms along with SYFPEITHI and BIMAS, and prediction to bind above the arbitrary thresholds described in the text in any of the algorithms was taken to indicate a potential binder. **(D)** A comparison of MAGE-A3 and WT1 proteome scan results. The total number of predicted binders identified in the human proteome and the percent of the binders identically found in the mouse proteome for both epitopes (searched without gaps) are highlighted in yellow.

Using the MHC-binding principles, an alternative strategy to BLAST is to scan the full proteome for sequence motifs that: (1) preserve critical residues (or conservative mutations) ideally identified by positional single-site substitutions of the peptide epitope, and (2) allow other residues to vary more widely. A similar strategy was used to identify potential positive-selecting ligands for the OT-1 TCR, scanning for MHC-binding motifs, and then scoring for similarity among the predicted TCR contacts to the H2-K^b-restricted ovalbumin peptide, OVA (87). For our current efforts, using previous alanine scan information for the SIY peptide

(SIYRYYGL) that stimulates the 2C TCR (39), a search motif was designed as “XX[YFW]/RKH/[YFW]/[YFW]/[GSAT]/[LIVM],” where “X” indicates that any residue would be acceptable at that position, and bracketed residues indicate that one of those limited set of residues would be acceptable. If every possible sequence were available in the proteome, the 2C homology search, as designed, would yield 518,400 sequences (20 × 20 × 3 × 3 × 3 × 3 × 4 × 4). Scanning the mouse proteome [the complete *Mus musculus* proteome from The Universal Protein Knowledgebase (UniProtKB), 73,947 entries] with this motif identified 43 peptides, of

which 33 were predicted to bind well to H2-K^b (in this case, defining binders using a SYFPEITHI cutoff of 17 or higher, see **Figure 2D**). Importantly, this strategy identified, among the 33 peptides, dEV8 from the NADH dehydrogenase, which as described above is known to react with 2C TCR and the higher-affinity m33 TCR.

We propose that the yield of identified, predicted MHC-binding peptides when searching the proteome in this manner provides a reasonable estimate of the possible number of self-reactive peptides, and a tractable number of candidates that could be tested for reactivity with a higher-affinity TCR (like m33) in CD8 T cells. Of course, the identified peptides are influenced by the design of the search string, as well as the accuracy of binding predictions. To improve the ability of searches like this to comprehensively identify all potentially cross-reactive peptides, an epitope of interest should be evaluated for the ability of substituted peptides to activate its specific TCR. Value can be obtained from simple, single-point alanine substitutions, as can be seen from the ability of the murine proteome scan to identify the dEV8 peptide (with only two amino acids in common with the agonist peptide, **Figure 2D**) when guided by alanine substitutions of SIY for binding and stimulation of the 2C TCR (39).

While the search strategies for structurally similar peptides may identify potential problematic cross-reactive epitopes, this strategy alone can not identify structurally dissimilar peptides which act as agonists. It is possible that such peptides could be identified using combinatorial peptide library techniques, where individual positions/residues are held constant in each peptide pool, and stimulation is evaluated (162, 163).

To evaluate *in silico* strategies for human tumor targeting, BLAST searches of the HLA-A2-restricted epitopes for MART-1, NY-ESO-1, MAGE-A3, and WT1 were performed in the human proteome; similarity was defined with an Expect value cut off of 5.0. Within this range of similarity, the MART-1 (27–35) and WT1 (126–134) described above were identified as unique within the proteome. NY-ESO-1 (157–165) yielded two additional peptides rated similar within these criteria; however, neither was predicted to bind to HLA-A2. By contrast, MAGE-A3 (112–120) yielded fourteen sequences that were similar within this range of Expect values, of which 10 were other members of the MAGE family. All of the MAGE-similar peptides were predicted to have some binding to HLA-A2 (SYFPEITHI score greater than 16 or BIMAS off-rate >30 s).

Two of the epitopes, MAGE-A3 and WT1, were used further as the basis for a scan for structurally similar peptide sequences, as done with SIY. The contribution of each peptide position to T cell recognition has not been systematically studied for these epitopes; however, some data on substitutions is available (106, 111, 164–166). Using these data, and striving to maintain structural homology/conservative mutations, proteome search strings were generated, and applied to both the human and murine proteomes. This strategy thus further aimed to determine what fraction of potentially cross-reactive, structurally similar epitopes would be represented in both the human and murine proteomes. This information could be useful in examining what fraction of potential cross-reactive epitopes might reveal toxicities in a mouse model (see below).

If every possible sequence were available in the proteome, the MAGE-A3, and WT1 searches would yield ~3,800,000 and ~1,040,000 sequences, respectively. Applying the searches to the human proteome (the complete *Homo sapiens* proteome downloaded from UniProtKB, 134,787 entries) yielded 134 sequences similar to the MAGE-A3 epitope and 13 sequences similar to the WT1 peptide. Thus, consistent with the BLAST search, WT1-like sequences were about 10-fold more rare in the human proteome than MAGE-A3-like sequences. We also allowed the search string to include a single random amino acid gap anywhere in the peptide sequence for WT1, yielding larger theoretical search maxima (e.g., 146,000,000 for WT1, almost 40-fold larger than the theoretical search size without gaps for MAGE-A3, 3,800,000). When even that search string for WT1 was applied to the human proteome, only 78 peptides were identified, still half as many as identified with the MAGE-A3 search string without gaps (134 peptides). It should be noted that for most TCRs, insertion of a single residue (i.e., “gap”) in the peptide may significantly alter the bound conformation of the peptide, resulting in a loss of recognition of the epitope. Using a combinatorial library scanning approach where peptide pools of different length were tested for the ability to stimulate different TCRs, it has been shown that TCRs have restricted length preferences in the peptide epitopes that they recognize (163). Thus, it remains to be seen whether the addition of “gaps” in a search string are of any value. This can be readily determined by activity analysis of cognate peptides that have the various single-amino acid insertions.

The MAGE-A3 and WT1-related peptides were further screened using the binding prediction algorithms listed above, and peptides predicted to bind by the ANN or SMM algorithms ($IC_{50} < 500$ nM) were designated as potential binders, resulting in 98 and 11 peptides for MAGE-A3 and WT1, respectively. Interestingly, of these epitopes, 41 and 64%, respectively, were identically represented in the mouse proteome, with many others having highly homologous sequences. The distribution of homologous sequences identified through these screens, and their presence uniquely in the human proteome, the mouse proteome, or identically in both is shown in **Figures 3B,C** (where the WT1-like peptides in **Figure 3C** also include those with single-amino acid gaps). A summary of the search results for these two epitopes can be seen in **Figure 3D**. The number of peptides identified by this type of search in all three cases (SIY, MAGE-A3, and WT1) is readily amenable to small-scale synthesis and *in vitro* testing for T cell stimulation by peptide-loaded, HLA-A2-positive APCs. We propose this straightforward screen to evaluate cross reactivities with structurally similar epitopes. Peptides with reactivities would be followed with more detailed analysis of gene transcript levels in different tissues, and studies of the ability of the gene-product to be processed and presented.

Proteome searches using a particular motif can not assess all potential cross-reactive peptide epitopes, especially those without structural similarity. Hence, we propose that an additional *in vitro* screening strategy may be useful. For example, an open reading frame (ORF) library (167, 168) covering genes from the human proteome would be transfected into HLA-A2-positive APCs. ORF libraries have been used in yeast two-hybrid systems toward mapping the protein “interactome,” (169) and recently the human ORFome is being developed in a lentiviral vector system, which

would allow for convenient application to mammalian cell transduction (168, 170). This would provide another opportunity to identify unpredicted cross reactivities, and such libraries would provide a resource available for screening virtually any TCR, restricted by the appropriate HLA alleles.

Finally, we propose expanded use of HLA transgenic mice to screen for safety. As mentioned above, 40–65% of peptides identified in MAGE-A3 and WT1 homology screens were identical in mouse and human, providing a rationale for using a mouse screen to identify at least some of the potentially adverse cross reactivities. The system would ideally involve the use of mouse T cells transduced with human TCRs (human V regions linked to mouse C regions), as these would provide syngeneic cell:cell adhesion systems for optimal activities. TCR-transduced mouse CD4 and CD8 T cells could be transferred to the transgenic HLA-A2/D^d hybrid MHCs (AAD, available from Jackson Labs) which allows cells to present HLA-A2 peptide epitopes while still engaging mouse CD8. This system could be tested with various affinity TCRs in order to push the limits of safety and efficacy.

A significant advantage of the mouse system would be the opportunity to also generate additional transgenic mice on the AAD background, where the tumor gene of interest (e.g., MART-1, MAGE-A3, or NY-ESO-1) is expressed under the relevant mouse promoter. Such models could reveal on-target/off-tumor activities due to uncharacterized expression of the target gene in normal tissue, either at low levels or by a low-frequency cell subset. As the WT1 [126–134] epitope is identical in the mouse and human proteins, this provides an opportunity to assess safety without the generation of the human WT1 transgene.

CONCLUDING REMARKS

While there will always remain a risk of unpredicted reactivities in patients receiving adoptive T cell therapies, we believe

that the use of TCRs with different affinities and specificities in an expanded set of pre-clinical approaches, as described here, will identify some of the possible problems. Proteome search approaches provide a measure of the number of related self-peptides that could pose safety concerns with adoptive T cell therapies. In addition, the number of peptides represented in the proteome predicted to be similar to a given epitope should correlate with the extent of central tolerance that might exist against a cancer peptide. In this regard, this type of analysis might be considered for peptide vaccines (e.g., lower numbers of homologous peptides may correlate with higher frequencies of peripheral T cells that have escaped negative selection). Further safeguards at the initial clinical stage, such as reduction in the number of T cells delivered, may be considered. Significant progress has also been made in the development of suicide genes or alternative approaches that could allow rapid deletion of T cells before a dangerous reaction reaches the critical stage (171, 172). Finally, transfer of only CD4 T cells may be desirable as they can mediate strong anti-tumor effects and potential for helping endogenous immune responses, but CD4 T cells may not exhibit the CD8-dependent cross reactivities that the same TCRs mediate in CD8 T cells.

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that could be construed as a potential conflict of interest.

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Structure-based, rational design of T cell receptors

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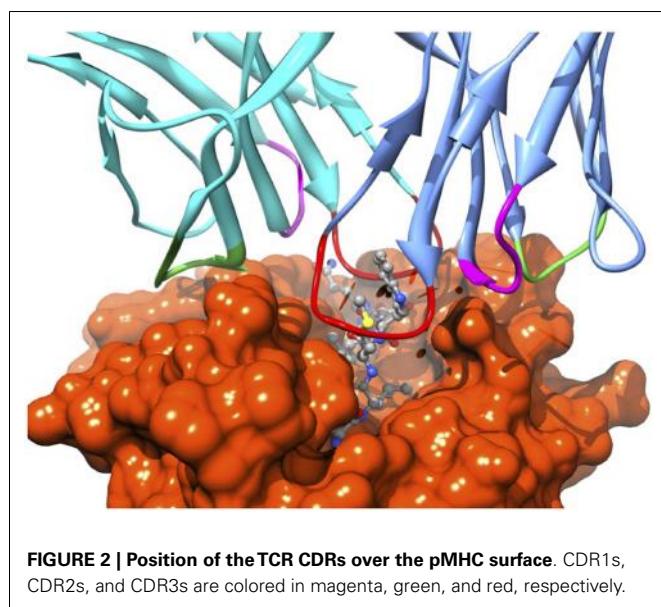
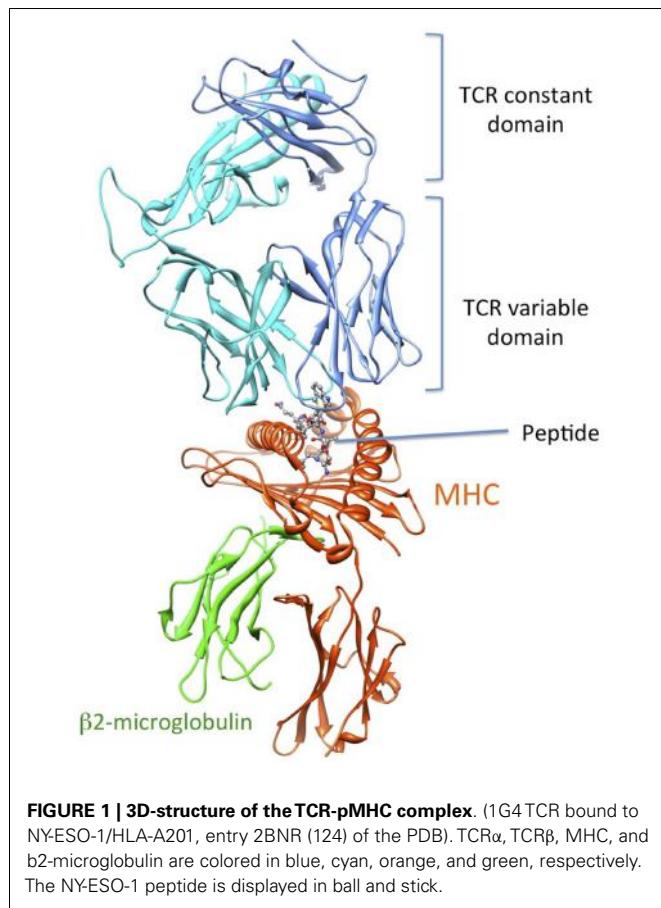
Adoptive cell transfer using engineered T cells is emerging as a promising treatment for metastatic melanoma. Such an approach allows one to introduce T cell receptor (TCR) modifications that, while maintaining the specificity for the targeted antigen, can enhance the binding and kinetic parameters for the interaction with peptides (p) bound to major histocompatibility complexes (MHC). Using the well-characterized 2C TCR/SIYR/H-2K(b) structure as a model system, we demonstrated that a binding free energy decomposition based on the MM-GBSA approach provides a detailed and reliable description of the TCR/pMHC interactions at the structural and thermodynamic levels. Starting from this result, we developed a new structure-based approach, to rationally design new TCR sequences, and applied it to the BC1 TCR targeting the HLA-A2 restricted NY-ESO-1₁₅₇₋₁₆₅ cancer-testis epitope. Fifty-four percent of the designed sequence replacements exhibited improved pMHC binding as compared to the native TCR, with up to 150-fold increase in affinity, while preserving specificity. Genetically engineered CD8⁺ T cells expressing these modified TCRs showed an improved functional activity compared to those expressing BC1 TCR. We measured maximum levels of activities for TCRs within the upper limit of natural affinity, $K_D = \sim 1 - 5 \mu\text{M}$. Beyond the affinity threshold at $K_D < 1 \mu\text{M}$ we observed an attenuation in cellular function, in line with the “half-life” model of T cell activation. Our computer-aided protein-engineering approach requires the 3D-structure of the TCR-pMHC complex of interest, which can be obtained from X-ray crystallography. We have also developed a homology modeling-based approach, TCRRep 3D, to obtain accurate structural models of any TCR-pMHC complexes when experimental data is not available. Since the accuracy of the models depends on the prediction of the TCR orientation over pMHC, we have complemented the approach with a simplified rigid method to predict this orientation and successfully assessed it using all non-redundant TCR-pMHC crystal structures available. These methods potentially extend the use of our TCR engineering method to entire TCR repertoires for which no X-ray structure is available. We have also performed a steered molecular dynamics study of the unbinding of the TCR-pMHC complex to get a better understanding of how TCRs interact with pMHCs. This entire rational TCR design pipeline is now being used to produce rationally optimized TCRs for adoptive cell therapies of stage IV melanoma.

Keywords: molecular modeling, protein-engineering, TCR, TCR-pMHC, immunotherapy, adoptive transfer, cancer

INTRODUCTION

Recognition by the CD8⁺ T cell receptor (TCR) of immunogenic peptide (p) presented by class I major histocompatibility complexes (MHC) is a key event in the specific immune response against virus-infected cells or tumor cells. Binding of the TCR to the pMHC complex leads to T cell activation and killing of the target cell (1). The TCR is composed of two chains, α and β , that pair on the surface of the T cell to form a heterodimeric receptor on the surface of the T cell. Each chain is composed of a constant domain that anchors the protein in the cell membrane and of a variable domain that confers antigen recognition (Figure 1). The TCR contacts pMHC molecules via the 6 complementarity-determining regions (CDR), three each from the α and β chains

(Figure 2). These CDRs constitute the hypervariable regions of the two V domains, called $V\alpha$ and $V\beta$ (2–4). They are generated by somatic gene rearrangement and negatively selected in the thymus against reactivity with endogenic pMHCs. CDR3 α and CDR3 β are the most diverse regions of the TCR and thus play a major role in antigen specificity. The CDR1 and CDR2 loops of the α and β chains predominantly make contact with the MHC molecule. The strength of the interaction between TCR and pMHC has been shown to play an important role in the T cell activation (5–9). However, the kinetics of the TCR/pMHC interaction is also determinant in T cell activation (10, 11). Consequently, understanding the biophysical properties of the TCR/pMHC interaction is of great interest for the prediction of the T cell activation, and for



the rational TCR optimization toward improved adoptive transfer cancer therapy (12, 13).

This review will focus on the different computer-aided techniques we developed and used to study the TCR-pMHC complex

from a structural and thermodynamic point of view. First we present the results obtained by steered molecular dynamics (SMD) simulations of molecular recognition events occurring during the TCR-pMHC complex formation. Second, we describe the different approaches we developed to derive structural models of TCR-pMHC complexes for a large TCR repertoire. Third, we summarize our approaches to estimate the binding free energy for the TCR association to pMHC. Finally, we present our *in silico* structure-based protein-engineering approach that enables the fine-tuning of TCR-pMHC binding parameters.

INVESTIGATING TCR-pMHC INTERACTIONS USING STEERED MD SIMULATIONS

The structure of ~66 TCR-pMHC complexes are known to date (14) among which ~25 are unique complexes. The compared features of these structures were the object of extensive reviews (1, 15, 16). In addition, binding kinetics and thermodynamics of many complexes have been measured (17). The general picture that emerges is that T cell activation requires TCR-pMHC binding to fall within a certain range of affinity and kinetics. However, clear structural determinants of TCR specificity have remained elusive. A single mutation can change a peptide from agonist to antagonist, but the same TCR can recognize various peptides with different binding modes. Thus TCRs can be exquisitely specific while displaying a high degree of cross-reactivity. In addition, TCRs use extremely varied thermodynamic strategies to bind to pMHCs, ranging from entropy-favored to entropy-opposed.

A system that exemplifies both the sensitivity of TCR recognition and its potential for cross-reactivity with different binding strategies is the Tax nonapeptide (LLFGYPVYV) from the HTLV-1 virus presented by the HLA-A201 MHC. This pMHC is a strong agonist for the A6 TCR but the P6A peptide mutant (Pro replaced by Ala at position 6, see Figure 3A) dramatically reduces the binding affinity and abrogates T cell activation (18). On the other hand, the B7 TCR, which has the same α chain as A6 but a different β chain, is also activated by the Tax peptide presented by the same MHC. B7 binds with an affinity similar to A6, but the binding is entropically opposed, whereas A6 binding is entropically favored, outlining a completely different binding mechanism (19) (see Figure 3B).

Detailed aspects of protein–protein interactions can be characterized by SMD simulations, in which the dissociation is actuated by an external force acting on the protein. A typical reaction coordinate for protein–protein dissociation is the distance between the centers of mass of each protein. In the following, we call this reaction coordinate ξ . In the case of the TCR-pMHC, we assumed that the dissociation happens in the direction perpendicular to the cellular membranes (see Figure 3C). We also assumed that there is no substantial conformational rearrangement upon dissociation (except possibly in the CDRs and in the peptide), which is supported by the similarity of X-ray structures of bound and unbound TCRs or pMHCs. To enforce these assumptions during the SMD, we devised the individual pulling scheme (20) in which each non-H atom (except CDRs and peptide) is subjected to an individual harmonic potential. As shown on Figure 3D, the center of mass distance ξ is increased by collectively shifting the reference positions of the individual potentials. For each TCR-pMHC complex,

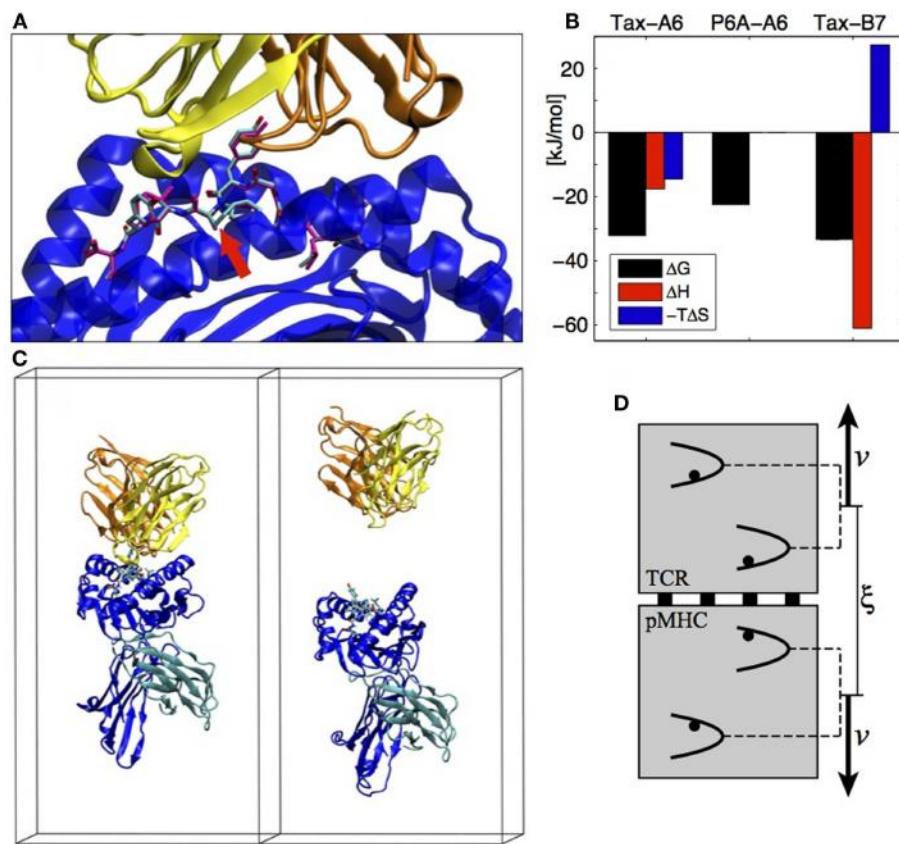


FIGURE 3 | (A) The A6TCR (yellow and orange) bound to the HLA-A2 MHC (blue) presenting the Tax peptide (cyan). The P6A peptide mutant is overlaid in magenta (PDB entry 1AO7). The site of the P6A mutation is indicated by a red arrow. **(B)** Experimental TCR binding thermodynamics. For P6A-A6, no entropy/enthalpy measurements are available. References are given in the text. **(C)** TCR-pMHC complex in its simulation box before

and after the SMD simulation (water molecules not shown). **(D)** The individual pulling scheme used to dissociate the proteins. The distance ξ between the centers of mass is increased at rate $2v$. Each backbone heavy atom is subjected to an individual harmonic potential. All individual potentials move in concert, with their relative positions fixed to their values in the crystal structure.

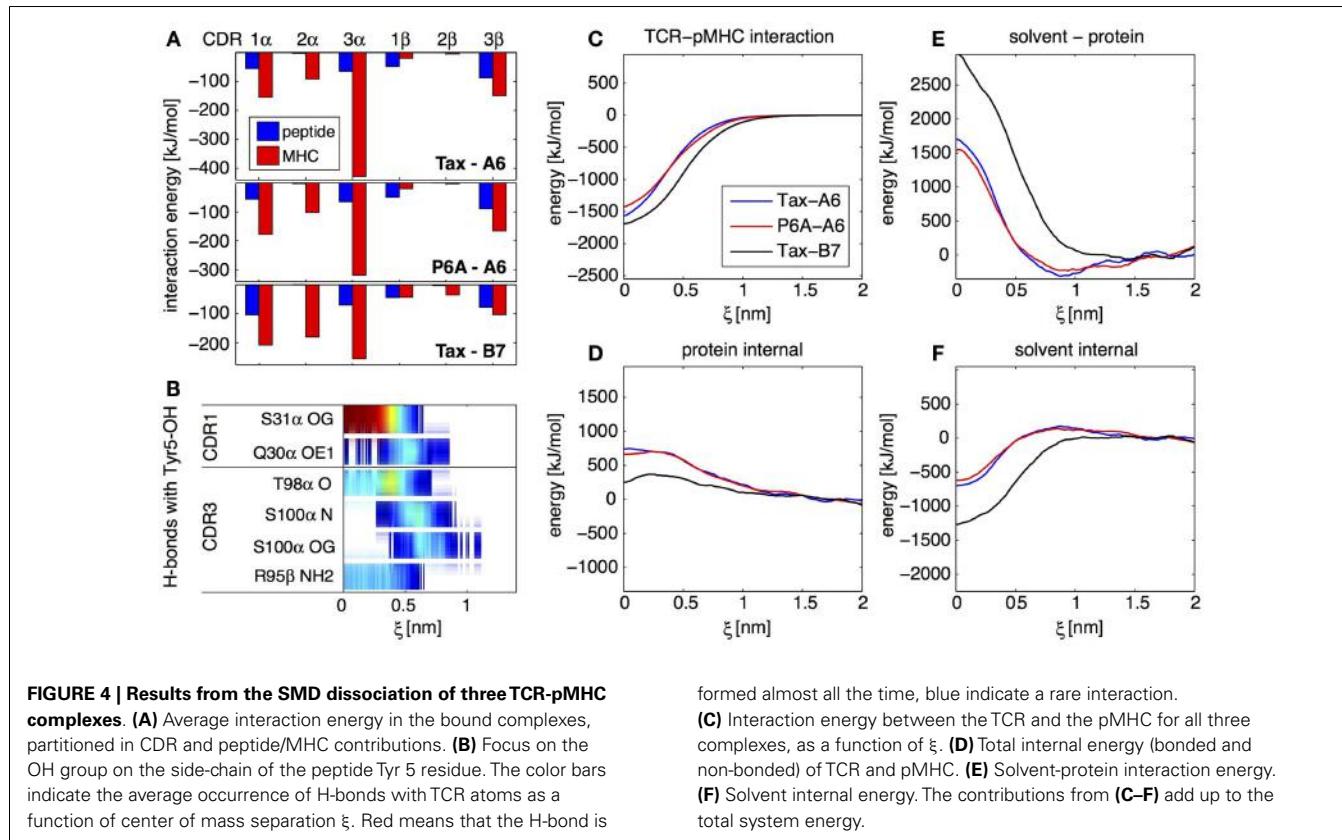
we performed about 150 unbinding trajectories of 4 ns each during which ξ is gradually increased by 2 nm from the bound state distance to reach a final conformation as shown on the right panel of **Figure 3C**. The simulations were performed with the Gromacs software (21) using the Gromos 45a3 force field (22) with explicit water molecules.

With about 150 trajectories we were able to obtain converged ensemble averages of many observables at any given protein–protein separation, including for highly fluctuating quantities involving solvent molecules. In particular, we established maps of H-bonds or of non-polar contacts for all residues of the TCR and pMHC as a function of ξ . As an example, **Figure 4B** shows an H-bond occurrence map for the OH group on the Tyr5 side-chain of the Tax peptide in the Tax-A6 system. We see that α -S31 is the main TCR H-bonding partner in the bound state, but that new H-bonds are formed with α -S100 in the transition state. Overall, our simulations have shown that the number and diversity of H-bonds occurring in a protein complex largely exceeds what is apparent from the crystal structures. Using this methodology, detailed maps such as **Figure 4B** can be established for any interaction to

any atom in the system, depending on the biological question of interest.

For all three TCR-pMHC complexes, we monitored energy variations in different parts of the system upon dissociation. **Figure 4C** shows that there are differences in TCR-pMHC interaction energies between the complexes. But these are largely compensated by effects in the internal protein reorganization energy (**Figure 4D**), solvent–protein interaction energy (**Figure 4E**), and solvent internal energy (**Figure 4F**). As a general lesson for protein–protein interactions, we retain that the solvent plays a key role in two different ways. First, variations of solvation energies exceed contributions from the proteins themselves upon binding. Second, specific water molecules trapped at the interface can influence the binding mechanism and thermodynamics (data not shown). In the present case, these two aspects happen to be also the two major factors differentiating A6 and B7 binding.

Focusing on the bound state of the TCR-pMHC complexes, the decomposition of the average interaction energy among CDRs brings valuable insights. **Figure 4A** illustrates the differences between the A6 and B7 binding modes, with a much less prominent



contribution of CDR3 α for B7. Generally for all complexes, Figure 4A shows that, while CDR2 interacts mostly with MHC, both CDR1 and CDR3 interact equally with peptide and MHC, even at large distance, as confirmed by our H-bond occurrence maps (not shown). Therefore, our simulations do not support the two-step model (23) for TCR engagement, in which the CDR1 and CDR2 preferentially contact the MHC at large distance, while the CDR3 establishes final contacts to “read” the peptide mainly at short distances.

Overall, although the P6A-A6 complex has a very different affinity compared to the wild-type Tax-A6, both complexes share very similar features in terms of specific H-bonds or energy contributions. On the other hand, the Tax-B7 complex has a binding affinity similar to that of Tax-A6, but uses a completely different binding mechanism. The B7 TCR creates a very different set of H-bonds and hydrophobic contacts to the pMHC and makes a very different usage of the solvent, which reflects in a different partition of the binding energy. In retrospect, as noted previously by Baker and coworkers (24), it is not so surprising to observe active TCRs with very different binding mechanisms. Indeed, if TCRs are issued from random sequence variation and selection upon pMHC binding affinity and kinetics only, each TCR is likely to adopt its own unique pMHC binding strategy as long as it matches these criteria.

TCR-pMHC HOMOLOGY MODELING

PIONEERING STUDY OF TCR-pMHC HOMOLOGY MODELING

The recent development and use of experimental techniques to determine sequences of TCRs that bind to a pMHC complex (25),

led to the collection of large repertoires of TCR sequences with given pMHC specificities (26, 27). Understanding the selection mechanism that causes this gene usage can be facilitated by the introduction of structural information regarding the underlying TCR-pMHC complexes. This information can be used to identify conserved 3D binding motifs that are not obvious from repertoire sequences alone (28), to suggest explanations regarding the impact of TCR mutation on its affinity for given pMHCs (29) and ultimately to support the rational engineering of TCRs with particular binding properties (7, 30). Experimental structural techniques such as X-ray crystallography or NMR provide direct and valuable information regarding the 3D-structures of macromolecules. Unfortunately, they require the production of the protein, can be time consuming, and are thus hardly applicable to the analysis of large repertoires of tens to hundreds of TCRs. According to the 3D-structure database of the international ImMunoGenetics information system [IMGT/3D-structure-DB (31, 32)] the 3D-structure of 66 TCR-pMHC complexes have been determined experimentally so far. This number is negligible compared to the vast TCR diversity created by genetic rearrangements of the TCR V, D, and J genes. Indeed, the number of unique TCR β chains in blood has been estimated to be of the order of 10^6 (33, 34). There is thus a need for tools able to predict the 3D-structure of TCR-pMHC complexes from the amino acid sequences of their components.

The pioneering work of Michielin et al. (29) provided a remarkable demonstration of the feasibility and the predictive ability of TCR-pMHC 3D-structure modeling. The authors used a murine

T1 TCR, specific for a photoreactive derivative of the Plasmodium berghei circumsporozoite (PbCS) 253–260 nonapeptide presented by the K_D class I MHC (35). Fifty mutants involving the TCR's CDR, the MHC's $\alpha 1$ and $\alpha 2$ helices and the peptide were prepared and the association constants between the TCR and the pMHC were measured (35). A first homology model was built for the wild-type TCR-pMHC complex with the MODELLER program (36–38), using the four TCR structures available at that time (39–42) and the structure of H-2Kb MHC (43) as templates. These structures provided good templates for the β -sheet framework of the TCR, and of the α helices/ β -sheet of the MHC grooves. The high secondary structure content of these regions imposed strong restrictions on their backbone conformation according to the MODELLER algorithm, which facilitates the modeling of those parts. In contrast, the CDR loops of TCRs have a very low level of sequence identity and no specific secondary structure, which obviously limits the efficiency of modeling by homology. The CDR loops conformation obtained in the first homology model were thus refined using a simulated annealing technique (44), followed by clustering the generated conformations based on their relative Cartesian coordinate root mean square deviation (RMSD). A final conformation was chosen from a well-populated, low-energy cluster, whose structure was compatible with the experimental mutational data. All but three of the 50 mutations found qualitative explanation in the model in terms of breaking of a significant TCR/pMHC interaction. In addition, the model suggested that a TCR pocket could form upon binding to accommodate the peptide hapten, explaining the high level of affinity of the T1 TCR for this pMHC ($K_D \sim 10$ nM), and demonstrating predictive capabilities for the modeling approach that go beyond reproducing only the structural features present in the templates. Since more X-ray structures of TCR-ligand complexes are continuously determined,

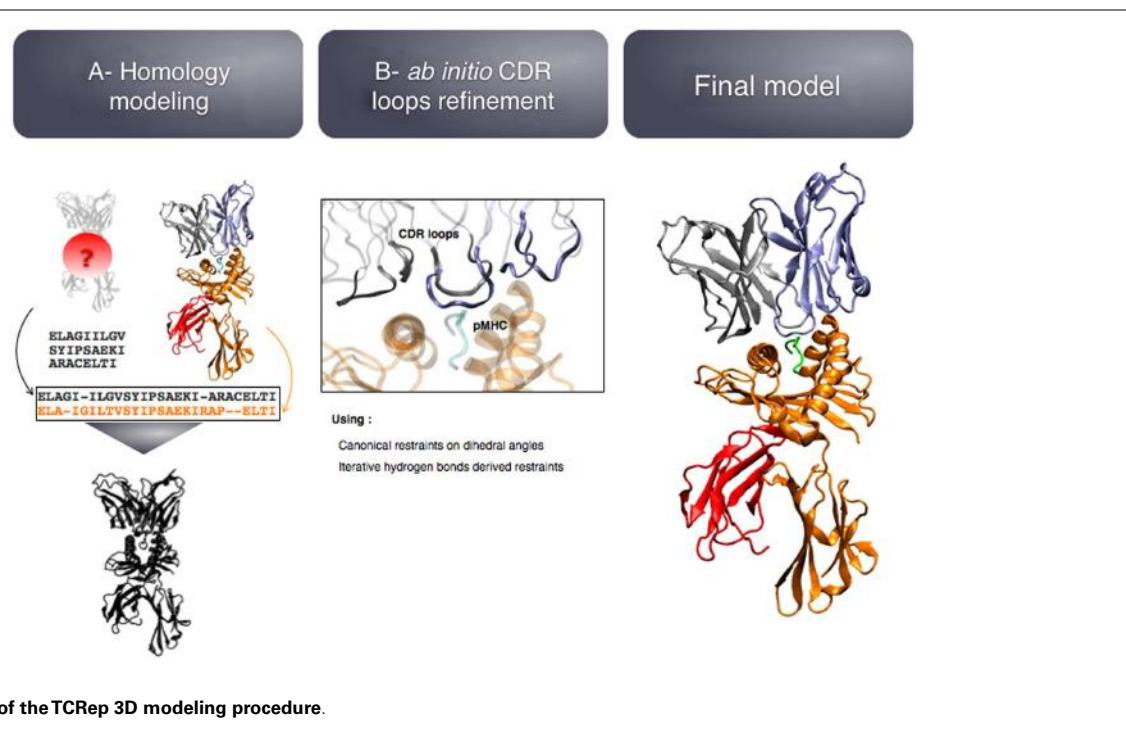
it could be expected that the range of applicability and the accuracy of such a modeling approach would improve, since there is no limitation to the number of simultaneous templates that can be used.

TCRep 3D

The first study described above (29) led to the development of TCRep 3D, as a generalization of the TCR-pMHC modeling approach (28). TCRep 3D is an approach dedicated to the prediction of high-quality 3D-structures that can provide a functional insight on the interaction between a TCR and a pMHC. It includes by design minimal input and optimal automation, to analyze wide sets of sequences of TCRs belonging to a common TCR repertoire.

The modeling pipeline is composed of two modules (see Figure 5): (i) homology modeling of the TCR-pMHC complex and (ii) *ab initio* CDR loops structure optimization. First, the user provides the sequence of the target complex and a list of preferred templates. By default, all the TCR-pMHC templates of the Protein Data Bank are used. The global structure of the complex is modeled by homology. It could be possible to couple this step to computer-aided approaches for the docking of peptide antigens into MHC molecules (45), in case the peptide binding mode could not be correctly predicted by homology modeling. Each CDR loop is then subsequently refined while the rest of the complex remains rigid. The MODELLER (38) software is used for the two modules.

The originality of TCRep 3D resides in the specific bias that we impose to the system during the structural sampling. Canonical restraints available from the literature (46) are added to the backbone dihedral angles of CDR1 and 2 to limit the conformational space accessible to the loops. We developed an iterative sampling



method that identifies potential hydrogen bonds between TCR and pMHC, and converts them into modeling restraints. The scoring function was adapted accordingly, to favor structures that satisfy most of the canonical restraints, and display potential hydrogen bonds. We demonstrated that TCRep 3D is significantly more efficient than common loop modeling approaches in predicting CDR loops conformations.

At the time of the study, TCRep 3D produced one TCR-pMHC structure in 7 days on a single CPU. However, the modeling can be parallelized on a computing grid, and the computation time scales efficiently with the number of CPUs used, allowing the user to quickly model a large number of sequences.

TCRep 3D has been successfully applied to experimentally determined sets of sequences of TCRs that recognize given cancer epitopes.

- (a) In a study on HLA-A*0201/Melan-A-specific CD8 T cells (47), the modeling of the TCR-pMHC 3D-structure revealed the structural feature that explained how two distinct sets of TCR performed differently in recognizing a naturally occurring decamer variant of the Melan-A peptide. One of the TCR subsets could not make proper interactions with the glutamic acid at position 1 of the peptide because of the location and structural properties of the CDR1 α (see **Figures 6A,B**).
- (b) The analysis of HLA-A*0201/NY-ESO-1_{157–165} specific CD8 $^{+}$ T cells from five melanoma patients showed a preferential usage of three V β genes. Additionally, experimental evidence on the importance of the Met4-Trp5 pair of the NY-ESO-1_{157–165} antigen were found, suggesting that those two contact residues make critical interactions with the TCR, regardless of the gene segment usage (26). The modeling of the corresponding TCR-pMHC structures revealed a striking mechanism of selection through the presence of a single conserved glycine residue situated in the center of all CDR3 β . An *in vitro* experimental functional study of mutations of this amino acid combined with *in silico* modeling of several mutants was performed. All mutations resulted in dramatic structural changes associated with complete experimental loss of affinity of the TCR to NY-ESO-1/HLA-A*0201 (28).

LONG-RANGE DRIVING FORCE FOR TCR ORIENTATION

Over the years, successive releases of TCR-pMHC crystal structures have revealed the variety of native binding orientations that the TCR can adopt. Recent studies reported a range of more than 45° in the TCR binding angles relative to the MHC (48), depending on the peptide, the MHC, and the α/β pairing of the TCR. Although the challenge of TCR binding mode prediction has been recurrently discussed, only a few studies have focused on predicting the actual binding mode of given TCR-pMHC (49, 50). Therefore, all methods and applications relied on the existence of at least one TCR-pMHC crystal structure.

In order to understand the molecular basis that governs TCR orientation upon binding, we tested a simplified rigid approach on all published TCR-pMHC crystal structures (48), which allowed scanning quickly multiple orientations of the TCR relative to the pMHC. In this approach, the TCR was moved 6–12 Å away from the pMHC molecule along the TCR principal axis (see **Figure 7A**). Subsequently, the TCR was rotated around that same axis until a complete revolution was obtained (see **Figure 7B**). The effective energy of the system was computed every 5°, as the sum of the intermolecular energy and the solvation free energy, using the CHARMM22 force field (51, 52) in combination with the FACTS implicit solvation model (53).

We demonstrated that the sum of the Coulomb interaction and the electrostatic solvation energies is sufficient to identify the native TCR orientation as the energetic minimum upon rotation (see **Figure 7C**). Importantly, despite the rigid-body simplification, the results were robust upon small structural variations of the TCR such as changes induced by MD simulations. We also tested our approach on crystal structures of unbound TCRs, which were confronted to pMHCs. Accurate energy minima were also identified, suggesting that perfect shape complementarity is not required to obtain a reliable signal. The long-distance interactions during the TCR approach appear to be independent of the binding process itself, since the binding orientation is reliably identified without considering either short-range energy terms or CDR induced fit upon binding.

Furthermore, we decomposed the effective energy into per-residue contributions, in an approach that is similar to the

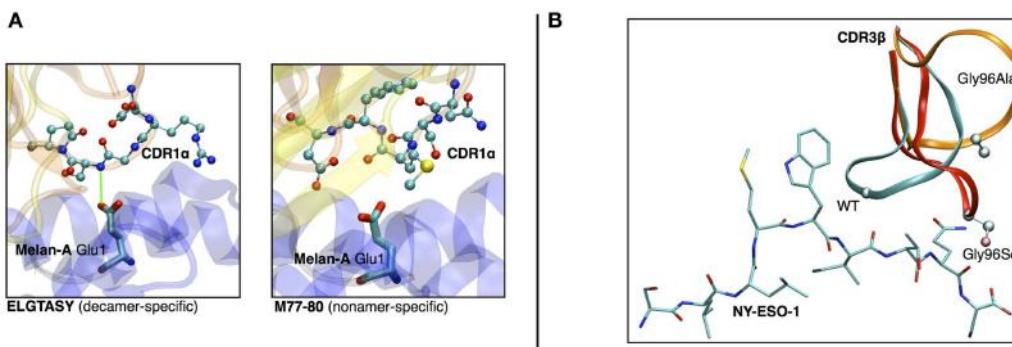
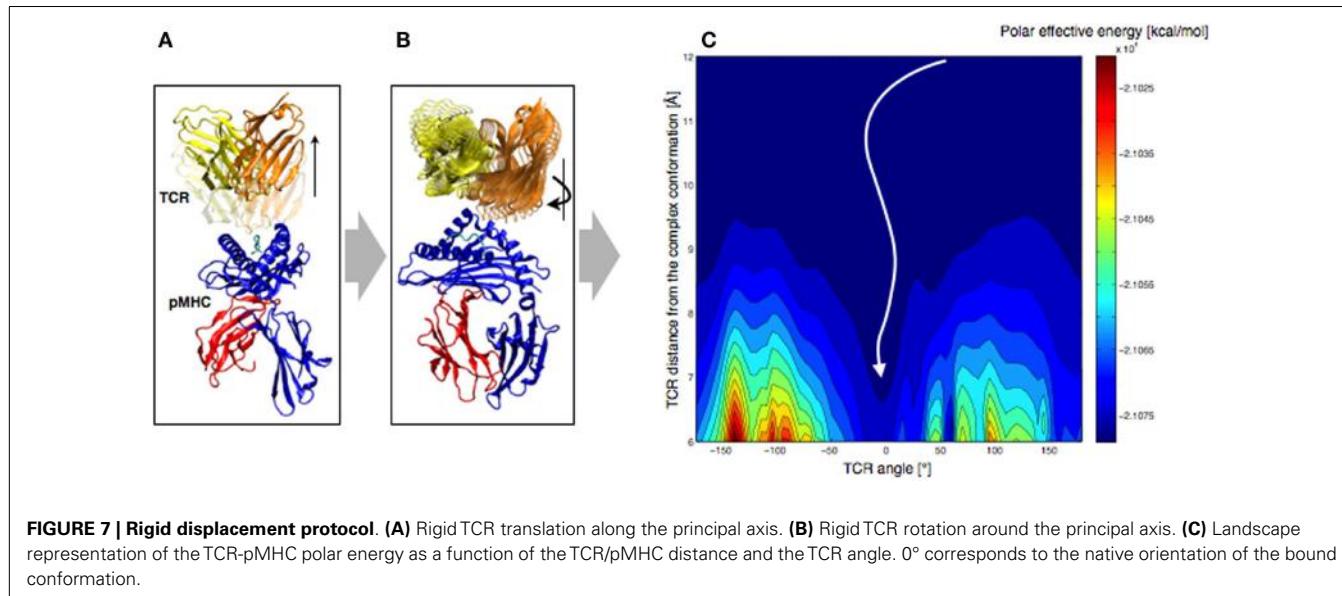


FIGURE 6 | (A) Structural differences between nona- vs. decamer-specific TCR bound to Melan-A(ELA)/HLA-A*0201. The green line identifies the hydrogen bond that is formed between CDR1 α and Glu1 of the peptide. No favorable interactions were identified between M77-80 and the decamer variant of Melan-A. **(B)** *In silico* mutation results in NY-ESO-1 repertoire. The dramatic structural rearrangement of the mutated CDR3 β confirms the importance of the central Gly.



Molecular Mechanics – Generalized Born Surface Area (MM-GBSA) energy decompositions (54). The contributions of structural sub-groups to the profile of the TCR/pMHC interaction energy during rotation were calculated, to estimate their role in the overall orientation. Results showed that most of the driving force (>90%) leading to the orientation of the TCR is defined by CDR1,2/MHC interactions. This is in agreement with previous observations, revealing a ring of charged residues at the pMHC interface, which interacts with CDR1,2 with complementary charges (48). We reported that the role of the CDR3/peptide interaction is of lesser importance at long-distance.

In turn, such knowledge of the structure may be used as a preliminary approach in the process of modeling protein/protein interactions. More specifically, the rigid search for an energetic minimum upon TCR rotation may become a complementary module of TCRep 3D, to search for the correct binding mode, after modeling the TCR and the pMHC independently. We attempted to predict the binding mode of the A6 TCR with tax/HLA-A*0201, after modeling the TCR by homology. The effective energy minimum upon rotation was computed for 500 homology models, and we obtained an average shift of 12.2° from the orientation of the crystal structure. This demonstrated the potential of the approach as a component of a TCR-pMHC structural prediction pipeline (55). The approach is also easily applicable to other types of protein complexes, provided that the association is also driven by long-range electrostatic interactions.

FREE ENERGY CALCULATIONS

APPLICATION OF THE THERMODYNAMIC INTEGRATION METHOD FOR TCR-pMHC BINDING FREE ENERGY DIFFERENCES

T cell receptor recognition can exhibit exquisite specificity upon single peptide mutation. In the A6/Tax/HLA-A0201 complex described above (Tax-A6), mutating the Ala at position 6 to a Pro (P6A) turns the Tax peptide from a strong agonist into a weak antagonist. These systems were extensively studied experimentally (18, 56) and the binding free energy difference

between the Tax-A6 and the P6A-A6 complexes was found to be $\Delta\Delta G = 2.90 \pm 0.20$ kcal/mol. (see Figure 3B). These results are difficult to rationalize from the structure alone, as there is almost no difference between the conformations of the Tax-A6 and P6A-A6 complexes (see Figure 3A, red arrow). To gain a better understanding of the effect of the mutation on TCR recognition, we used free energy simulation to analyze in detail the origin of the binding free energy difference (57).

As we will see below, calculating the binding free energy by simulating the entire TCR-pMHC unbinding process itself is difficult. Instead, the present method uses the thermodynamic cycle shown in Figure 8A to reformulate the problem,

$$\Delta\Delta G_{\text{P6A}}^{\text{Binding}} = \Delta G_{\text{P6}}^{\text{Binding}} - \Delta G_{\text{A6}}^{\text{Binding}} = \Delta G_{\text{Bound}}^{\text{P6} \rightarrow \text{A6}} - \Delta G_{\text{Unbound}}^{\text{P6} \rightarrow \text{A6}}. \quad (1)$$

This means that we can obtain $\Delta\Delta G_{\text{P6A}}^{\text{Binding}}$ by computing the P6 → A6 mutation free energy in both the unbound and the bound states. Among the different methods available to calculate mutation free energy differences (58, 59), we chose thermodynamic integration (60, 61). We define an interpolated potential energy function $U(r, \lambda)$ that is equivalent to the potential energy function of the wild-type for $\lambda = 0$ (Pro) and to that of the P6A mutant for $\lambda = 1$ (Ala). The free energy difference can be obtained through

$$\Delta G_{\text{Bound}}^{\text{P6} \rightarrow \text{A6}} = \int_0^1 \left\langle \frac{\partial U(r, \lambda)}{\partial \lambda} \right\rangle_\lambda d\lambda.$$

Here, $\langle \cdot \rangle_\lambda$ represents an ensemble average at fixed λ . In practice, we perform a set of simulations at discrete λ -values and evaluate the integral above numerically. The number and position of λ -values required for accurate integration depends on the smoothness of the $\langle \frac{\partial U}{\partial \lambda} \rangle_\lambda$ function. To construct an appropriate interpolating potential energy function, a dual topology scheme was used, as shown on Figure 8B. For vanishing atoms, only the non-bonded interactions are scaled, while the bonded interactions are left unchanged (62). As we did not use soft-core potentials

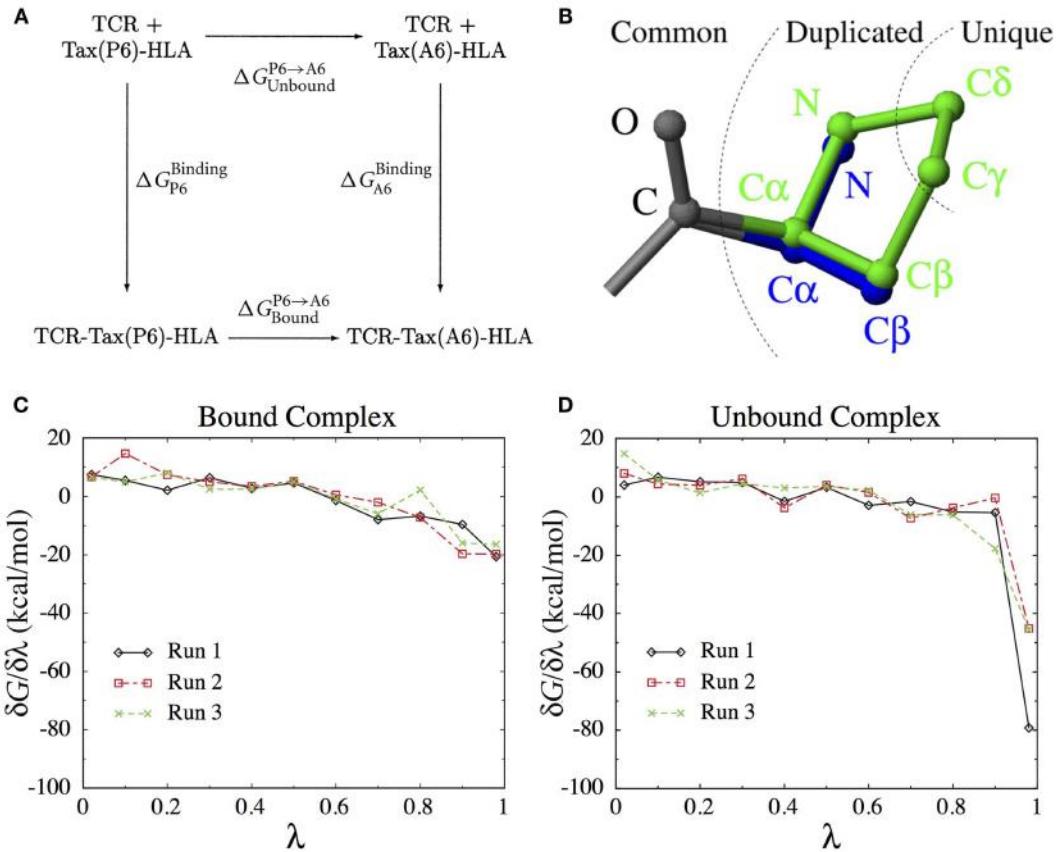


FIGURE 8 | (A) The thermodynamic cycle underlying Eq. 1. **(B)** The dual topology scheme used to interpolate the potential energy function between the Pro side-chain (green) and the Ala side-chain (blue). Common atoms are unaffected, Duplicated atoms change type and non-bonded parameters.

Unique atoms vanish, with their non-bonded interactions switched to zero and their bonded interactions unchanged. **(C)** Derivatives of the free energy obtained for each λ -value in three independent runs for the bound complex. **(D)** Idem for the unbound complex.

(63) for vanishing atoms, special care was taken in the limit of $\lambda \rightarrow 0$ and $\lambda \rightarrow 1$ to deal with the singularities of the Coulomb and Lennard-Jones potentials.

Starting from the crystal structures for both the unbound (64) and the bound complex (18), MD simulations were performed in the CHARMM program (51) with the CHARMM22 force field (52). The proteins were locally solvated in a sphere of 16 Å surrounding the peptide using the stochastic boundary method (65). The set of λ -values used were $\lambda = 0.02, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 0.98$. After 100 ps of initial equilibration, data was collected for 30 ps at each λ -value, separated by 10 ps equilibration time after each λ update. Three simulations with independent initial velocities were produced.

The simulations were structurally stable with average RMSD of non-H atoms no greater than 0.8 Å with respect to the crystal structures. The average free energy derivatives obtained in the three different runs are shown on Figures 8C,D for the bound and unbound states, respectively. In the unbound state (Figure 8D), the derivative takes very large values close to $\lambda = 1$, due to the interaction of the vanishing Pro atoms with the solvent molecules. This does not happen in the bound state, because the vanishing

atoms are concealed from the solvent in the void created by the protein pocket.

Three different schemes were tested for the extrapolation to $\lambda = 0$ and $\lambda = 1$, using linear, quadratic or $\lambda^{-3/4}$ functions. The integration over all λ -values was performed using the trapezoidal rule. The final result is $\Delta \Delta G_{\text{P6A}}^{\text{Binding}} = 2.9 \pm 1.1$ kcal/mol, which compares very favorably with the experimental value of 2.9 ± 0.2 kcal/mol.

One of the major strengths of the method lies in the linearity of Eq. 1, which allows decomposing as a sum of contributions from different types of interactions and/or of different parts of the system. Given that the total free energy difference is in good agreement with experiment, there is a good chance that the decomposition provides meaningful insights on the mechanisms leading to TCR specificity.

A notable contribution to $\Delta \Delta G$ (+0.64 kcal/mol) arises from the difference in solvation free energy of the mutated residue: in the unbound structure, the Tax P6 residue is solvent-exposed with around 35% of its surface accessible to water molecules. The A6 mutant has fewer exposed hydrophobic groups, which entails a more modest solvation penalty in the unbound state.

This stabilizes the unbound state of the P6A mutant relative to the wild-type, which in turn makes P6A-A6 binding less favorable. The rest of the peptide contributes a modest 0.38 kcal/mol to $\Delta\Delta G$.

The total contribution of the TCR in the bound state is around +0.8 kcal/mol destabilizing the P6A mutant, the most significant part of which is due to the CDR3 α loop. Most of this energy arises from the van der Waals term, in accord with the fact that the TCR provides good surface complementarity for the hydrophobic side-chain of the Pro residue. Since the pocket is already present in the Tax-A6 complex, there is no large free energy cost needed to induce it, in contrast to what is found in the solvent. The TCR residues that contribute the most to the TCR specificity for the wild-type peptide are N30 from the CDR1 α , D99, and S100 from the CDR3 α , and G97, L98, A99, G100, G101 from the CDR3 β .

The most important contribution to $\Delta\Delta G$ (1.26 kcal/mol) arises from the difference in interactions with the MHC. This is due to a conformational change that takes place in the Tax P6 region upon TCR engagement. In the Tax system, the cost of this conformational change is balanced by a very favorable interaction of the Pro ring with hydrophobic residues of the MHC groove, which does not take place with the shorter Ala side-chain. This is an example of how a conformational change taking place along the physical binding pathways translates into a free energy contribution along the alchemical pathways.

Overall, it emerges that the total binding free energy difference between the wild-type and the mutant peptide consists of four contributions that are similar in magnitude. The self-interaction of the peptide and the change in the interaction between the peptide and the three portions of its environment (TCR, HLA-A2, and solvent) all contribute between 0.5 and 1.2 kcal/mol to stabilizing the wild-type complex. This important result was not evident from the X-ray structures or the experimental data. Interestingly, these calculations show that accurate free energy differences could be obtained although most of the complexity of this system was ignored in our relatively short simulations including only the mutated side-chain and its local environment.

ASSESSING THE APPLICABILITY OF THE JARZYNSKI IDENTITY TO CALCULATE TCR-pMHC BINDING FREE ENERGY PROFILES

In classical thermodynamics, the dissipative work $W_{A \rightarrow B}$ needed to bring a system from state A to state B is greater than the free energy difference ΔG_{AB} between the two states, with equality only in adiabatic conditions. Conversely, a recent result in non-equilibrium statistical mechanics, the Jarzynski identity (JI) states that (66, 67)

$$e^{-\beta\Delta G_{AB}} = \langle e^{-\beta W_{A \rightarrow B}} \rangle_0.$$

Here, $\beta = (k_B T)^{-1}$, with T the temperature and k_B the Boltzmann constant. The average $\langle \cdot \rangle_0$ is taken over canonically distributed initial conditions in state A. The JI was proven to hold in the case of thermostated molecular dynamics (66–69). The JI was applied with some success to simulations of small molecular systems (68–71). Given the biological importance of the TCR-pMHC system, we were compelled to determine if the JI could be employed to calculate protein–protein binding free energy profiles from SMD trajectories of large protein–protein complexes

(72). The free energy profile, or potential of mean force (PMF), is very relevant because in addition to the total binding free energy it provides estimates of k_{on} and k_{off} from the free energy barrier height.

In SMD, an external potential of the form $u(\xi(r), t)$ is used to steer the system from position A at time 0 to B at time t along the reaction coordinate ξ . This potential can be a simple harmonic potential or take a more complex form such as in the individual pulling scheme (72) presented in Section “Investigating TCR-pMHC Interactions Using Steered MD Simulations.” If we are interested in the PMF along ξ , the JI can be written as (73),

$$e^{-\beta G(\xi)} \alpha \langle \delta[\xi(r) - \xi] e^{-\beta W(t)} \rangle_0.$$

Importantly, $W(t)$ is the work accumulated by the perturbed system (including u), defined as (74),

$$W(t) = \int_0^t dt \frac{\partial u}{\partial t} (\xi(r), t).$$

For each of the Tax-A6 and P6A-A6 complexes, we performed 150 trajectories starting from independent conformation of the bound complex. The TCR and pMHC centers of mass separation was increased by 2 nm over 4 ns. The resulting work profiles are shown on Figure 9A and the distribution of final work values is shown on Figure 9B. As expected if the system is not too far from equilibrium (71, 75), the distribution (in its central part) close to a Gaussian. To obtain the PMF, the work profiles $W(t)$ collected from multiple SMD simulations have to be postprocessed with three distinct operations:

1. Reduce from the biased system to the physical system (unbias);
2. Average over all $\xi(r)$ visited during the evolution to recover $G(\xi)$;
3. Estimate the exponential average $\langle e^{-\beta W} \rangle_0$.

Operations 1 and 2 are performed within a modified weighted histogram scheme (73) adapted to the case of the individual potentials (72). Operation 3 can be more problematic. If we apply direct exponential averaging, the estimated PMF is very close to the lowest measured work profile at a given ξ . In the case where the true free energy value lies in the unsampled lower tail of the work distribution, direct averaging will result in a large overestimation. Indeed, for the Tax-A6 complex, the work profiles of Figure 9A would result in a dissociation free energy around 250 kJ/mol, compared to the experimental value of 32.2 kJ/mol (18).

Instead of direct averaging, if we assume that the work distribution is Gaussian, we can estimate $G(\xi)$ with a second-order cumulant expansion (71, 75),

$$G(\xi) = \bar{W}(\xi) - \frac{\beta}{2} \sigma^2(\xi).$$

Here, $\bar{W}(\xi)$ is the mean and σ the standard deviation of the work values at ξ obtained by applying operations 1 and 2 above to each trajectory independently. The resulting PMFs are shown on Figure 9C. The final dissociation free energies are -110 and

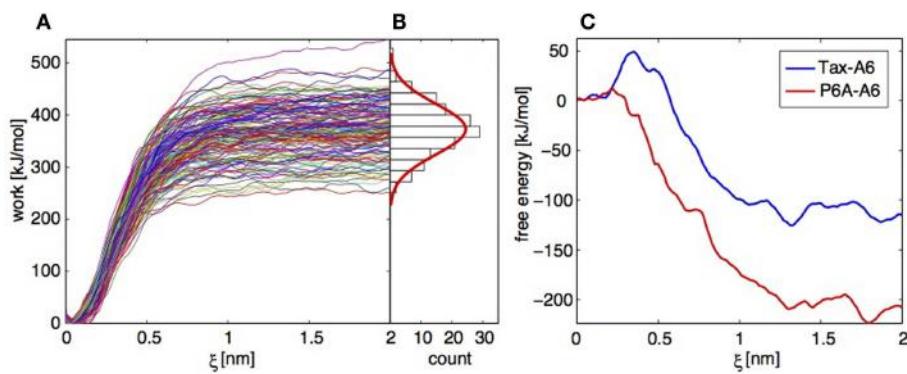


FIGURE 9 | Application of the Jarzynski identity to TCR-pMHC dissociation. (A) Work profiles collected in 152 independent trajectories for the Tax-P6A complex. (B) Histogram of the work distribution at

$\xi = 2$ nm, with a fitted Gaussian distribution. (C) Free energy profiles calculated using the Jarzynski identity and the cumulant expansion method.

−210 kJ/mol for Tax-A6 and P6A-A6, respectively, which is a severe underestimation of the experimental values of 32.5 and 22.5 kJ/mol. We note that the calculated values should be corrected for translational and rotational entropic contributions to be compared to standard state free energy measurements, but this does not improve the results.

Our results show that direct averaging produces a strong overestimation of $G(\xi)$, which could be fixed only by sampling an extremely large number of trajectories to get enough low work values. Conversely, the cumulant expansion produces a strong underestimation of $G(\xi)$, which shows that the real work distribution has a shorter lower tail than the Gaussian distribution. Repeating the calculations with datasets of similar sizes and slightly different conditions showed the reproducibility of these findings.

Overall, this example illustrates the severe sampling difficulties that hamper the application of the JI to systems with sizeable dissociation. These difficulties have been evidenced by other researchers in systematic convergence studies of the JI method (76–79).

ESTIMATION OF RESIDUE CONTRIBUTION TO THE BINDING FREE ENERGY OF THE TCR/pMHC ASSOCIATION USING MM-PB(GB)SA

In the Molecular Mechanics – Poisson Boltzmann Surface Area (MM-PBSA), or its variant the MM-GBSA, the binding free energy, ΔG_{bind} , is written as the sum of the gas phase contribution, $\Delta H_{\text{bind}}^{\text{gas}}$, the desolvation free energy of the system upon binding, ΔG_{desolv} , and an entropic contribution, $-T\Delta S$ (80):

$$\Delta G_{\text{bind}} = \Delta H_{\text{bind}}^{\text{gas}} + \Delta G_{\text{desolv}} - T\Delta S.$$

The term $\Delta H_{\text{bind}}^{\text{gas}}$ is constituted by the van der Waals (ΔH_{vdw}) and electrostatic (ΔH_{elec}) interaction energies between the two partners in the complex, and their conformational energy change upon binding, ΔH_{intra} :

$$\Delta H_{\text{bind}}^{\text{gas}} = \Delta H_{\text{elec}} + \Delta G_{\text{vdW}} + \Delta H_{\text{intra}}.$$

ΔG_{desolv} is the difference between the solvation free energy, ΔG_{solv} , of the complex and that of the isolated parts. ΔG_{solv}

is divided into the electrostatic, $\Delta G_{\text{elec,solv}}$, and the non-polar, $\Delta G_{\text{np,solv}}$, contributions,

$$\Delta G_{\text{solv}} = \Delta G_{\text{elec,solv}} + \Delta G_{\text{np,solv}}.$$

$\Delta G_{\text{elec,solv}}$ is calculated by solving the exact Poisson or the Poisson–Boltzmann (PB) equation (81, 82) in MM-PBSA, and the much faster but approximate Generalized Born (GB) model (83) in MM-GBSA (84). The term $\Delta G_{\text{np,solv}}$, which can be considered as the sum of a cavity term and a solute–solvent van der Waals term, is assumed to be proportional to the solvent accessible surface area (SASA) (85),

$$\Delta G_{\text{np,solv}} = \gamma \text{SASA} + b.$$

The entropy term can be decomposed into translational, S_{trans} , rotational, S_{rot} , and vibrational S_{vib} , contributions. These terms are calculated using standard equations of statistical mechanics (54, 86).

In the standard MM-PB(GB)SA protocol, all these energy terms are typically averaged over several hundreds of frames extracted from multi-nanosecond MD simulation trajectories, generally performed in explicit solvent. Those explicit water molecules are removed prior to energy calculations. In principle, three trajectories should be performed, one for the complex and one for each of the isolated partners, and the energy terms calculated using the corresponding simulation. However, a frequent, less computationally demanding, approximation consists in performing only one MD simulation for the complex (54). The terms relative to one isolated partner are then calculated after removing the atoms of the other partner in the frames extracted from the MD simulation of the complex. In this variant, the conformational energy change upon association is therefore neglected ($\Delta H_{\text{intra}} = 0$), and the influence of conformational changes on the other energy terms are not captured.

MM-PB(GB)SA has been used successfully to identify the hot-spots of protein–protein association and to determine the effect of mutations on association processes (84, 87–90). Two approaches can be considered. The computational alanine scanning (CAS)

approach (91) is directly comparable to its experimental counterpart. In this approach, ΔG_{bind} values are calculated for the wild-type system, as well as for several mutants in which one residue has been replaced by an alanine. The difference in ΔG_{bind} between the wild-type system and the mutants can be used to estimate the role played by each residue in the association process. Alternatively, it is possible to perform a binding free energy decomposition (BFED) for the wild-type system (84). In this approach, the contributions to ΔG_{bind} arising from groups of atoms, typically single residues, or even backbone or side-chain, are estimated from the wild-type system by performing a pairwise decomposition of the MM-PB(GB)SA terms (88, 90). BFED, which requires only one binding free energy calculation, is faster than CAS. In addition, BFED provides the possibility to study contributions from non-mutable groups of atoms, such as the backbone. However, contrarily to CAS, BFED results cannot be compared directly to an experimental alanine scanning.

To assess the ability of the MM-GBSA approach to identify quantitatively the hot-spots residues for the TCR/pMHC association, we performed a study of the 2C TCR/SIYR/H-2Kb system using both the CAS and BFED methods (90). This system was chosen because both the experimental 3D-structure and the results of an experimental alanine scanning were available at that time (92). A very good correlation was found between the residue contributions to ΔG_{bind} from both methods, with a correlation coefficient of 0.94, highlighting the interest in the faster BFED approach. A correlation coefficient of $R = 0.67$ was found between experimentally determined activity differences for alanine mutants and the calculated binding free energy changes upon mutation.

Our results also showed that BFED provided a more detailed and reliable description of the interactions between the TCR and pMHC molecules when including entropic terms. When the entropy was taken into account, the correlation coefficient was increased to 0.72. It was noticeable that the correlation obtained when neglecting the entropy term, which is very computationally expensive to calculate, was sufficient to quantify and rank the importance of the residues for TCR/pMHC association. Altogether, these pioneering results suggested that the BFED for the TCR-pMHC system provides a detailed and reliable enough description of the interactions between the molecules to be used as an *in silico* investigation tool in TCR protein-engineering.

COMPUTER-AIDED PROTEIN ENGINEERING

BACKGROUND

Patients with diverse types of cancer develop tumor-specific CD4⁺ and CD8⁺ T cell responses. Although these responses are typically unable to contain solid tumor growth or hematological malignancies, clinical studies have revealed the adoptive transfer of *ex vivo* expanded autologous tumor-specific T cells to be a promising immunotherapeutic approach to cancer treatment (13). A limitation, however, is that TCRs which bind tumor associated/self antigen are often of relatively low affinity. TCRs generally bind pMHC in the range of $K_D = 1-100 \mu\text{M}$ (93). However, as a result of the thymic negative selection whereby T cells with high-affinity TCRs for "self" antigens are eliminated to prevent autoimmunity, TCRs specific for "self" tumor associated antigens tend to be weaker-binders compared to TCRs specific for "non-self" peptides (94).

Thus, the development of tumor-targeting TCRs to endow them with optimal binding properties, both in terms of fine-specificity against the targeted pMHC, and kinetic/affinity parameters that confer maximum cellular responsiveness, is a field of intense research toward cancer immunotherapy development (95).

The relative importance of the roles played by the TCR/pMHC binding affinity (K_D), and individual kinetic parameters (k_{on} and k_{off}), on T cell activation, has been intensively studied recently (11). The emerging consensus hypothesizes the existence of a TCR/pMHC "dwell-time" (96) enabling the sequential interaction of TCRs with a rare antigenic pMHC complex – a process known as "serial triggering" – and conferring an optimal T cell activation (97). It has also been demonstrated that both k_{on} and k_{off} define the "effective half-life" of a TCR/pMHC interaction (98). Thus, K_D , k_{on} , and k_{off} do all contribute to T cell activation and their optimizations should be addressed concomitantly by TCR engineering techniques for cancer immunotherapy.

As introduced earlier, TCRs contact pMHC antigens via the six CDRs (Figure 2), with CDR3 α and CDR3 β mainly bound over the peptide, and CDR1 and CDR2, α and β , making more contacts with MHC. It could thus be expected that mutations of CDR3s would be more likely to maintain peptide specificity than mutants of CDR1s and CDR2s. Indeed, several mutagenesis studies produced high-affinity TCRs bearing mutations on CDR3 α and CDR3 β that were found to be peptide-specific (99–102). However, other studies also discovered high-affinity mutants in CDR1 and CDR2 retaining peptide specificity despite the close proximity of the mutated region to MHC residues (99, 103). This indicates that all six CDRs can serve as a focus for mutagenesis to generate higher-affinity TCRs, while still potentially retaining substantial peptide specificity (2, 93).

Several efforts have been performed to optimize TCRs (93), which mainly consist in experimental yeast (100, 101, 104–106), phage (18, 102, 103, 107), and mammalian cell (108, 109) display techniques. These approaches were able to increase the affinity of the TCR by a factor of 100–10⁶, leading to K_D as low as 26 pM (102). Although very efficient to increase the K_D , these techniques lead to TCRs bearing multiple mutations, without providing a straightforward control of the effect of each one. Such TCRs are prone to alloreactivity due to peptide-independent binding of MHCs (95, 110).

Detailed control of the effect of each mutation at the atomic level can be provided by *in silico* rational protein-engineering techniques (111–115). Recently, Haidar et al. (116) engineered the human A6 TCR for enhanced affinity toward the Tax peptide/HLA-A2 MHC complex. Rapidly, the authors created a set of 219 fitted scoring functions, aiming to reproduce the binding affinity change upon 648 mutations of the ovomucoid turkey inhibitor molecule, using energy and statistically derived potential terms. Each function was then tested against the affinity changes of a first set of 11 A6 TCR mutants, and evaluated by correlation. The function reproducing the best the affinity changes on A6 TCR (named ZAFFI score) was retained to suggest new mutations. Due to the significant number of non-binding mutations generated using only the ZAFFI score, the authors further developed the ZAFFI filter function. The latter, trained by a Monte Carlo method on the 36 first A6 TCR point mutations, was employed to filter out mutations

with potentially bad electrostatic contacts. In total 59 mutants were tested. Twelve were found to be better binders than the wild-type TCR, as measured experimentally by Surface Plasmon resonance (SPR). It must be noted that some non-binders were generated on purpose to help training the ZAFFI score and filter. All mutations found in this study to increase the binding were hydrophobic substitutions that enhanced the interface complementarity. No mutation introducing new significant electrostatic contacts, and thus potentially increasing the selectivity of the TCR/pMHC binding, was found positive. Despite the use of fitted scoring function and filter that hampers its straightforward translation to non-TCR systems, this interesting study illustrated the feasibility of a rational *in silico* approach to design TCR with higher affinities. It opened the road to new approaches, with physically sound and non-fitted universal free energy estimates, straightforward transferability, and high success rates.

METHODOLOGY AND RESULTS ON BC1 TCR

Encouraged by the results of our BFED method in reproducing the outcome of a alanine scanning experiment on the TCR/SIYR/H-2Kb system (90), we decided to develop a new structure-based approach, based on MM-GBSA free energy calculations, to rationally design new TCR sequences. Our approach can be divided into several steps. First, the importance of each wild-type TCR residue for the TCR/pMHC association is estimated using a MM-GBSA BFED. Then, based on the TCR-pMHC structure and the residue contributions to the binding, mutations are designed for the residues showing the most promising opportunities of enhancement for the interaction with the pMHC. These putative sequence modifications are finally selected for experimental testing based on the estimated binding free energy gain, $\Delta\Delta G_{\text{bind}}$. The latter was obtained by calculating the contribution of each residue to the binding free energy change upon a given mutation, $\Delta\Delta G_{\text{bind}}^{\text{res}}$, as the difference between the residue contribution for the mutated complex, $\Delta G_{\text{bind}}^{\text{res,mut}}$, and that for the wild-type complex, $\Delta G_{\text{bind}}^{\text{res}}$:

$$\Delta\Delta G_{\text{bind}}^{\text{res}} = \Delta G_{\text{bind}}^{\text{res,mut}} - \Delta G_{\text{bind}}^{\text{res}}.$$

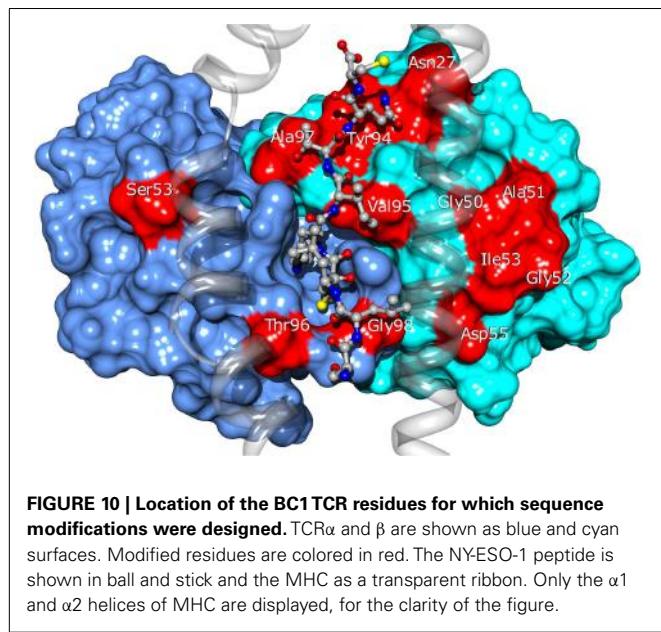
$\Delta G_{\text{bind}}^{\text{res}}$ and $\Delta G_{\text{bind}}^{\text{res,mut}}$ values were calculated from MD simulations of the wild-type and the corresponding mutated TCR-pMHC, respectively. The binding free energy difference upon a given mutation, $\Delta\Delta G_{\text{bind}}$, was finally obtained by summing the $\Delta\Delta G_{\text{bind}}^{\text{res}}$ values over the mutated residue and all the residues in contact with it. This local summation was preferred to a summation over all residues of the TCR-pMHC complex since it suppresses the errors arising from residues far from the site of the sequence modification, and making no contribution to the mutation effect.

We have applied our computer-aided protein-engineering approach to the BC1 TCR (26, 117). This TCR was discovered from a long-surviving melanoma patient (LAU #155) with a naturally occurring CD8⁺ T cell response against the immunodominant cancer-testis epitope NY-ESO-1_{157–165} (SLLMWITQC), presented by the commonly expressed MHC class I allele HLA-A*0201. The expression profile of NY-ESO-1 makes it an attractive target for cancer immunotherapy. It is indeed expressed by several

solid tumors, including melanoma, as well as hematological malignancies (myelomas, lymphomas, and leukemias), while in normal tissue its expression is limited to the testis cells (118–122). Interestingly, recent studies have shown that an immune response against NY-ESO-1 can convey an important clinical benefit for the patient. Seventy-seven percent of patients treated with the CTLA-4 blocking antibody ipilimumab showed favorable clinical outcome if they had a detectable CD8⁺ T cell responses against NY-ESO-1, compared to only 14% otherwise (123). Our *in silico* approach was facilitated by the existence of the crystal structure of the 1G4 TCR bound to NY-ESO-1/HLA-A*201 (124), PDB ID 2BNR, available in the protein databank (125). The sequence of the latter differs only from that of the BC1 TCR by four residues: Thr95 α , Ser96 α , Asn97 β , and Thr98 β of the 1G4 TCR, are replaced by Gln95 α , Thr96 α , Ala97 β , and Ala98 β in BC1 TCR, respectively. These sequence modifications were introduced in the 1G4 X-ray structure before applying our approach. Noticeably, three crystal structures of free and bound 1G4 TCR were solved after we performed our simulations (126). These affinity-enhanced TCRs contain mutations in the CDR3 loops or in both the CDR2 and CDR3 loops, which were obtained by *in vitro* directed evolution (102). They revealed that the binding mode for the high-affinity TCRs was identical to that of the wild-type TCR, with only limited changes in the mutated CDRs. A previous assessment by Zhao et al., of six phage library-derived 1G4 TCR variants demonstrated three categories of TCR specificity related to affinity; (i) super-high-affinity TCR (26 pM) which completely lacked specificity, (ii), mid-range affinity TCR (5 and 85 nM) that were specific only in the absence of CD8 co-engagement, and, (iii), intermediate-range affinity TCR (0.4 and 4 μ M) that maintained specificity (107). By taking a rational computer-aided approach to TCR engineering, we were able to design a new original set of TCR variants. We enhanced the TCR/pMHC binding interactions in a “step-by-step” manner, targeting change to specific kinetic parameters, and limiting overall gain in affinity as well as potential for cross-reactivity.

Twenty four of the most promising mutations identified using this approach, spanning 11 different residues of the CDRs (see Figure 10), were introduced into the BC1 TCR sequence. The engineered proteins were produced using a mammalian cell expression system, purified and tested by titration ELISA. We found a qualitative agreement between the calculated $\Delta\Delta G_{\text{bind}}$ values and the experimental results. Thirteen (54%) of the mutations proposed by our approach showed improved affinity for the pMHC, compared to the wild-type TCR (Zoete et al., in preparation). We obtained an excellent correlation of $R = 0.85$ between the calculated $\Delta\Delta G_{\text{bind}}$ and the measured co-logarithm of the optical density measured by ELISA titration. Only three outliers were found: V β A51V, Y94N, and A51D. Their presence might be explained by conformational rearrangements upon mutation, minimally accessible during the MD simulations. This correlation illustrates the efficiency with which the $\Delta\Delta G_{\text{bind}}$ calculated with our method allows for the rational selection of TCR sequence modifications potentially increasing its affinity for pMHC.

For quantitative TCR/pMHC binding measurements some of the TCRs were refolded from bacterial inclusion bodies and analyzed by SPR. We began by producing TCR



having one amino acid change. The best single replacement TCR had 52-fold improvement in K_D (Zoete et al., in preparation). Then, we gradually increased affinity by step-wise combination of rationally selected replacements in both the β -chain and the α -chain. As predicted by modeling and assessed by SPR binding assays, we found the following progression in K_D (Table 1): WT (21.4 μM) $>$ β -G50A (4.6 μM) $>$ β -G50A + A51E (1.9 μM) $>$ β -G50A + A51E + A97L (0.91 μM) $>$ β -G50A + A51E- β A97L + α -S53W (0.14 μM) (Zoete et al., in preparation) (7). Our method thus allows for the design of TCRs with fine tuned K_D values, potentially leading to T cells with optimal activity (7). This is in contrast with experimental display approaches which tend to select the tightest-binding TCRs that can potentially surpass an “affinity threshold of specificity,” as has been reported for the 1G4 TCR (107). We found a very satisfying correlation between the calculated energies and the pK_D and k_{off} values measured by SPR (Zoete et al., in preparation).

Importantly, we found that our approach was able to predict successfully mutations toward both non-polar and polar residues, contrary to previous studies where only designed mutations toward non-polar residues were successful in increasing the experimental affinity (116). To take place, polar interactions, such as hydrogen bond or ionic interaction, require an appropriate match of chemical functionalities and precise geometrical constraints between interacting partners. Therefore, they provide an essential contribution to the directionality and the specificity of molecular recognition (127). This point is critical to the development of TCRs for immunotherapies. It is indeed essential that TCRs maintain their specificity, and do not acquire novel antigen specificities that might cause off-target toxicity upon the adoptive transfer of genetically engineered T cells to patients. Two rationally designed sequence modifications provide interesting examples regarding the detailed control provided by our approach on the binding process. Mutation β -A51E introduces an ionic interaction between the new glutamate side-chain and MHC Arg75

Table 1 | Binding affinity of rationally designed BC1 TCR variants [from Ref. (7)] measured by SPR.

TCR mutations	K_D (μM)
β -V49I	Near non-binding
Wild-type	21.4
β -G50A	4.62
β -A97L	2.69
β -G50A + A51E	1.91
β -G50A + A51E + A97L	0.91
α -S53W + β -G50A + A51E	0.4
α -S53W + β -G50A + A51E + A97L	0.14
β -G50A + A51E + G52Q + I53T	0.015

(Figure 11A), translating into a calculated favorable $\Delta\Delta G_{\text{bind}}$. This replacement produced a fourfold experimental improvement in K_D as measured by SPR (8). This mutation thus increases the affinity for the pMHC through better interactions with the MHC molecule, and therefore cannot be expected to increase the selectivity for the peptide antigen. Interestingly, we also designed the β -A97D variant, which introduces a new hydrogen bond between TCR and the peptide Thr7 side-chain (Figure 11B). The overall effect of this modification was somewhat unfavorable to the binding, as shown by the experimental titration ELISA and calculated $\Delta\Delta G_{\text{bind}}$ values (Zoete et al., in preparation). However, despite the decreased activity, this mutation is interesting for the putative gain in selectivity for the NY-ESO-1 system thanks to the new polar interaction taking place between the TCR and the peptide.

An experimental alanine scanning of the peptide supported this idea. The affinity of several TCR mutants (β -G50A/A51E, A97L, A97D, I53W, V95L, and Y94N) for the different mutated pMHC were measured by titration ELISA and compared to the results for the wild-type TCR. All mutated TCR showed affinities similar to the wild-type TCR for all the peptide mutants. The only exception was the β -A97D TCR mutant, which did not bind to the peptide T7A mutant contrary to the wild-type TCR. This experimental result is in good agreement with the *in silico* data showing that all those TCR mutations modified the interactions with the MHC rather than with the peptide with the exception of β -A97D which exchanges a hydrogen bond with the peptide Thr7 side-chain. The β -A97L TCR mutant, which makes non-polar contact with the side-chain of peptide Thr7 but no hydrogen bond, is less affected by the Thr7 mutation to Ala than the β -A97D TCR variant. Thus, although the β -A97D modification is somewhat unfavorable to the binding, it could be useful to introduce it in engineered TCRs targeting NY-ESO-1, in addition to other variations increasing the affinity, in order to obtain more selective TCRs.

The selectivity of TCRs designed by our approach was tested experimentally. We observed no interaction of our TCRs with any non-cognate pMHC complexes. In addition, tetramer binding studies with an extensive panel of non-cognate pMHC revealed that the TCR variants expressed at the cell-surface were also HLA-A*0201/NY-ESO-1_{157–165} –specific (7).

The binding free energy calculations used in our approach are physics-based and not reliant on *ad hoc* model fitting. We

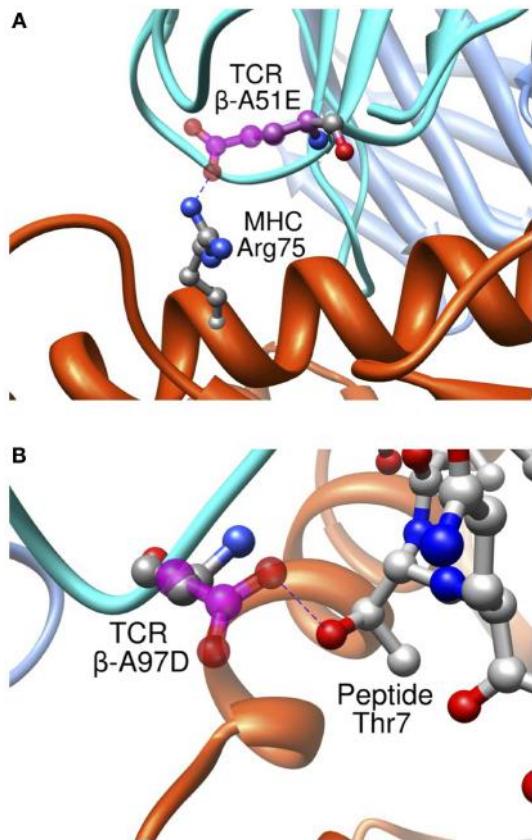


FIGURE 11 | Interaction between pMHC and the modified residues of the β -A51E (A) and β -A97D TCR variants (B). TCR wild-type residues are shown in ball and stick, colored according to the atom types. Modified residues are colored in magenta. New hydrogen bonds are shown as dotted blue lines.

can thus expect that our design strategy is highly transferable to any protein/protein interaction of known structure and biological interest.

INTERMEDIATE TCR/pMHC BINDING PARAMETERS CONFER MAXIMUM BIOLOGICAL RESPONSES

In order to efficiently screen our extensive panel of modeled BC1 TCR variants we began by establishing a mammalian cell expression system for TCR production. HEK-293 cells were PEI co-transfected with pHYK8 plasmids encoding the α - and β -chains, each under control of the CMV promoter. Following the strategy of Chang et al. an acidic-basic zipper was incorporated to facilitate heterodimeric chain pairing (128). Following 5–7 days culture in serum-free medium, the TCR variants were HIS-tag purified from the supernatants (yields were up to \sim 3 mg/L) and compared to the wild-type TCR by titration ELISA for binding plate-captured pMHC. Single and multiple amino acid replacements were assessed in the α - and β -chains. Over 60% showed enhanced pMHC binding as compared to the wild-type TCR. Further, binding of the TCR variants against an Ala replacement scan of the NY-ESO-1_{157–165} peptide (SLLMWITQC) revealed near identical patterns of recognition, suggesting a conserved

mechanism of binding. For all TCR variants, M160, W161, I162, and Q164 were critical contact residues as their replacement with Ala abolished TCR/pMHC binding (7, 8).

The TCR chains were subcloned into pGMT7 for their expression as inclusion bodies in BL21(DE3)pLys bacterial cells and subsequent TCR refolding by dialysis (129). The binding affinity and kinetics for a panel of TCRs having various combinations of amino acid replacements in CDR2- β , CDR3- β , and CDR2- α , were measured by SPR using the BIAcore 3000. Most natural TCRs bind pMHC with weak affinity, in the range of 1–100 μ M, as a result of slow association (10^3 – 10^4 $M^{-1}s^{-1}$), and fast rates of dissociation (typically a half-life of seconds at 37°C) (1, 10, 130), reflecting the need for T lymphocytes to detect a virtually limitless repertoire of foreign epitopes while avoiding autoreactivity, and the fact that they do not undergo somatic hypermutation as do antibodies. The eight TCRs chosen amongst our panel incrementally increased in affinity from 21.4 μ M for the wild-type one, to extreme physiologic affinity at 15 nM (summarized in Table 1).

Lentiviral constructs were built to assess the activity of the rationally designed TCRs in transduced primary human CD8 $^{+}$ T cells and identify those able to confer maximum activity levels. A range of functions were assessed for the transduced CD8 $^{+}$ T cells including Ca^{2+} flux, intracellular signaling, cell-surface TCR clustering, target-cell killing, and cytokine/chemokine release. All activity levels for affinity-enhanced TCRs uniformly increased from that of the wild-type TCR, reaching a peak for TCRs within the upper range of natural affinity, 1–5 μ M. Beyond this affinity, in the supraphysiological range, however, activity levels began to drop, both in the presence and absence of CD8 co-receptor engagement, usually reaching a minimum for the extreme supraphysiological affinity TCR (1, 7, 8, 10, 130). However, under experimental conditions in which the T cells were exposed to target-cells pulsed with high concentration of NY-ESO-1_{157–165} peptide this attenuation in activity for the supraphysiological affinity TCR was no longer observed. This phenomenon is illustrated in Figure 12. Importantly, no non-specific reactivity was observed for any of the rationally designed TCRs (7, 8).

Over the years two main models of T cell activation have emerged [reviewed in Stone et al. (11)]. For the “affinity model” (101, 131) the total number of TCRs bound to pMHCs at equilibrium is thought to regulate T cell activity levels. The “half-life model” proposes that the TCR must stay bound with sufficiently strength/duration for productive signaling and also enable the serial engagement of the “rare” antigenic pMHC complex by adjacent TCR for the amplification of signal (i.e., the TCRs must have an optimal dwell-time) (96, 97, 132–134). Although the half-life of a TCR/pMHC interaction has traditionally been calculated from k_{off} values ($t_{1/2} = \ln 2/k_{off}$), recent work by Aleksic et al. has elegantly demonstrated that for TCRs having faster association rates there can be rapid TCR/pMHC re-engagement rather than a lateral diffusion of TCRs in the cell membrane to prolong the effective half-life of the TCR/pMHC interaction. Thus, both k_{off} and k_{on} can contribute to TCR/pMHC dwell or confinement time (135). Overall our findings correspond to the “half-life” model of T cell activation. Presumably the supraphysiological affinity TCRs are limiting the serial engagement but at high peptide concentrations this is not an issue as the TCR can find more pMHC molecules

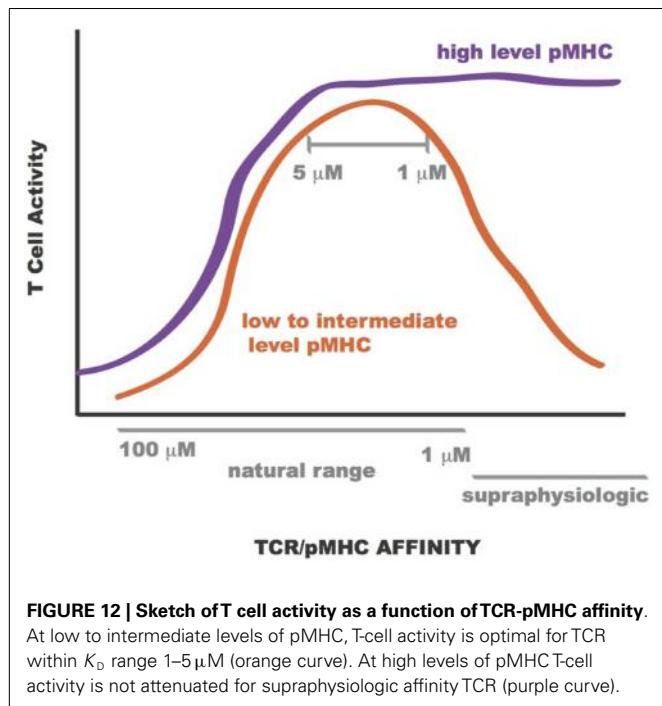


FIGURE 12 | Sketch of T cell activity as a function of TCR-pMHC affinity.

At low to intermediate levels of pMHC, T-cell activity is optimal for TCR within K_D range 1–5 μM (orange curve). At high levels of pMHC T-cell activity is not attenuated for supraphysiologic affinity TCR (purple curve).

on the target-cell-surface. This is demonstrated in **Figure 13**. Our work also demonstrated the value of a modeling approach because TCRs with particular binding parameters, i.e., falling within an optimal affinity range, either through faster on-rates or slower off-rates, can be developed to enable maximum T cell activity levels.

CONCLUSION

We found that the synergy between *in silico* design, and *in vitro* testing using both soluble molecules and transfected cells is key to the design of improved TCRs to be used in adoptive cell transfer therapies. Using an innovative structural approach based on recent *in silico* techniques, we have developed a method to rationally optimize the surface of the TCR to increase its affinity for pMHC. As opposed to library screening, we propose a step-wise, incremental optimization of the TCR. This allows the preservation of the favorable initial properties of the TCR through the various cycles. Our optimization method can selectively target contacts involving the MHC or the peptide, thus maintaining a good balance between overall affinity and specificity. In addition, modifying only a few amino acids reduces the risk of obtaining undesired cross-recognition or raising anti-TCR antibodies *in vivo*. This rational optimization approach is therefore very promising to the design of TCRs for adoptive T cell immunotherapy clinical trials.

The application of our TCR engineering approach to the tumor-targeting BC1 TCR targeted to the A2/NY-ESO-1_{157–165} antigen identified several original single mutations of the CDR loops conferring increased experimental affinity for pMHC compared to the wild-type TCR. T cells expressing some of the affinity-enhanced TCR showed better overall functionality, including improved killing of both peptide-loaded T2 cells and melanoma

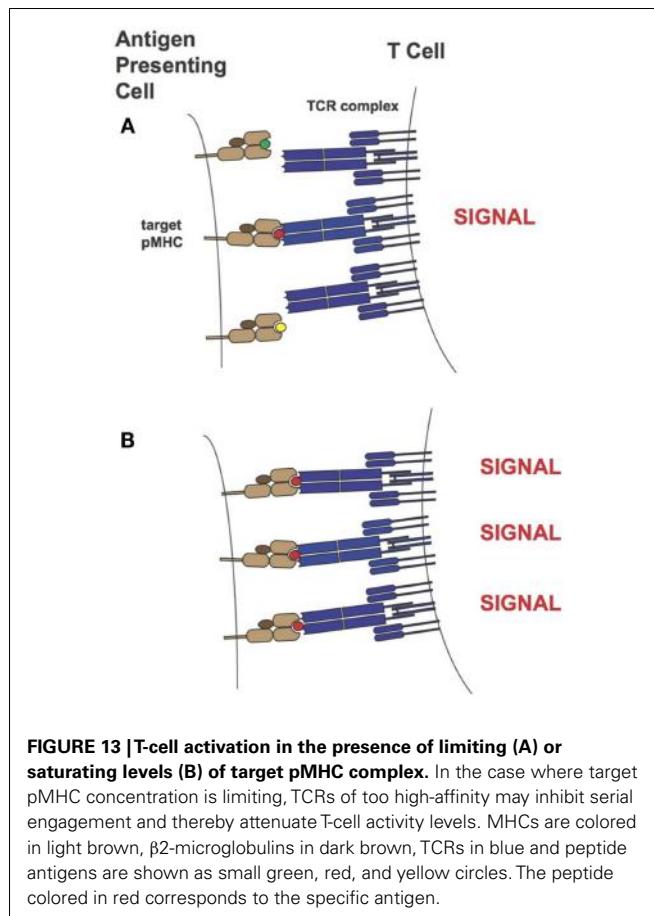


FIGURE 13 | T-cell activation in the presence of limiting (A) or saturating levels (B) of target pMHC complex. In the case where target pMHC concentration is limiting, TCRs of too high-affinity may inhibit serial engagement and thereby attenuate T-cell activity levels. MHCs are colored in light brown, $\beta 2$ -microglobulins in dark brown, TCRs in blue and peptide antigens are shown as small green, red, and yellow circles. The peptide colored in red corresponds to the specific antigen.

tumor cell lines, higher proliferative capacity, and increased levels of cytokine/chemokine secretion, as compared to wild-type TCR expressing T cells. For all functional assays, we observed a gain in CD8⁺ T cell activity level with increase in affinity, with a peak at an affinity of ~ 1 –5 μM . Beyond this affinity we observed a progressive decrease in activity levels. We are currently testing the relative activity of the different TCRs *in vivo* in a mouse model. The successful candidates are planned to enter a phase I clinical trial program for stage IV melanoma. As the methods presented here are general and transferable to any TCR-pMHC complex, other cancer types will follow shortly.

In parallel to direct applications of the existing approach, we will take advantage of the methodological work presented above to improve our *in silico* TCR optimization method. The development of homology-based approaches to model the 3D-structure of TCR-pMHC complexes potentially extends the use of our method to TCR repertoires for which no X-ray structure is available. The detailed picture of the TCR/pMHC interaction emerging from the MD simulations showed the presence of single water molecules trapped at the interface (20). Including these interfacial waters in the BFED scheme would improve accuracy in some cases. Close residues at protein–protein interfaces can display collaborative effects that result in the non-additivity of their contributions to the binding free energy, which will have to be taken into account in the next generation of TCR optimization

methods. Noticeably, because the binding free energy decomposition method used is physics-based, without any *ad hoc* parameters, our *in silico* techniques are straightforwardly transferable to other types of macromolecular protein complexes.

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Increased peptide contacts govern high affinity binding of a modified TCR whilst maintaining a native pMHC docking mode

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INTRODUCTION

CD8⁺ $\alpha\beta$ T cells recognize mainly intracellularly expressed antigens through an interaction mediated by the cell surface expressed T cell receptor (TCR). Intracellular proteins, processed by the proteasome into short peptides (generally 8–13 amino acids in length), are presented to CD8⁺ T cells on the surface of almost all nucleated human cells by class I major histocompatibility complex proteins (pMHC_I). TCR recognition of pMHC_I initiates CD8⁺ T cell activation and the adaptive immune response. The ability of CD8⁺ T cells to scrutinize the intracellular environment provides an important mechanism to target aberrant disease epitopes that would be otherwise hidden from the immune system. Thus, CD8⁺ T cells play an important role during viral infections (Miles et al.,

Natural T cell receptors (TCRs) generally bind to their cognate pMHC molecules with weak affinity and fast kinetics, limiting their use as therapeutic agents. Using phage display, we have engineered a high affinity version of the A6 wild-type TCR (A6wt), specific for the human leukocyte antigen (HLA-A*0201) complexed with human T cell lymphotropic virus type 1_{11–19} peptide (A2-Tax). Mutations in just 4 residues in the CDR3 β loop region of the A6wt TCR were selected that improved binding to A2-Tax by nearly 1000-fold. Biophysical measurements of this mutant TCR (A6c134) demonstrated that the enhanced binding was derived through favorable enthalpy and a slower off-rate. The structure of the free A6c134 TCR and the A6c134/A2-Tax complex revealed a native binding mode, similar to the A6wt/A2-Tax complex. However, concordant with the more favorable binding enthalpy, the A6c134TCR made increased contacts with the Tax peptide compared with the A6wt/A2-Tax complex, demonstrating a peptide-focused mechanism for the enhanced affinity that directly involved the mutated residues in the A6c134TCR CDR3 β loop. This peptide-focused enhanced TCR binding may represent an important approach for developing antigen specific high affinity TCR reagents for use in T cell based therapies.

Keywords: human T leukocyte virus type 1, crystal structure, peptide-major histocompatibility complex, surface plasmon resonance, T cell, T cell receptor, A6TCR, high affinity TCR

2010), which typically elicit strong CD8⁺ T cell responses, many of which have been well-characterized, including those to HTLV-1 (Bieganowska et al., 1999; Vine et al., 2004), although some viruses can escape CD8⁺ T cell mediated clearance (Klenerman and Zinkernagel, 1998; Overbaugh and Bangham, 2001). Cancers too, as a result of their malignant transformation, have altered protein expression causing the presentation of tumor-associated peptide antigens (TAPAs) (Renkvist et al., 2001; Cole et al., 2009). However, although these TAPAs can elicit a host CD8⁺ T cell response, this is often insufficient to cause tumor rejection (Blohm et al., 2002; Parkhurst et al., 2004). CD8⁺ T cell responses are also integral to the initiation and progression of many autoimmune diseases, possibly through the unwanted recognition of self-peptide antigens (Bulek et al., 2012). The TCR/pMHC interaction, which governs CD8⁺ T cell responses, is therefore an attractive therapeutic target in many varied diseases, particularly

Abbreviations: pMHC, peptide-major histocompatibility complex; RU, response unit; SPR, surface plasmon resonance.

in cases where a disease-associated peptide antigen “target” has been unambiguously established.

However, unlike antibodies that can undergo somatic hypermutation and bind with high affinity ($K_D = \text{nM-pM}$) and long half-lives (typically hours), TCRs are selected in the thymus to bind with weak affinity ($K_D = 100 \text{ nM-270 } \mu\text{M}$) and short half-lives (typically seconds) (Cole et al., 2007; Bridgeman et al., 2012). Why TCRs are selected to bind within this weak affinity range is not fully understood, but may represent a balance between self-tolerance and a requirement for T cell cross-reactivity (Mason, 1998; Sewell, 2012; Wooldridge et al., 2012). However, the weak affinity and short half-lives of natural TCR/pMHC interactions imposes limitations on the use of TCRs for targeting cell surface expressed pMHCs, primarily because the short half-life is not adequate for the delivery of therapeutic interventions to target antigens. In order to overcome this limitation, we have recently implemented phage display to generate TCRs with an enhanced affinity for cognate antigen (Li et al., 2005; Dunn et al., 2006; Sami et al., 2007; Varela-Rohena et al., 2008; Liddy et al., 2012) that can be used to target cell surface expressed MHC molecules displaying any disease epitope of interest. Using this technique, we have generated several high affinity TCRs derived from the $\alpha\beta$ TCR A6 (A6wt) that are specific for the HTLV-1_{11–19} peptide, presented by HLA-A*0201 (A2-Tax) (Li et al., 2005). High affinity TCRs generated using this method can be used for two distinct type of therapies. The first involves genetically reprogramming host T cells so that they express a modified TCR (adoptive therapy) (Morgan et al., 2006; Varela-Rohena et al., 2008). The second involves using a soluble high affinity TCR to deliver a therapeutic payload intravenously (soluble therapy) (Liddy et al., 2012). The optimal TCR affinity for these two types of therapy, in terms for retaining specificity and reactivity, will probably be distinct and will likely be lower for adoptive therapy because of the polyvalent nature of T cell antigen recognition at the cell surface versus a soluble therapy in which the TCR reagent will likely require a longer half-life to effectively target intended disease markers. Understanding how these reagents function at the molecular level is key to determining these parameters and optimizing these types of T cell directed therapies.

Previous structural investigations of TCR/pMHC interactions have shown that TCRs bind with a relatively conserved diagonal orientation (Garboczi et al., 1996; Rudolph et al., 2006), with the α -chain focused toward the N-terminus of the peptide and the β -chain toward the C-terminus. Although exceptions occur (Burrows et al., 2010), this orientation enables the TCR complementarity determining region (CDR)2 loops to be positioned over mainly the MHC surface, the CDR3 loops to be positioned primarily over the peptide and the CDR1 loops positioned in between. This binding mode, and the low native TCR binding affinity, is presumably important for maintaining T cell specificity and antigen sensitivity, and is possibly important in T cell signaling (Adams et al., 2011). However, we have previously shown that just a small number of mutations in the TCR CDR loops can improve the low natural TCR/pMHC binding affinity dramatically (Li et al., 2005; Dunn et al., 2006; Hawse et al., 2012). Thus, it is likely that high affinity TCRs are generated in the thymus, but they are not selected for release into the periphery. In order to better understand the

consequences of high affinity TCR interactions, and to provide further insight into: (1) how high affinity TCR binding is mediated and (2) what effects this binding is likely to have on TCR specificity, we solved the atomic structures of a high affinity TCR, A6c134, free, and in complex with A2-Tax. By comparing this structure with the previously published structures for the A6wt TCR (Garboczi et al., 1996; Scott et al., 2011), we provide a molecular explanation for the improved binding of this high affinity TCR.

RESULTS

DECONSTRUCTION OF HIGH AFFINITY A6 TCR VARIANTS SPECIFIC FOR A2-TAX

In order to generate a high affinity version of the A6wt TCR, we implemented phage display as previously described (Li et al., 2005). This process generated a number of high affinity TCRs, including the mutant A6c134 that varied from the A6wt TCR parental sequence at only four codons, all located within the CDR3 β loop (Table 1). It has been previously determined that the A6wt TCR binds to A2-Tax with an affinity of $\sim 1\text{--}3 \mu\text{M}$ and an off-rate ($t_{1/2}$) of $\sim 7\text{--}10 \text{ s}$ (Davis-Harrison et al., 2005; Armstrong and Baker, 2007; Cole et al., 2007). In contrast, the engineered high affinity A6c134 TCR bound to A2-Tax with an affinity of 4 nM (nearly 1000 times greater than the A6wt TCR, or $\Delta\Delta G^\circ = -3.96 \text{ kcal/mol}$) and an off-rate ($t_{1/2}$) of 3900 s (>400 times longer than the A6wt TCR), as determined by surface plasmon resonance (SPR) (Table 1). A6c134 did not bind to other HLA-A2 restricted peptides that were used as negative controls in different SPR experiments, including as A2-ILAKFLHWI, A2-ELAGIGILTV, and A2-YLEPGPVTA demonstrating that A6c134 retained peptide specificity (data not shown). In order to investigate the molecular basis for how this high affinity was generated, we used reverse engineering to construct a range of A6wt-based TCRs containing different combinations of amino acids from the A6c134 CDR3 β sequence and, conversely, a range of A6c134-based TCRs containing different combinations of amino acids from the A6wt CDR3 β sequence (Table 1). All of these TCRs exhibited very similar on-rates, but showed marked differences in their off-rates (Table 1). In general, the mutations appeared to act cooperatively to enhance affinity. The mutation of the A6wt TCR from R₁₀₂ to Q₁₀₂ had only a small effect upon binding affinity and may have been selected because of the lower toxicity of the TCR to the TG1 phage host (Li et al., 2005).

A6c134 TCR BOUND TO A2-TAX USING A SIMILAR CONFORMATION TO THE A6wt TCR

In order to determine the structural basis of the high affinity binding for the A6c134 TCR, we solved the A6c134/A2-Tax complex structure to 2.74 Å. Molecular replacement was successful only in space group C121, consistent with the presence of one molecule of the complex per asymmetric unit, and the resolution was sufficiently high to show that the interface between the two molecules was well ordered and contained well defined electron density. The crystallographic R/R_{free} factors were 22 and 26%, within the accepted limits shown in the theoretically expected distribution (Tickle et al., 2000) (Table S1 in Supplementary Material). The overall buried surface area (BSA) of

Table 1 | Biophysical analysis of different combinations of “knock-in” and “knock-out” mutations (in bold type and underlined) of A6wt and A6c134TCRs binding to A2-Tax.

Mutant	CDR3 β	K_D	k_{on} (s $^{-1}$ M $^{-1}$)	k_{off} (s $^{-1}$)	$t_{1/2}$ (s)	ΔG° (kcal/mol)	$\Delta \Delta G^\circ$ (kcal/mol)
A6wt	AGGR	3.2 μ M	2.3×10^4	7.4×10^{-2}	9.3	-7.49	n/a
A6c134M	M GGGR	1.9 μ M	1.8×10^4	3.5×10^{-2}	20	-7.80	-0.31
A6c134S	<u>A</u> SGR	1.8 μ M	2.3×10^4	4.1×10^{-2}	17	-7.83	-0.34
A6c134AE	<u>A</u> SAE	9.4 nM	2.3×10^4	5.2×10^{-4}	1320	-10.94	-3.45
A6c134E	M SAE	4.4 nM	5.5×10^4	2.2×10^{-4}	3120	-11.39	-3.90
A6c134R	M SA <u>R</u>	8 nM	1.9×10^4	1.5×10^{-4}	4500	-11.04	-3.55
A6c134	M SA <u>Q</u>	4 nM	4.5×10^4	1.8×10^{-4}	3900	-11.45	-3.96

Off-rates were determined by least squares fitting of single-component exponential decay equation to the decay curve following TCR binding to A2-Tax.

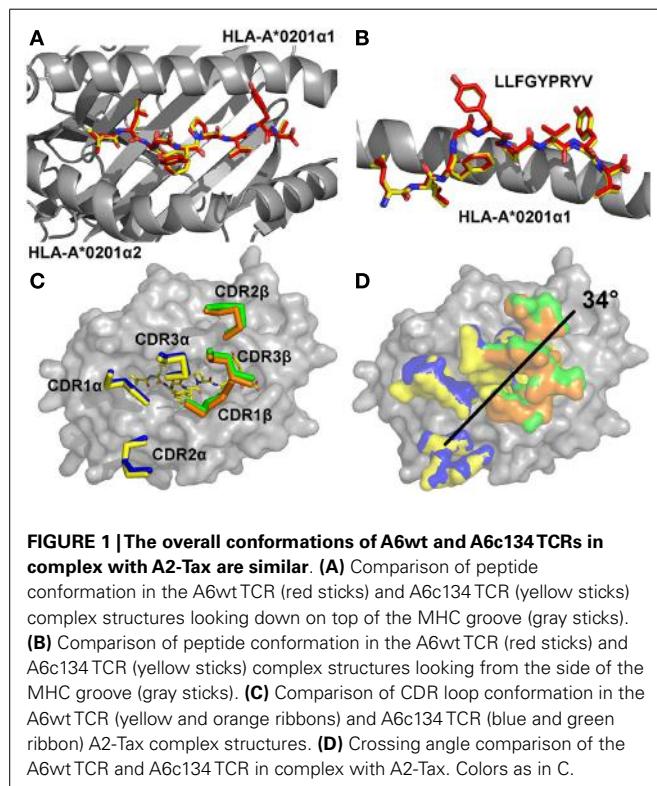
Half-lives were calculated using the equation $t_{1/2} = \ln 2/k_{off}$.

$\Delta \Delta G^\circ = \Delta G^\circ$ of high affinity A6c134 variant binding to A2-Tax $- \Delta G^\circ$ of A6wt binding to A2-Tax.

2445.4 Å (TCR/pMHC) was within the range observed for previously characterized TCR/pMHC interactions (Rudolph et al., 2006). The high affinity A6c134 TCR bound with a diagonal docking geometry to A2-Tax and showed one to one stoichiometry as previously reported of other TCR/pMHC complexes (Rudolph et al., 2006). We observed a high level of similarity between the A6wt/A2-Tax and A6c134/A2-Tax complexes, suggesting that overall conformation was unaffected by the mutations in A6c134. Importantly, the Tax peptide conformation was virtually identical in both complexes, discounting the possibility that structural changes in the peptide contributed to the high affinity observed (Figures 1A,B). Similarly, the architecture of the CDR loops was unaffected by the mutations in the A6c134 TCR (Figure 1C), and the crossing angle of both TCRs was identical at 34° (Figure 1D) and fell within the previously observed range (Rudolph et al., 2006). Thus, differences in binding affinity between the A6wt and A6c134 TCRs could not be explained by a large conformational change in geometry, consistent with observations in similar studies with other systems (Dunn et al., 2006; Sami et al., 2007; Madura et al., 2013).

THE A6c134 TCR CDR LOOPS UNDERGO LARGE CONFORMATIONAL ADJUSTMENTS DURING A2-TAX ENGAGEMENT

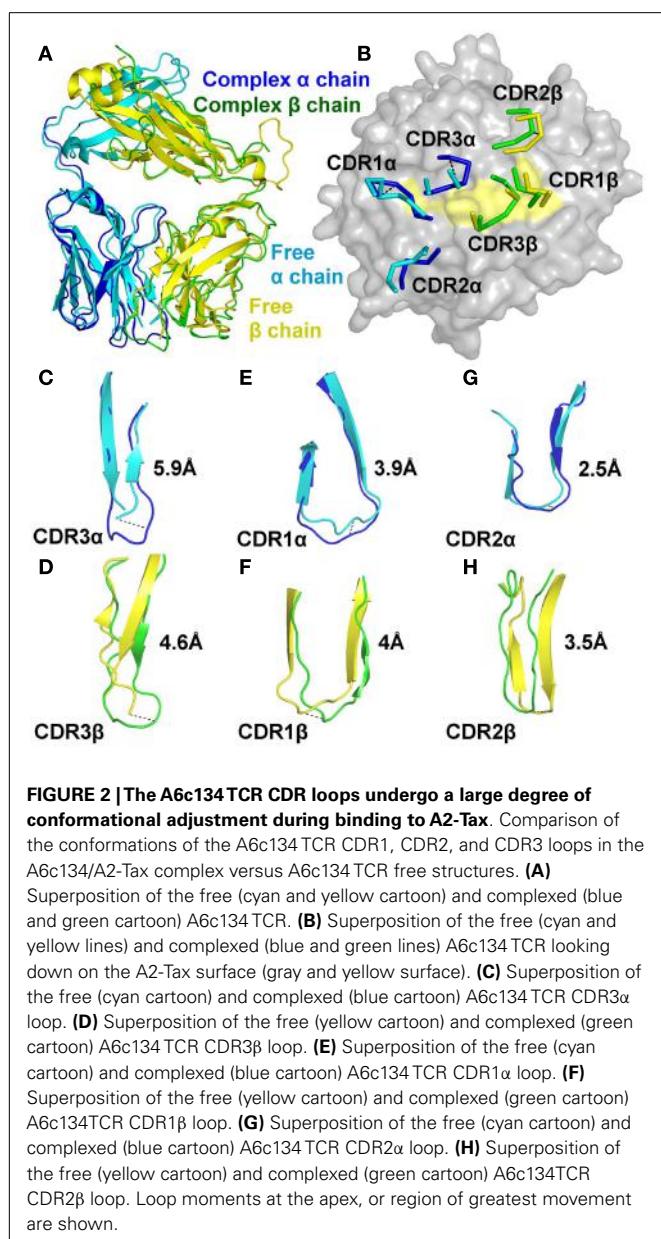
We next solved the structure of the A6c134 TCR at 2 Å (Table S1 in Supplementary Material; Figure 2). The crystallographic R/R_{free} factors were 23.4 and 29.5%, consistent with the expected ratio range (Tickle et al., 2000). In many cases, although not all, (Borbulevych et al., 2011b; Holland et al., 2012), TCR CDR loops have been shown to undergo numerous, and sometimes large, conformational changes upon pMHC binding (Armstrong et al., 2008b). Superposition of the free and the bound A6c134 TCR showed that, although the overall conformation of the TCR was virtually identical (Figure 2A), the CDR loops underwent substantial movements (Figure 2B). The CDR3 loops of both chains of the A6c134 TCR were poorly ordered and could not be fully resolved, indicating a large amount flexibility in this region of the TCR. Although the apex of the CDR3 α and CDR3 β loops that were visible underwent large hinge movements of ~5.9 and ~4.6 Å, respectively (Figures 2C,D). These changes were substantial compared to other structural studies in which the largest loop



movement observed for a human MHC I restricted TCR was 5.6 Å (Kjer-Nielsen et al., 2002; Stewart-Jones et al., 2003). Interestingly, a nearly identical observation was made for A6wt TCR (Scott et al., 2011), for which the dynamics of the CDR3 loops were shown to have a large influence on the specificity and cross-reactivity of the TCR. This occurrence with A6c134 leads to the somewhat counterintuitive conclusion that substitution of the sequence AGGR with MSAQ in CDR3 β does not greatly impact the overall dynamics of the loop, and leads us to suggest that the enhanced affinity of A6c134 was not attributable to “preorganization” of CDR3 β , i.e., the MSAQ mutation did not alter the non-bound form of the A6c134 TCR to a conformation closer to that of the bound form. This conclusion is consistent with the observation that the binding

of A6c134 to A2-Tax is characterized by a much more favorable enthalpy change along with a less favorable entropy change (as pre-organization would have resulted in a more favorable binding entropy) (Armstrong and Baker, 2007; Piepenbrink et al., 2009).

The CDR1 and CDR2 loops underwent a smaller rigid body shift and hinge movements of 2.5–4 Å (Figures 2E–H). On average, the A6c134 TCR CDR loops moved by 4.1 Å. Altogether, this degree of conformational plasticity is high compared to other TCRs (Armstrong et al., 2008b), and demonstrated that the A6c134 TCR undergoes a high degree of conformational melding during binding, as does the A6wt TCR (Borbulevych et al., 2011a). These movements enabled direct contacts between the A6c134 TCR and A2-Tax and resolved steric clashes that would have occurred between the unbound TCR and the MHC surface, as observed for the A6wt TCR (Scott et al., 2011).



THE HIGH AFFINITY BINDING OF THE A6c134 TCR WAS GOVERNED BY INCREASED PEPTIDE CONTACTS

As the overall free and bound conformations of A6wt and A6c134 were nearly identical, we decided to investigate differences in atomic interactions at the A6wt and A6c134 interfaces. The binding footprints of the A6wt and A6c134 TCRs on A2-Tax were similar, but not identical, resulting in the involvement of different peptide and MHC residues at the interface (Figures 3A,B). Although the A6c134 TCR α -chain contained no mutations, it utilized a different combination of TCR residues for binding A2-Tax compared with A6wt. As a result, the A6c134 TCR α -chain made a number of new and different contacts with A2-Tax compared with the A6wt TCR (Tables S2 and S3 in Supplementary Material; Table 2; Figures 3C,D). Thus, the mutations in the A6c134 TCR β -chain mediated a knock-on, or indirect effect resulting in a modified binding mode for the TCR α -chain.

In contrast with the A6c134 TCR α -chain, the A6c134 β -chain containing the mutated MSAQ motif formed a virtually identical footprint on A2-Tax compared to the A6wt TCR (Figure 3D). The A6c134 β -chain made a similar number of contacts with the MHC compared to the corresponding residues in the A6wt TCR (AGGR) (Figures 4A,B). This was reflected by the observation that the A6c134 TCR made only 11 more contacts with the MHC surface compared to the A6wt/A2-Tax complex. However, the A6c134 TCR made 26 extra contacts with the Tax peptide compared to the A6wt TCR, suggesting a TCR-peptide mediated mechanism for the enhanced affinity observed. This observation was also supported by the increase in shape complementarity index (Lawrence and Colman, 1993; Reinherz et al., 1999) ($SC = 0.74$) for the A6c134/A2-Tax complex compared to the A6wt/A2-Tax complex ($SC = 0.63$), and is consistent with the

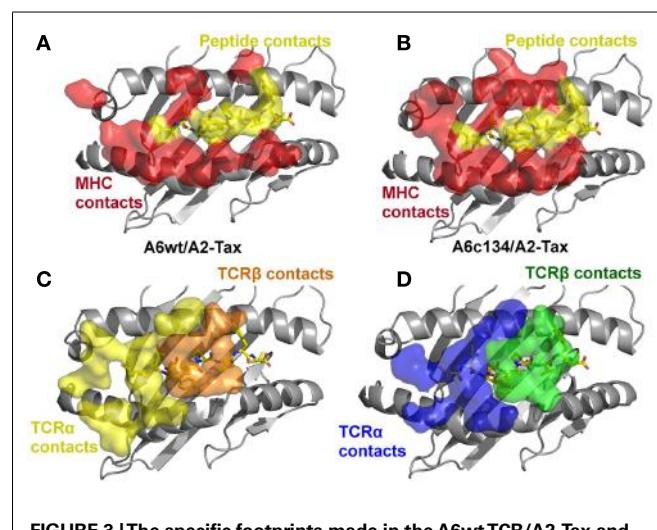


FIGURE 3 |The specific footprints made in the A6wt TCR/A2-Tax and A6c134 TCR/A2-Tax complex structures are unique. **(A)** MHC (red surface) and peptide (yellow surface) residues that are contacted by the A6wt TCR. **(B)** MHC (red surface) and peptide (yellow surface) residues that are contacted by the A6c134 TCR. **(C)** A6wt TCR residues (orange and yellow surface) that contact the pMHC. **(D)** A6c134 TCR residues (blue and green surface) that contact the pMHC. Although the overall contact footprint is similar, the A6c134 TCR makes new interactions with both the MHC surface and the peptide.

Table 2 | Direct contacts made by the A6wt TCR, or A6c134 TCR CDR3 loops.

	TCR	vdW (3.2–4 Å)	H-bonds (≤ 3.4 Å)
A6wt TCR	Thr93	0	1
	CDR3 α	Thr98	2
		Asp99	15
		Ser100	7
		Trp101	5
		Gly102	0
A6c134 TCR	Thr93	1	0
	CDR3 α	Thr98	2
		Asp99	15
		Ser100	12
		Trp101	9
		Gly102	3
A6wt TCR	Arg95	0	1
	CDR3 β	Leu98	14
		Ala99	0
		Gly100	1
		Gly101	6
		Arg102	14
A6c134 TCR	Arg95	2	1
	CDR3 β	Leu98	17
		Met99	4
		Ser100	6
		Ala101	17
		Gln102	7
		Pro103	11
		Pro103	0

Mutated residues are shown in red.

observation that the enhanced affinity of A6c134 was enthalpically driven as noted above (Armstrong and Baker, 2007; Piepenbrink et al., 2009).

Overall, the mutated MSAQ motif directly accounted for 11 of the 37 new contacts with the surface of A2-Tax (Figures 4C,D; Table 2). For instance; the A99–M99 mutation generated 4 additional van der Waals contacts (Figure 5A), G100–S100 generated an additional 5 additional van der Waals contacts (Figure 5B) and G101–A101 generated 10 additional contacts (Figure 5C), with A2-Tax. Interestingly, the R102–Q102 mutation resulted in the loss of seven van der Waals contacts and one hydrogen bond (Figure 5D). However, the overall affinity was stronger for A6c134 (MSAQ) compared to A6c134R (MSAR) suggesting that the R102–Q102 mutation contributed indirectly to binding. Thus, 26 new contacts were generated through indirect interactions with non-mutated residues in the c134 TCR. The majority of these (21 new contacts) were made between non-mutated residues in the c134 TCR CDR3 α and CDR3 β loops demonstrating that the proximity of residues to the mutated MSAQ motif in the CDR3 β loop was probably important for enabling the formation of these new interactions (Table 2).

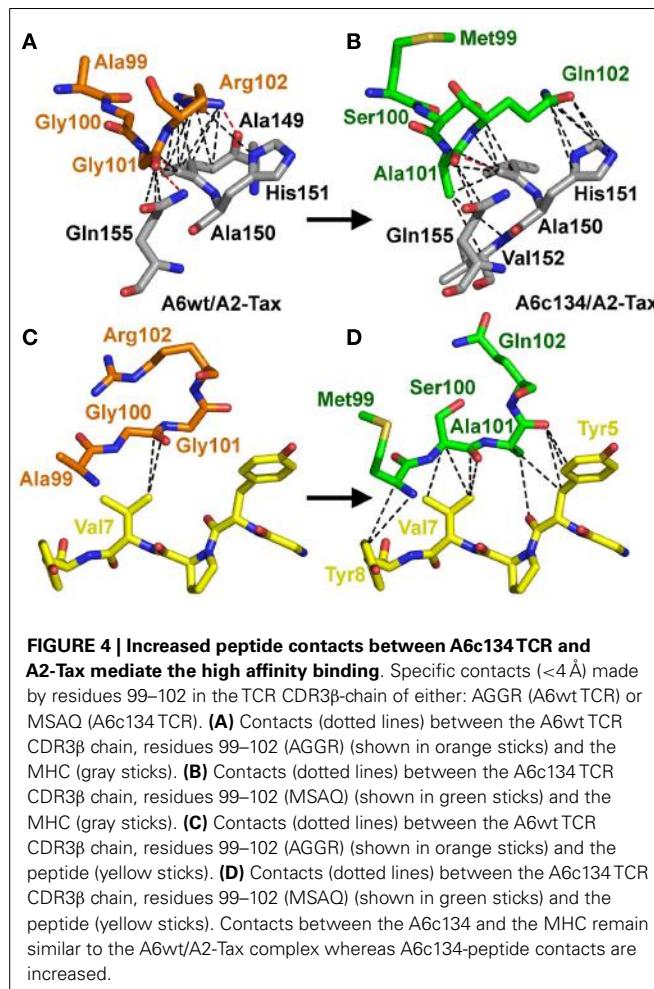
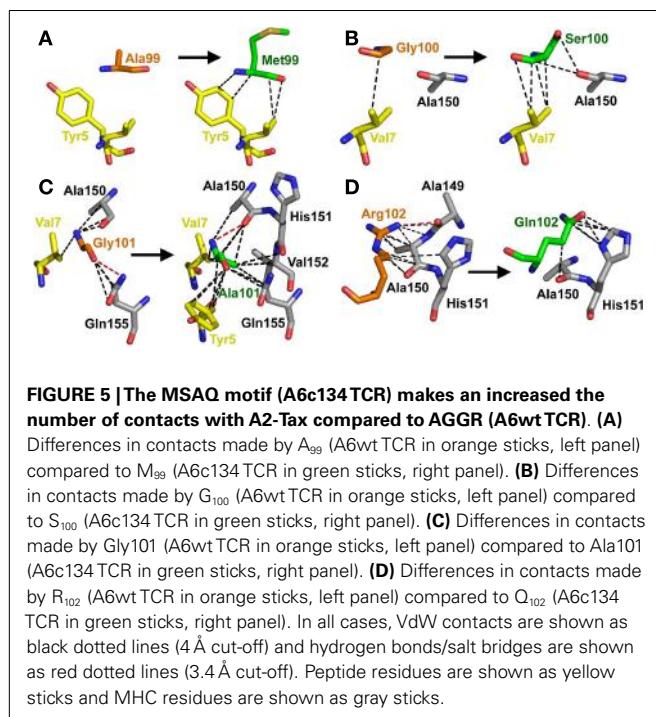


FIGURE 4 | Increased peptide contacts between A6c134 TCR and A2-Tax mediate the high affinity binding. Specific contacts (<4 Å) made by residues 99–102 in the TCR CDR3 β -chain of either: AGGR (A6wt TCR) or MSAQ (A6c134 TCR). **(A)** Contacts (dotted lines) between the A6wt TCR CDR3 β chain, residues 99–102 (AGGR) (shown in orange sticks) and the MHC (gray sticks). **(B)** Contacts (dotted lines) between the A6c134 TCR CDR3 β chain, residues 99–102 (MSAQ) (shown in green sticks) and the MHC (gray sticks). **(C)** Contacts (dotted lines) between the A6wt TCR CDR3 β chain, residues 99–102 (AGGR) (shown in orange sticks) and the peptide (yellow sticks). **(D)** Contacts (dotted lines) between the A6c134 TCR CDR3 β chain, residues 99–102 (MSAQ) (shown in green sticks) and the peptide (yellow sticks). Contacts between the A6c134 and the MHC remain similar to the A6wt/A2-Tax complex whereas A6c134-peptide contacts are increased.

DISCUSSION

Thymic selection generates T cells that express TCRs with a weak binding affinity ($K_D = 100$ nM–270 μ M) for cognate antigen (Cole et al., 2007; Bridgeman et al., 2012). Presumably, this affinity range is important to ensure host protection against foreign invaders, whilst maintaining tolerance to self-antigens from a T cell repertoire of around 25 million (Arstila et al., 1999). In order to perform this function, growing evidence suggests that TCRs must be highly cross-reactive within the confines of MHC-restriction (Mason, 1998; Sewell, 2012; Wooldridge et al., 2012). We have previously shown that it is possible to enhance TCR binding affinity using phage display (Li et al., 2005). These engineered high affinity TCRs represent a potentially useful tool to target specific disease molecules, such as cancer (Liddy et al., 2012) or HIV (Varela-Rohena et al., 2008). However, how enhanced affinity affects TCR binding and specificity is not fully understood, and there are likely multiple mechanistic routes through which affinity can be enhanced. A greater understanding of such mechanisms is particularly important when developing T cell therapies that involve genetically modifying T cells with enhanced affinity TCRs or when using soluble high affinity TCR therapies, to limit potentially dangerous self-reactivity.



In order to explore how TCR CDR3 loop mutations could influence TCR binding and enhance affinity, we generated a modified TCR with nearly 1000-fold enhanced binding affinity by mutating just four residues in the TCR CDR3 β loop. Despite the enhanced binding affinity, the A6c134 TCR utilized a native binding mode with diagonal binding geometry as observed for other TCR/pMHC complexes (Rudolph et al., 2006). This observation is similar to other high affinity TCR structures that have been reported previously (Dunn et al., 2006; Sami et al., 2007; Madura et al., 2013). Furthermore, comparing the structures of the free and complexed A6c134 TCR demonstrated that the TCR CDR3 loops underwent a reduction in conformational flexibility upon ligand binding similar to that observed with the A6wt TCR. Thus, despite the mutation of two glycines in the CDR3 β loop, high affinity binding did not seem to result from preorganization of the TCR binding site.

We observed that the total number of contacts across the interface was greater for A6c134/A2-Tax than for A6wt/A2-Tax. For instance, in the A6c134/A2-Tax complex, there were a total of 154 contacts, including 72 to peptide and 82 to the MHC. In the A6wt/A2-Tax complex, there were total of 117 contacts, including 46 to peptide and 71 to the MHC. Both the MHC and peptide were involved in generating these new contacts, and they were mediated by both mutated and non-mutated TCR residues. However, the majority of the new contacts arose from interactions between the peptide and the c134 TCR CDR3 loops. Thus, we concluded that higher affinity was mediated predominantly by new TCR-peptide interactions. This conclusion, that new contacts mediated the stronger binding affinity, is consistent with previous thermodynamic analyses, which showed that the A6c134 TCR bound to A2-Tax with a substantially more favorable enthalpy compared to the A6wt TCR ($\Delta\Delta H = -10$ kcal/mol) (Armstrong et al., 2008a; Piepenbrink et al., 2009).

Our generation of a high affinity TCR that contained mutations at only four residues in the CDR3 β loop compared to the wt TCR sequence raises the question of why high affinity TCRs are not naturally selected in the thymus (Holler et al., 2003). Clearly, the structural framework of the TCR allows for high affinity binding, and the mutations we have identified fall within the rearranged gene segment of the TCR rather than the pre-defined germline encoded segments. Furthermore, the mutations in the TCR CDR3 β chain generated an increase in peptide contacts within the boundaries of the native A6wt TCR binding mode and would thus be unlikely to alter MHC-restriction. Therefore, it seems very likely that high affinity TCR variants could be generated during the process of TCR rearrangement in the thymus. Yet such high affinity TCRs have not been observed during the peripheral immune response, implying that they are negatively selected (Holler et al., 2003). Presumably, this process is designed to limit self-reactivity. However, weaker affinity TCRs may also be selected to ensure a level of T cell cross-reactivity capable of fully protecting the host against all possible disease epitopes (Mason, 1998; Sewell, 2012; Wooldridge et al., 2012). In support of this notion, our data indicate that A6c134 and other high affinity TCRs can retain extremely high levels of specificity, and may be more specific than their wild-type parents (Laugel et al., 2005; Dunn et al., 2006; Madura et al., 2013). Generally, therapeutic TCRs do not need the capacity to be cross-reactive as they are designed to target a single disease epitope. This difference in desired function (immune response versus specific therapy) may represent an opportunity to improve the affinity of natural TCRs in a safe manner. Thus, our structural investigation of A6c134/A2-Tax, showing that the high affinity interaction was mediated by a native binding mechanism that was peptide-focused, may represent an important approach for developing antigen specific high affinity TCR reagents for use in T cell based therapies.

MATERIALS AND METHODS

PHAGE DISPLAY

Selection of high affinity A6wt TCR variants was performed as previously described (Li et al., 2005).

PROTEIN PURIFICATION

A2-Tax peptide-MHC complexes was prepared as previously described (Garboczi et al., 1992), by expressing HLA-A*0201 heavy-chain truncated at residue Pro-276 and β 2 microglobulin separately in *E. coli* in the form of inclusion bodies, followed by *in vitro* refolding with synthetic peptide. pMHC for binding analysis was prepared similarly, but with the MHC fused to a biotinylation tag (Cull and Schatz, 2000) which was biotinylated *in vitro* by the BirA enzyme (O'callaghan et al., 1999). Disulfide-linked A6c134 TCR was prepared as previously described (Boulter et al., 2003; Li et al., 2005).

BINDING ANALYSIS BY SURFACE PLASMON RESONANCE (BIACORETM)

Binding analysis was performed on a BiacoreTM 3000 machine using a CM-5 (research grade) chip as previously described (Cole et al., 2008; Miles et al., 2011). Streptavidin was immobilized on all flow cells using amine coupling to a level of >1000 RU (response units). Biotin tagged peptide-MHC was flowed over

the streptavidin coated surface at a concentration of approximately 10 µg/ml until ~150 RU pHLA was bound. Control surfaces were coated with non-cognate pMHCs (A2-ILAKFLHWL, A2-ELAGIGILTV, and A2-YLEPGPVTA) or were left coated with streptavidin. Kinetic binding data were generated using the KINJECT program to inject 10 nM TCR over the flow cells. Data were analyzed using BIAevaluation™ software by kinetic fitting to calculate k_{on} and k_{off} rates. Binding affinities were calculated using the following equation: $K_D = k_{off}/k_{on}$.

CRYSTALLIZATION AND X-RAY DATA COLLECTION

A6c134/A2-Tax crystals were grown in MES 25 mM pH 6.5, 24% PEG 3350 and 10 mM NaCl; A6c134 free crystals were grown in MES 25 mM pH 6.5, 24% PEG 3350 and 10 mM NaCl. All crystals were soaked in 30% ethylene glycol before cryo-cooling. Data were collected at 100 K at the Advanced Photon Source at Argonne National Laboratory, USA. Reflection intensities were estimated with the XIA2 package (Winter, 2010) and the data were scaled, reduced, and analyzed with SCALA and the CCP4 package (Collaborative Computational Project, Number 4, 1994). Structures were solved with molecular replacement using PHASER (McCoy et al., 2007). Sequences were adjusted with COOT (Emsley and Cowtan, 2004) and the models refined with REFMAC5. Graphical representations were prepared with PYMOL (Delano, 2002). The reflection data and final model coordinates were deposited

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- SUPPLEMENTARY MATERIAL**
- The Supplementary Material for this article can be found online at http://www.frontiersin.org/T_Cell_Biology/10.3389/fimmu.2013.00168/abstract
- Table S1 | Data collection and refinement statistics (molecular replacement).**
- Table S2 | Structural analysis of A6wt/A2-Tax contacts.**
- Table S3 | Structural analysis for A6c134/A2-Tax contacts (mutant residues in red).**
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Young T cells age during a redirected anti-tumor attack: chimeric antigen receptor-provided dual costimulation is half the battle

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Adoptive therapy with chimeric antigen receptor (CAR)-redirected T cells showed spectacular efficacy in the treatment of leukemia in recent early phase trials. Patient's T cells were *ex vivo* genetically engineered with a CAR, amplified and re-administered to the patient. While T cells mediating the primary response were predominantly of young effector and central memory phenotype, repetitive antigen engagement irreversible triggers T cell maturation leaving late memory cells with the KLRG1⁺ CD57⁺ CD7⁻ CCR7⁻ phenotype in the long-term. These cells preferentially accumulate in the periphery, are hypo-responsive upon TCR engagement and prone to activation-induced cell death. A recent report indicates that those T cells can be rescued by CAR provided CD28 and OX40 (CD134) stimulation. We discuss the strategy with respect to prolong the anti-tumor response and to improve the over-all efficacy of adoptive cell therapy.

Keywords: adoptive cell therapy, chimeric antigen receptor, memory T cells, CD28, OX40

ADOPTIVE THERAPY WITH CAR ENGINEERED T CELLS SHOWED SPECTACULAR EFFICACY IN EARLY PHASE TRIALS

Recent success in the immune therapy of malignant diseases has sustained the promise that the immune system can control cancer in the long-term. On the other hand, tumor-specific T cells are rare in cancer patients making their isolation and *ex vivo* amplification to therapeutic numbers necessary. To overcome the situation strategies were developed to engraft T cells with defined specificity by genetic engineering; the so-called "T-body" strategy equips patient's T cells with a recombinant trans-membrane receptor molecule which is composed in the extracellular part of an antibody-type recognition domain for MHC-independent binding and in the intracellular part of T cell activating domains, mostly the TCR CD3 ζ endodomain linked to a costimulatory domain like CD28, OX40, or 4-1BB (Gross and Eshhar, 1992; Eshhar, 2008). Such a chimeric antigen receptor (CAR) redirects T cells in an antigen-specific fashion producing specific T cell activation toward defined targets.

Second generation CAR's providing CD28 costimulation along with the primary CD3 ζ signal more effectively activate T cells than CAR's with CD3 ζ signaling only. This is basically due to the CD28 mediated improvement of T cell effector functions and the protection from activation-induced cell death resulting in prolonged persistence *in vivo* (Savoldo et al., 2011). Other costimulatory domains of the CD28 family, like 4-1BB (CD137), also improves T cell persistence (Milone et al., 2009; Song et al., 2011). Each individual costimulatory signal, however, differentially orchestrates the effector functions including cytokine secretion, amplification, and cytolytic activity (Hombach and Abken, 2011) which allows to modulate the anti-tumor response in a fine-tuned fashion.

Current clinical trials are utilizing second generation CARs to ensure prolonged persistence of engineered T cells *in vivo*. T cells engineered with a 4-1BB- ζ CAR targeting CD19 recently produced spectacular efficacy toward refractory leukemia in patients with high tumor burden (Kalos et al., 2011; Grupp et al., 2013). Further studies in other centers are also reporting encouraging clinical responses using CD28- ζ CAR T cells (Brentjens et al., 2011; Kochenderfer et al., 2012). The general success of these studies is likely due to repetitive tumor cell killing by CAR T cells; additionally, the targeted CD19 is also expressed by healthy B cells which re-stimulate the CAR T cells independently of the targeted tumor cells. The situation in targeting solid tumors, however, is more complex, in particular with respect to immune repression, and may require supporting strategies as discussed in a recent review (Gilham et al., 2012). We here address an additional aspect which is attracting increasing attention and which is of equal relevance for the success in adoptive cell therapy: the progression of CAR redirected T cells into terminal maturation upon repetitive antigen encounter.

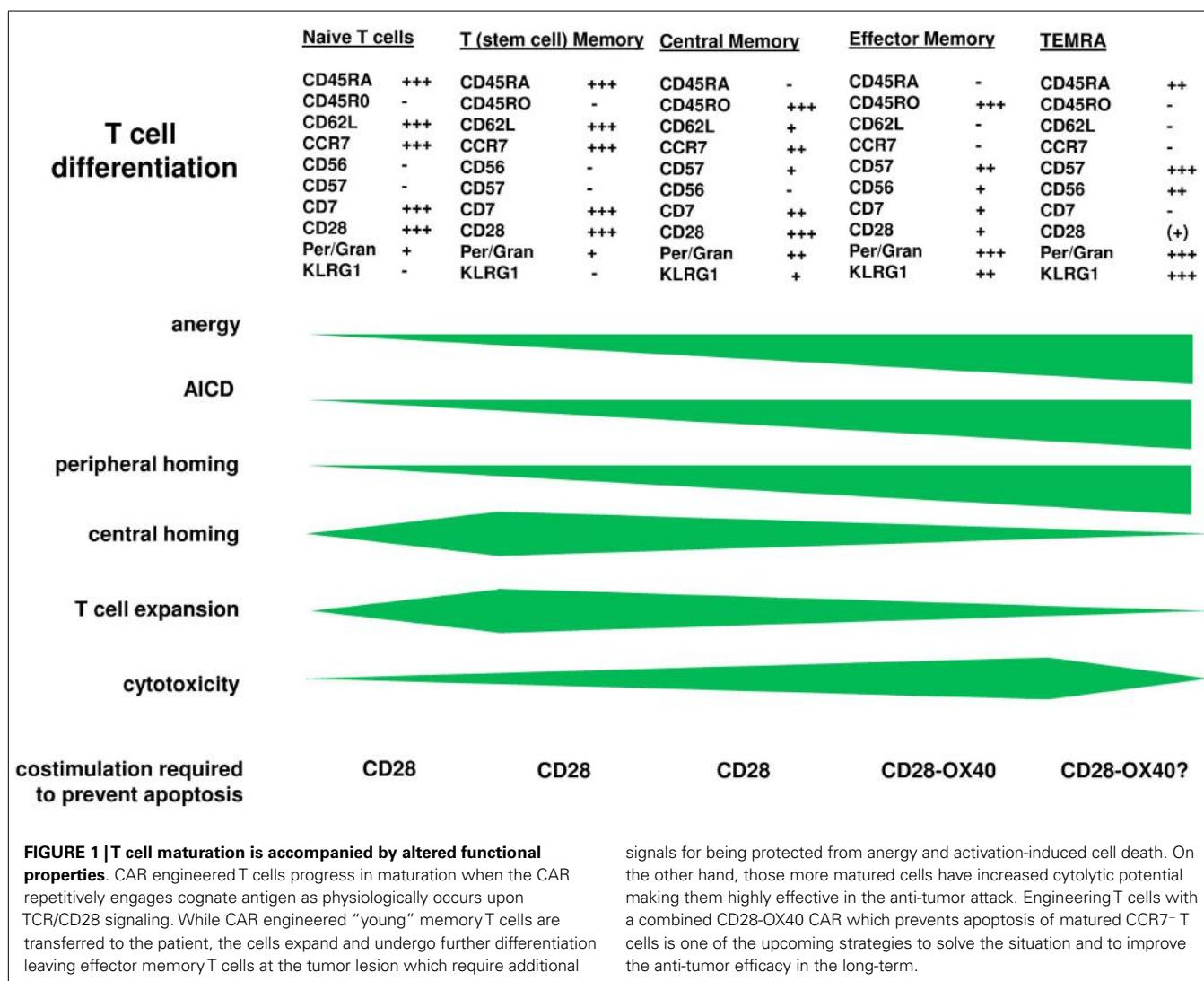
REPETITIVE CAR ENGAGEMENT PRODUCES LATE EFFECTOR MEMORY CELLS WITH ALTERED FUNCTIONAL CAPACITIES

Chimeric antigen receptor engineered young T cells, the majority of them with central memory phenotype, are adoptively transferred to the patient since these cells showed superior in mediating an anti-tumor response in pre-clinical models (Klebanoff et al., 2005). Repetitive binding to cognate antigen, however, induces CAR T cells to amplify, as T cells physiologically do upon TCR/CD28 engagement or TCR stimulation in presence of IL-2. Extensive amplification, however, substantially impacts the anti-tumor efficacy of CAR T cells in the long-term. After

<2 weeks of cell divisions *in vitro*, T cells progress in maturation and alter their functional properties, associated by a change in phenotype (Figure 1). Repetitive antigen engagement converts naïve and central memory T cells to cells with a CCR7[−] CD62L^{low} CD57⁺ KLRG1⁺ effector memory phenotype with CD45RO^{high} CD45RA^{low} and CD27^{low} CD28^{low} expression. CAR mediated maturation occurs in both CD8⁺ and CD4⁺ T cells and does not happen when cell division is blocked. The process is observed in a mouse tumor model in which after adoptive transfer of young CCR7⁺ CAR T cells the majority of tumor infiltrating CAR T cells are of CCR7^{low} phenotype (Hombach et al., 2013). One consequence is that the capacity of those cells to re-enter the lymph and to re-circulate is diminished since CCR7 is required for T cell homing into secondary lymphoid organs (Sallusto et al., 1999; Müller and Lipp, 2003; Bromley et al., 2005; Klebanoff et al., 2005; Moschovakis and Förster, 2012). Inability of CCR7[−] T cells to re-circulate, on the other hand, may be of advantage since most solid cancer lesions occur in the periphery. The assumption is sustained by the observation that CCR7[−] CAR T cells persist in

higher numbers in the tumor lesion although both the CCR7⁺ and CCR7[−] subset T cells equally efficiently target to the tumor (Hombach et al., 2013). Paradoxically, the anti-tumor response of CCR7[−] CAR T cells is less efficient than that of CCR7⁺ T cells when redirected by a CD28- ζ CAR. This is moreover unexpected since CCR7[−] T cells secrete higher amounts of pro-inflammatory cytokines like IFN- γ and harbor higher levels of cytolytic effector molecules like perforin and granzymes compared to CCR7⁺ T cells. Detailed analyses revealed that CCR7[−] T cells are prone to spontaneous and activation-induced cell death which is insufficiently prevented by CAR mediated CD28 costimulation (Hombach et al., 2013). Similar observations were reported for CD57⁺ T cells (Chattopadhyay et al., 2009) and which is in contrast to CCR7⁺ T cells.

Simultaneous CD28 and OX40 costimulation reduces the high propensity of CCR7[−] T cells to undergo apoptosis (Hombach et al., 2013). OX40 promotes Bcl-xL and Bcl-2 expression, enhances the survival of antigen-experienced effector T cells and improves the generation of antigen-specific T cell memory (Rogers



et al., 2001). In combination with CD28 in a so-called “third generation” CD28-OX40 CAR, combined costimulation improved survival and cytolytic activities of CCR7[−] T cells toward targeted cancer cells (Hombach et al., 2013). Similar results were obtained with CD62L[−] T cells which mature from CCR7[−] CD62L⁺ T cells upon stimulation (Hombach and Abken, 2011). The combination of costimulatory signals achieves the effect since OX40 alone does not provide benefit in this context whereas CD28 costimulation alone, which prevents CCR7⁺ T cell apoptosis, does not reduce the number of apoptotic CCR7[−] cells. Taken together adoptive cell therapy will benefit from redirecting T cells by a CD28-OX40 CAR to provide protection from apoptosis when young cells progress in maturation.

PERSPECTIVES: HOW TO MAINTAIN AN ANTI-TUMOR RESPONSE IN THE LONG-TERM?

Several factors together are required to rescue matured CAR T cells for the anti-tumor response in the long-term.

First, any T cell subset converts to effector memory cells upon repetitive antigen engagement and amplification and in each stage of maturation requires appropriate costimulation to escape cell death; consequently, harnessing young T cells with a CD28-OX40 CAR will be beneficial and will rescue CCR7[−] cells when produced during an anti-tumor attack.

Second, CCR7[−] T cells persist in peripheral lesions due to their inability to re-enter the lymph, thereby increasing the probability for successful cancer cell killing. To improve their accumulation at the tumor site T cells were additionally engineered with the CCR2 receptor (Moon et al., 2011). Alternatively, CAR engineered T cells are injected into the tumor lesion taking advantage of the plethora of different effector T cell subsets in fighting cancer while circumventing the limitations in T cell trafficking. In contrast, i.v. injected T cells become stuck in the lungs for hours and subsequently accumulate in liver, spleen, and lymph nodes, while regional application produces T cell persistence at the injected tumor site with only local diffusion within the following days (Parente-Pereira et al., 2011). The strategy, however, requires good accessibility by direct puncture or by endoscopy and is currently evaluated in the treatment of head and neck cancer (EudraCT 2012-001654-25, NCT01722149) or will be applied to the treatment of cutaneous lymphoma (EudraCT 2011-003125-10).

Third, effector memory T cells produce increased levels of pro-inflammatory cytokines and cytolytic molecules, both contributing to improve cancer cell killing. Late memory T cells,

however, are TCR hypo-responsive which is due to an inefficient formation of the TCR synapse as a result of galectin-3 anchoring of TCR components. Interestingly, formation of a transgenic CAR synapse in those cells is not affected making them fully responsive to CAR targets (Rappl et al., 2012).

Since T cell expansion is mandatory to establish adoptively transferred T cells in the long-term, sufficient space is provided to transferred T cells by non-myeloablative lympho-depleting preconditioning followed by IL-2 administration to sustain expansion; other cytokines like IL-7 and IL-15 are also explored (Weber et al., 2011). Extensive T cell amplification, on the other hand, produces effector memory T cells which then need to be protected from activation-induced cell death. Other costimulation than by CD28 and OX40 may also provide benefit to those cells, for instance 4-1BB (Song et al., 2011). Co-signaling by 4-1BB and CD28 may also provide an advantage to matured T cells, however, needs to be evaluated in detail. The effect of each combination of costimulatory signals, however, cannot be predicted since the CAR with its linked endodomains provides simultaneous signaling while in the physiological situation the signals occur individually in a well-defined temporal and spatial order. An elegant solution of the dilemma is the use of virus-specific T cells which are further matured by the immune system and have some significant properties needed for effective anti-cancer therapy. These cells obtain survival and costimulatory signals when engaging virus-infected cells by their TCR. Current trials use EBV or CMV specific, autologous T cells engineered with a first or second generation CAR, for instance directed against ErbB2 (NCT01109095), CD30 (NCT01192464), CD19 (NCT00709033; NCT01475058; NCT01430390; NCT00840853; NCT01195480), or GD-2 (NCT00085930). Virus-specific T cells are long-lived in pre-clinical models *in vivo*, have a great capacity to amplify and are particularly applied in the context of allogeneic stem cell transplantation where they protect from virus re-activation and tumor relapse while having low risk of inducing graft versus host disease.

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Molecular insights for optimizing T cell receptor specificity against cancer

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Cytotoxic CD8T cells mediate immunity to pathogens and they are able to eliminate malignant cells. Immunity to viruses and bacteria primarily involves CD8 T cells bearing high affinity T cell receptors (TCRs), which are specific to pathogen-derived (non-self) antigens. Given the thorough elimination of high affinity self/tumor-antigen reactive T cells by central and peripheral tolerance mechanisms, anti-cancer immunity mostly depends on TCRs with intermediate-to-low affinity for self-antigens. Because of this, a promising novel therapeutic approach to increase the efficacy of tumor-reactive T cells is to engineer their TCRs, with the aim to enhance their binding kinetics to pMHC complexes, or to directly manipulate the TCR-signaling cascades. Such manipulations require a detailed knowledge on how pMHC-TCR and co-receptors binding kinetics impact the T cell response. In this review, we present the current knowledge in this field. We discuss future challenges in identifying and targeting the molecular mechanisms to enhance the function of natural or TCR-affinity optimized T cells, and we provide perspectives for the development of protective anti-tumor T cell responses.

Keywords: cytotoxic T cells, TCR-affinity, melanoma, immunotherapy, TCR engineering, TCR signaling, T cell activating receptors, T cell inhibitory receptors

QUANTITATIVE ASPECTS OF ANTIGEN RECOGNITION BY CD8 T LYMPHOCYTES

Cytotoxic CD8 T lymphocytes recognize through their T cell receptors (TCRs) an antigenic peptide that is presented by MHC class I molecules (peptide-MHC, pMHC) on the surface of an infected or transformed cell. TCR triggering activates in T cells a signaling cascade, which leads to the release of effector molecules and to the cytolytic elimination of the cell that stimulated the T cell. The efficiency of triggering a T cell response critically depends on how well a TCR binds to a stimulating pMHC complex and stronger interactions are thought to cause more vigorous T cell activation than weaker interactions (Stone et al., 2009; Zehn et al., 2009). The dissociation constant K_D is a physical parameter that is generally used to describe the strength with which a TCR binds to a given pMHC complex (Zehn et al., 2012) and to which we usually refer to as the affinity of TCR and pMHC interaction.

Peripheral CD8 T cells express TCRs that only weakly react with self-peptide presenting pMHC and the K_D values of these interactions are in the range of 100–10 μ M (Cole et al., 2007). In contrast, TCRs that interact with foreign-peptide presenting MHC with a K_D of up to 1 μ M are frequently found among T cells that respond to pathogens (Davis et al., 1998). In fact, it is well established that immune responses to pathogen are dominated by cytotoxic T cells that express high affinity TCRs (Figure 1), and these cells are thought to be superior in executing effector function than low affinity T cells (Speiser et al., 1992; Alexander-Miller et al., 1996). Nonetheless, recent observations

indicate that also a larger number of lower affinity T cell clones participate in immune responses. Moreover, it is well established that anti-tumor immune responses critically rely on lower affinity T cells, as most high affinity self/tumor-antigen specific T cells are usually thoroughly eliminated by both central and peripheral tolerance mechanisms. Within the subsequent sections, we will present key findings regarding the biology of cytotoxic CD8 T cells that respond with high or low affinity to antigen, we will describe how differences in affinity impact the outcome of a T cell response, and we will discuss several strategies to bypass the limitation that are linked to T cell responses mediated by low affinity T cells.

EVIDENCE FOR THE PARTICIPATION OF LOW AFFINITY CD8 T CELLS IN IMMUNE RESPONSES TO PATHOGENS

To characterize how TCR-pMHC affinity impacts T cells in an infection, we expressed in pathogens a set of altered peptide ligands that gradually differ in the strength of binding to the OT-1 TCR. By infecting mice with pathogens expressing these ligands, we can mimic high, intermediate, or low affinity stimulation, as it would be the case with polyclonal cytotoxic T cells of which some respond with high and others with low affinity to pathogen-derived antigen (Zehn et al., 2009). Unexpectedly, we found that the OT-1 T cells initially responded similarly to pMHC complexes that very differently stimulated the OT-1 TCR. Even very low affinity complexes induced the same initial rapid T cell proliferation as high affinity ones. Low affinity-stimulated OT-1 CD8 T cells were early on phenotypically indistinguishable from cells stimulated by

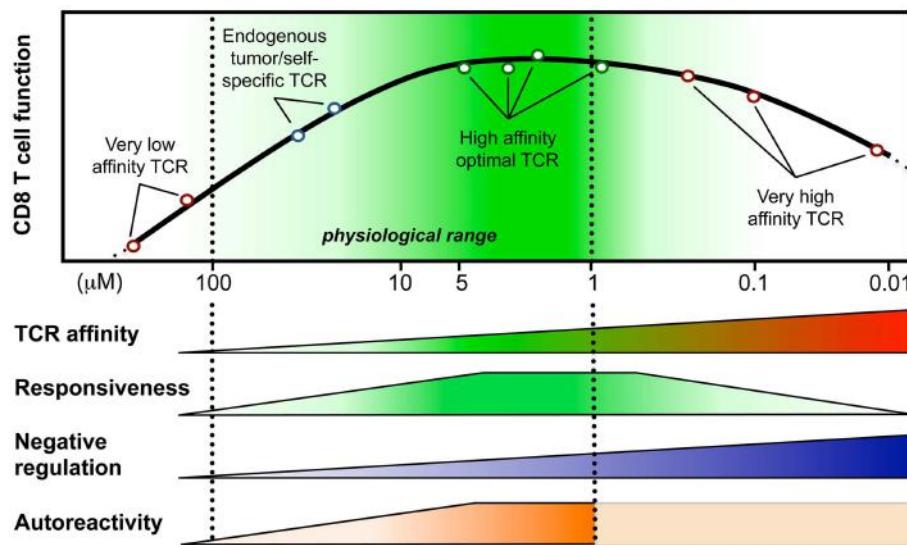


FIGURE 1 | Model integrating the relationship between T cell responsiveness (e.g., cell signaling, gene expression, and functionality) and TCR-affinity (in K_D , μM) of human CD8 T lymphocytes engineered with anti-tumor TCR variants of optimized affinities (Irving et al., 2012; Hebeisen et al., 2013; Zhong et al., 2013). Optimal/maximal T cell effectiveness is observed with cells expressing affinities in the upper natural

limit (K_D from 5 to 1 μM ; dark green). Negative regulation mechanisms may counteract T cell responsiveness in T cells bearing very high affinities (depicted as blue gradients) (Corse et al., 2010; Slansky and Jordan, 2010; Hebeisen et al., 2013). Moreover, Zhong et al. (2013) recently described an affinity threshold (K_D around 10 μM) for maximal anti-tumor activity and autoreactivity (depicted as orange gradients).

high affinity complexes. Expression of effector molecules such as granzyme B, as well as effector and memory T cell functions were surprisingly efficient (Zehn and Bevan, 2006; Enouz et al., 2012). It has also been shown that very low affinity stimulated T cells support pathogen elimination (Turner et al., 2008). Together, these findings indicate that lower affinity CD8 T cells fully participate in the immune response.

However, there is a major difference between low and high affinity CD8 T cells. Namely, the former undergo fewer rounds of division and decline in numbers faster than high affinity stimulated T cells. Thus, while undergoing full differentiation, low affinity primed effector T cells reach lower numbers. Therefore the high affinity T cells dominate in numbers at the time when T cell expansion is at its maximum.

Given their low numbers, one may question the importance of low affinity CD8 T cells. The large numbers of high affinity T cells at the peak of the immune response have so far distracted from exploring the relevance of low affinity T cells during infection. Several kinetic aspects may suggest that low affinity T cells could perhaps be more important than previously appreciated. In the naïve T cell repertoire, high affinity T cell clones specific to any given antigen are rare. In contrast, it is likely that low affinity T cell clones are more frequent. As low and high affinity clones expand equally at the beginning, there should be a larger number of low than high affinity effector T cells in the early phase of the T cell response, as we found in our experiments. The dominance of high affinity CD8 T cells develops later, because these cells overgrow the lower affinity T cells in the late T cell expansion phase (Zehn et al., 2009). Importantly, we noticed that low affinity T cells leave secondary lymphoid organs earlier than high affinity

T cells, suggesting that the earliest wave of effector T cells that enter peripheral organs predominately consists of low affinity T cells. Thus, the critical early phase of pathogen elimination may be primarily achieved by low affinity cytolytic T cells (Zehn et al., 2009).

The number of low affinity T cells responding to one particular epitope is perhaps small. However, there could be many unknown epitopes recognized by low affinity T cells, which cumulatively might result in a reasonably sized T cell population. These considerations suggest that low affinity CD8 T cells play a more important role during infection than previously anticipated, which may have been underestimated in the past.

ANTI-SELF AND -TUMOR IMMUNE RESPONSES ARE FREQUENTLY MEDIATED BY LOW AFFINITY CD8 T CELLS

Anti-tumor immune response targets tumor-associated antigens such as cancer testis antigens (e.g., NY-ESO-1 or MAGEs) expressed by several tumors or differentiation antigens (e.g., Melan-A/MART-1, gp100, or tyrosinase) expressed in melanoma cells (Romero et al., 2002; Van Der Bruggen et al., 2002; Boon et al., 2006). Most of these antigens are expressed in the thymus (Kyewski and Klein, 2006) and accordingly, T cells with high affinity become negatively selected. As a backup, tumor-antigen reactive T cells can be eliminated in the periphery through mechanisms of peripheral tolerance (Kurts et al., 1997). However, it has been convincingly shown, that these mechanisms spare cytotoxic T cells that react with lower affinity to self- or tumor-antigens (von Herrath et al., 1994; Zehn and Bevan, 2006; McMahan and Slansky, 2007; Turner et al., 2008). Although, it is still often rather difficult to judge how effectively lower affinity CD8 T cells execute effector T cell

functions, several strong lines of evidence indicate that low affinity auto-reactive T cells are able to eliminate tumors and play a critical role in autoimmunity (von Herrath et al., 1994; Zehn and Bevan, 2006; McMahan and Slansky, 2007; Bulek et al., 2012). In fact, it becomes more and more clear that most self/tumor-specific cytotoxic T cells express low affinity TCRs and there is increasing evidence that the self/tumor-specific T cells are indeed capable to destroy cancer cells *in vivo* (Boon et al., 2006; Rosenberg et al., 2008). Moreover, it has been shown that self/tumor-antigen specific CD8 T cells can undergo considerable clonal expansion in cancer patients, differentiate to memory and effector cells, and persist during several years at relatively high frequencies (Speiser et al., 2011; Baitsch et al., 2012). These observations are well in line with the aforementioned findings that low affinity CD8 T cells participating in the response to pathogens may have great implications for anti-cancer immunity.

However, researchers must still deal with several challenges associated with activating low affinity CD8 T cells. For example, these cells require higher numbers of presented pMHC complexes than high affinity T cells before they become activated and for mounting an effector T cell response. Also, requirements for interactions with DCs by CD8 T cells of low TCR affinities are likely higher, to achieve sufficient TCR triggering and co-stimulation (Liechtenstein et al., 2012; Chen and Flies, 2013). Furthermore, lower affinity CD8 T cells undergo, as mentioned above, shorter clonal expansion following stimulation than high affinity T cells which means that fewer of such cells will be obtained following vaccination. Given these limitations, we need to find better ways to more effectively activate these T cells, to enhance their function, and to selectively interfere with the mechanisms, which prevent these cells from responding to tumors. One possible way to do this is to alter the kinetics with which the TCR of a tumor-specific T cell binds to its peptide-pMHC complex. Another approach would be to manipulate the signaling cascades downstream of the TCR.

TCR-AFFINITY OPTIMIZATION AGAINST CANCER ANTIGENS

Immunotherapy aims at mobilizing the body's immune cells to fight against tumor cells in a highly specific manner. There are two biological strategies to achieve immune activity: active immunization with the use of vaccination and passive immunization. A form of passive immunotherapy is the adoptive cell transfer (ACT) of autologous T lymphocytes to patients with metastatic cancer (Restifo et al., 2012). This approach uses autologous TIL (tumor infiltrating lymphocytes), which are isolated from metastatic lesions, expanded *in vitro*, and selected for tumor reactivity. Remarkably, about 50 to 70% of patients with metastatic melanoma experience objective clinical responses, and up to 20% even have complete and durable responses (Rosenberg et al., 2011). Nevertheless, further improvements are necessary.

A limiting factor is the relatively low affinity of tumor-antigen reactive T cells. For improvement, T cells can be engineered with TCRs of increased affinity for tumor-antigens before transfer to patients (Figure 1). Indeed, this approach may augment the functional and protective capacity of tumor-antigen reactive CD8 T cells (Robbins et al., 2008, 2011; Bendle et al., 2009; Bowerman et al., 2009; Chervin et al., 2009; Johnson et al., 2009). In turn,

TCR engineering also bears the risk that the normal tissue could be harmed. It has been demonstrated that T cells, whose TCR binds to pMHC complexes with very high affinities ($K_D < 1 \text{ nM}$) lose antigen specificity and can become cross-reactive or allo-reactive (Holler et al., 2003; Zhao et al., 2007; Robbins et al., 2008). Importantly, genetically engineered T lymphocytes expressing very high affinity self/tumor-specific TCRs also target normal tissues expressing the cognate antigen (e.g., melanocytes in the skin, eye, and ear for Melan-A-specific T cells and neurons for MAGE-A3-specific T cells), and can mount harmful cytotoxic immune responses *in vivo* (Johnson et al., 2009; Morgan et al., 2013). Moreover, TCR mispairing between introduced and endogenous TCR α and β chains has also been shown to lead to off-target toxicity (Bendle et al., 2010; van Loenen et al., 2010). Therefore, TCR optimization through affinity alteration must include the evaluation of optimal T cell responsiveness and lack of cross-reactivity to ensure the safety of TCR-engineered T cells in clinical trials. Moreover, it must further include the development of new strategies to minimize the extent of mispairing (reviewed in Govers et al., 2010; Daniel-Meshulam et al., 2012), as elegantly shown by Aggen et al. (2012), describing the use of stabilized V α V β single-chain TCRs (scT ν ; Figure 2). Unfortunately, unexpected auto-reactive responses may never be completely excluded. In that regard, it is important to further study the tissue distribution of self/tumor-antigen expression, to optimize the choice of antigens targeted by ACT therapy (e.g., cancer testis versus differentiation antigens) (Offringa, 2009).

TCR-AFFINITY THRESHOLD FOR MAXIMAL ANTI-TUMOR CD8 T CELL RESPONSE

During recent years we established a panel of human CD8 T cells expressing engineered TCRs of optimized affinities against the tumor-antigen NY-ESO-1 presented in the context of HLA-A2. They were obtained through structure-based rational predictions (Zoete and Michielin, 2007; Zoete et al., 2010). The functional potential of these T cells (Schmid et al., 2010; Irving et al., 2012) showed that T cells expressing TCRs with affinities in the upper natural range (K_D from 5 to $1 \mu\text{M}$) displayed greater biological responses when compared to those expressing intermediate affinity wild-type TCR (K_D at $21.4 \mu\text{M}$) or very low affinity ($K_D > 100 \mu\text{M}$) (Figure 1). Unexpectedly, we noticed that T cells which express TCRs beyond the natural affinity range ($K_D < 1 \mu\text{M}$) showed a severe decline in their gene expression profile, signaling, and functionality (Irving et al., 2012; Hebeisen et al., 2013), despite retaining their antigen specificity and showing no broad cross-reactivity as observed in other studies (Holler et al., 2003). Major findings revealed that maximal T cell effectiveness was limited by at least two mechanisms (Figure 1). The first one was characterized by the preferential expression of the inhibitory receptor programmed cell death-1 (PD-1) within T cells of the highest TCR affinities and this correlated in those cells with full functional recovery upon PD-1 ligand 1 (PD-L1) blockade (Hebeisen et al., 2013). The second one contrasted to PD-1 expression with the gradual upregulation of the Src homology 2 domain-containing phosphatase 1 (SHP-1) in CD8 T cells with increasing TCR affinities. Consequently, pharmacological inhibition allowed further incremental gaining of cell function in

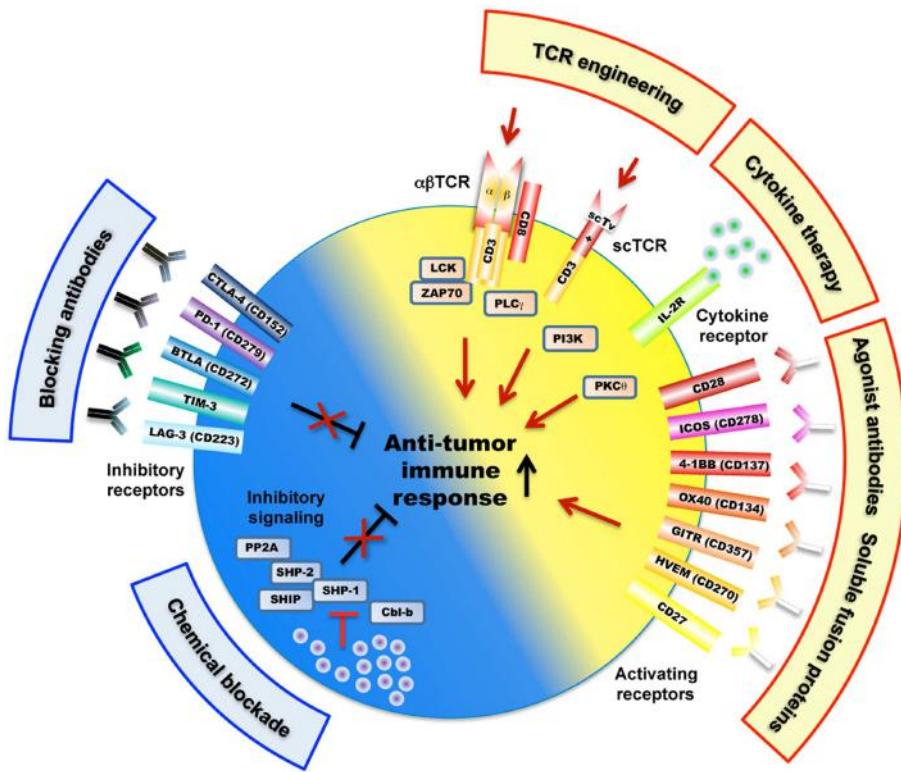


FIGURE 2 | Overview of mechanisms and potential therapeutic targets to improve tumor-antigen reactive T lymphocytes. These include a large variety of receptors (e.g., engineered TCRs, activating/inhibitory surface receptors, cytokine receptor) as well as TCR-downstream signaling molecules (e.g., SHP-1,

SHP-2, PP2A) that regulate T cell activation, signaling, and function (e.g., killing, cytokine secretion) against cancer antigens. Of note, the scFv single $\text{V}\alpha\text{V}\beta$ chain TCRs may be linked to intracellular signaling domains such as Lck and CD28, independently of the CD3 subunits (Aggen et al., 2012).

all engineered T cells, according to their TCR-binding affinities (Hebeisen et al., 2013).

Our observations provide new evidence that T cell activation and signaling may be limited to a given affinity threshold for the TCR-pMHC interaction and that above this threshold, T cells may not develop productive functions. They also nicely fit with other *in vitro* and *in vivo* studies that reported maximal T cell responses at an optimal TCR-pMHC off rate (k_{off}) or K_D while functional attenuation was observed when kinetic parameters extended above the natural range (Kalergis et al., 2001; Gonzalez et al., 2005; McMahan et al., 2006; Carreno et al., 2007; Corse et al., 2010; Thomas et al., 2011). Furthermore, Krogsgaard and colleagues (Zhong et al., 2013) recently evaluated the TCR-affinity threshold defining the optimal balance between effective anti-tumor activity and autoimmunity *in vivo*, using human melanoma gp100₂₀₉₋₂₁₇ – specific TCRs spanning within the physiological affinity range. Their results show the presence of an affinity threshold (around 10 μM) for maximal anti-tumoral activity and autoreactivity, suggesting that a relatively low-affinity threshold is necessary for the immune system to avoid self-damage (Zhong et al., 2013). Altogether, we and others propose that the rational design of improved self-specific TCRs for adoptive T cell therapy may not need to be optimized beyond the natural TCR-affinity range to achieve optimal T cell function and avoidance of unpredictable

risk of cross-reactivity (Schmid et al., 2010; Slansky and Jordan, 2010).

Recently, Liddy et al. (2012) described the use of novel reagents termed immune-mobilizing monoclonal TCRs (or ImmTACs) against tumor-antigens including NY-ESO-1, which are fused to a humanized CD3-specific single-chain $\alpha\beta$ fragment (scFv). These ImmTACs comprise TCRs of picomolar affinity range and allow to effectively redirect T cells to kill *in vivo* cancer cells expressing very low surface epitope densities. In line with previous studies from the same group (Li et al., 2005; Dunn et al., 2006), soluble monomeric TCRs possessing affinity $\approx 10^6$ -fold higher than native TCRs showed a remarkable high degree of specificity for the cognate pMHC molecules. Possibly, soluble monomeric TCRs may allow circumventing the two major limitations associated with TCR engineering within CD8 T cells. First, the loss of target cell specificity associated with T cells expressing extremely high affinity TCRs ($K_D < 1 \text{ nM}$) (Zhao et al., 2007; Robbins et al., 2008). And second, the functional defects of T cells with supraphysiological TCR affinities ($K_D < 1 \mu\text{M}$) (Kalergis et al., 2001; Gonzalez et al., 2005; McMahan et al., 2006; Corse et al., 2010; Thomas et al., 2011).

At present, what remains intriguing is how super affine TCRs modulate cell activation and responsiveness. One likely explanation is that in contrast to soluble TCRs, the cellular TCR

expression integrates and potentiates the effect of several variables/parameters including TCR density, multivalent TCR clustering, and basal cell activation state (Stone et al., 2009). Furthermore, several observations including ours (Hebeisen et al., 2013) now indicate that T cell activation and signaling is also finely tuned by the proximal TCR-signaling complex as well as by activating or inhibitory co-receptors, and will be discussed in detail below.

LOW AND HIGH AFFINITY ANTIGEN RECOGNITION DEPENDS ON THE PROXIMAL TCR-SIGNALING COMPLEX

The TCR complex is composed of the TCR $\alpha\beta$ chains, which are directly involved in the pMHC recognition, and of the invariant CD3 proteins, that contain in their cytosolic domains the immunoreceptor tyrosine-based activation motifs (ITAM) (Hedrick et al., 1984; Malissen et al., 1984; Letourneur and Klausner, 1992). TCR triggering elicits a series of membrane-associated events, leading to the transduction of signal across the plasma membrane and phosphorylation of key residues in the TCR-associated CD3 ITAM domains (Stefanova et al., 2003; James and Vale, 2012). Phosphorylation of CD3 ζ -associated ITAM is mediated by the Src family kinases Lck and Fyn (Acuto et al., 2008) and form docking sites for several protein tyrosine kinases (PTKs) including the Syk-family kinase ζ -associated protein of 70 kDa, ZAP-70. Activation of ZAP-70 by Lck in turns results in phosphorylation and activation of other proteins and recruitment of adaptors (e.g., LAT and SLP-76). This initiates the formation of multi-molecular signalosomes, leading to the subsequent generation of secondary messengers and of multiple distal signaling cascades (Acuto et al., 2008; Smith-Garvin et al., 2009).

CD8 T cells may further adapt these signaling pathways to different stimulation conditions and different requirements for antigen sensitivity. Several lines of evidence indicate that differential patterns of CD3 ζ ITAM phosphorylation directly modulate TCR-pMHC mediated downstream signaling and that ITAMs can act as both positive (ITAMs) and negative (inhibitory ITAMi) cell signaling regulators (Blank et al., 2009). For instance, resting peripheral T cells have a constitutive pattern of phosphorylated ITAMs, and incomplete CD3 ζ ITAM phosphorylation after TCR triggering can by itself become inhibitory depending on the nature of the TCR ligand (Kersh et al., 1999). Thus, it is of great importance to further explore whether distinct CD3 ζ ITAM phosphorylation states could also influence cell activation and responsiveness along the range of TCR-affinity and particularly in engineered CD8 T cells of supraphysiological affinity TCRs.

Lck represents another key regulatory element involved in the modulation of proximal TCR activation and signaling, and Lck activation stage may currently be viewed as a sensor of the strength of TCR engagement. On the one hand, weak binding of the TCR triggers Lck-dependent activation and recruitment of SHP-1, which in a classical feedback loop inactivates Lck and downregulates TCR signaling. On the other hand, stronger TCR activation induces an Erk-dependent Lck phosphorylation that impairs the inhibitory SHP-1 recruitment and in contrast reinforces TCR signaling by decreasing the threshold of T cell activation (Stefanova et al., 2003). Interestingly, as mentioned above, we recently used a panel of CD8 T cells engineered with TCRs of incremental

affinities for an NY-ESO-1 derived peptide and saw that SHP-1 phosphatase was upregulated in a TCR-affinity-dependent manner, with the highest levels in T cells of the supraphysiological TCRs (Hebeisen et al., 2013). These observations further suggests that SHP-1 may play a dual role and restricts not only T cell signaling at the very low range of TCR stimulation (e.g., antagonist ligands) as described by Stefanova et al. (2003), but also at the higher range.

Other phosphatases have been shown to act on the proximal TCR signaling such as Lyp, a PTPN22 encoded phosphatase, and together with Csk inhibit T cell activation, likely through dephosphorylation of the activating tyrosine on Lck and ZAP-70 (Cloutier and Veillette, 1999). The importance of PTPN22 is highlighted by the observation that PTPN22 deficient mice have augmented TCR-induced phosphorylation and activation (Hasegawa et al., 2004). Furthermore, a point mutation in PTPN22 has been found associated with several autoimmune diseases (Mustelin et al., 2005). The precise role of PTPN22 in T cell activation remains unknown and there is contradictory data on the effect of the polymorphism found in autoimmune patients and whether or not it causes a loss or gain of function (Vang et al., 2005).

These TCR-affinity-dependent feedback mechanisms are likely part of a tunable instrument that enables T cells to adapt their reactivity to different stimulatory conditions, and we have just began to understand how those are achieved. For instance specific microRNAs such as miR-181a are thought to be critical in augmenting TCR-signaling sensitivity during positive selection in the thymus (Li et al., 2007). The expression of miR-181a has been shown to decrease the amount of several phosphatases, resulting in an elevated steady-state level of phosphorylated proteins of the TCR-signaling cascade and therefore a reduction in the TCR-signaling threshold (Li et al., 2007; Ebert et al., 2009). TCR activation and signaling transduction may also be negatively regulated by SHP-1 phosphatase and contributes to the settings of threshold during thymocyte selection (Plas et al., 1996; Acuto et al., 2008). Moreover, SHP-1 and SHP-2 can be recruited by multiple inhibitory surface receptors in T cells, and inhibit TCR signaling through dephosphorylation of proximal targets including Lck and ZAP-70 (Lorenz, 2009). In line with this concept, Yokosuka et al. (2012) recently showed that ITIM-containing PD-1 could directly inhibit TCR-mediated signaling by recruiting SHP-2 phosphatase in a TCR stimulation strength-dependent manner.

CYTOTOXIC CD8 T CELL RESPONSES ARE REGULATED BY ACTIVATING AND INHIBITORY SURFACE RECEPTORS

Co-stimulatory and inhibitory membrane receptors have great influence on T cell responses (Chen and Flies, 2013). T cell co-stimulation prevents T cell anergy, a state of unresponsiveness that is induced after TCR stimulation in absence of co-stimulation (Figure 2). This was first observed when studying co-stimulation via CD28 that binds to its ligands B7.1 (CD80) and B7.2 (CD86) expressed on antigen-presenting cells (APC). This interaction also lowers the threshold for T cell activation, thus allowing increased IL-2 production and promoting cell proliferation and survival (Sharpe and Freeman, 2002). CD28 ligation stimulates T cell function by activating phosphoinositol-3-kinase (PI3K) and protein kinase C theta (PKC θ), and the downstream Akt, mTOR, and Ras

signaling pathways, which eventually synergize with TCR signaling (Smith-Garvin et al., 2009). T cell activation also leads to surface expression of CTLA-4, which has a much higher binding avidity to B7.1 and B7.2, and thus outcompetes CD28 (Greene et al., 1996). Possibly, this may be the main reason for CTLA-4 mediated T cell inhibition. In addition, it has been shown that CTLA-4 directly triggers inhibitory signaling by interacting with SHP-1, SHP-2, and PP2A phosphatases, with the consequence of down-regulating TCR-signaling pathway (Scalapino and Daikh, 2008). CTLA-4 inhibition also occurs indirectly via retro-signaling through B7.1 and B7.2 and production of IDO in APCs (Grohmann et al., 2002) or by a process of trans-endocytosis of its ligands (B7.1 and B7.2) from APC (Qureshi et al., 2011). CTLA-4 may preferentially inhibit T cells with strong TCR signaling, as suggested by observations that accumulation of CTLA-4 at the immunological synapse depended on the strength of TCR triggering (Egen et al., 2002).

Programed death-1 is also highly upregulated in T cells following TCR stimulation, similarly to CTLA-4. Expression of PD-1 is not restricted to T cells, suggesting a broader role in immune regulation (Greenwald et al., 2005). PD-1 interacts with the two ligands PD-L1 and PD-L2, expressed non-redundantly in different tissues and cell types. CTLA-4-deficient mice have lymphoproliferative disorders and early fatal multi-organ tissue destruction (Tivol et al., 1995; Waterhouse et al., 1995), whereas PD-1-deficient mice spontaneously develop milder forms of autoimmune diseases (Nishimura et al., 2001). Based on the observed differential expression of CTLA-4 and PD-1 ligands, it is assumed that CTLA-4 plays a preferential role in limiting T cell function early during thymocyte development and in secondary lymphoid structures, whereas PD-1 may mediate inhibition in the periphery, for example in maintaining long-term peripheral tolerance to self-antigens by preventing activation of self-reactive T cells that have escaped negative selection (Fife and Pauken, 2011). TCR down-modulation through TCR/CD28 signaling transduction represents a fundamental process regulating the initial events of T cell activation. Recently, the interaction of PD-L1 on DCs and PD-1 on CD8 T cells has been shown to contribute to ligand-induced TCR down-modulation (Karwacz et al., 2011). Furthermore, interference with PD-L1/PD-1 signaling inhibited TCR down-modulation, leading to hyper-activated and proliferative CD8 T cells in an arthritis model (Karwacz et al., 2011).

In humans, a regulatory polymorphism in PD-1 is associated with susceptibility to systemic lupus erythematosus and multiple sclerosis (Prokunina et al., 2002; Kroner et al., 2005), while polymorphisms of the CTLA-4 have been linked to multiple autoimmune diseases including asthma, systemic lupus erythematosus, Graves' disease, and autoimmune thyroid diseases (Kristiansen et al., 2000). The induction of PD-L1 ligand expression was observed in several tumor cells as a mechanism of cancer immune evasion (Schreiber et al., 2011). A specific polymorphism of CTLA-4 was found to be protective for autoimmune disease, but associated with risk of multiple types of cancer (Sun et al., 2009).

Members of the tumor necrosis factor receptor (TNFR) superfamily represent further important co-stimulatory molecules,

mediating survival signals to T cells after initial CD28-B7 interactions (Acuto and Michel, 2003) (Figure 2). Multiple members of TNFR/TNF ligand pairs have been shown to directly impact T cell function following TCR activation, namely OX40/OX40L, 4-1BB/4-1BBL, GITR/GITRL, CD27/CD70, and CD30/CD30L (Watts, 2005). These receptors and their ligands are expressed on a variety of immune and non-immune cells and are inducible and non-ubiquitous, suggesting that they are involved in modulating and coordinating global immune responses (Croft, 2009). Intense translational and clinical research in this field aims at modulating T cell function in pathological settings such as autoimmunity and cancer (Figure 2). TNFR/TNF family member ligation often induces bi-directional activating signaling pathways in both the APC and the T cell. The recruitment of TNFR-associated factors (TRAF) activate the NF- κ B signaling pathway and increase the expression of anti-apoptotic molecules, thus promoting the survival of CD4 and CD8 T cells (Croft, 2009). Like CD28, TNFR signaling can also synergize with the TCR pathway to promote cell cycle progression and cytokine production. Finally, ligation of OX40 and 4-1BB may concomitantly block the generation of inducible regulatory T cells (Tregs), and may inhibit their suppressive activity (So et al., 2008).

A particularly unique and interesting member of the TNFR superfamily is HVEM (Herpes virus entry molecule). It binds to the TNFR ligands LIGHT and lymphotoxin $\text{L}\alpha\text{t}\alpha 3$, which are predominantly co-stimulatory and pro-inflammatory in T cells. Curiously, HVEM also binds to BTLA and CD160, which are structurally similar to PD-1 and CTLA-4 and transduce inhibitory signals, in part through recruitment of SHP-1 and SHP-2 phosphatases (Watanabe et al., 2003; Sedy et al., 2005). The individual effects of HVEM interaction with its different ligands are particularly complex to elucidate since both receptor and ligands can be expressed on the same T cell, as well as on other immune and epithelial cell types (Shui et al., 2011). *Hvem*^{-/-} and *Btla*^{-/-} T cells were found to be hyper-responsive to TCR stimulation *in vitro*. Furthermore, *Hvem*^{-/-} and *Btla*^{-/-} knockout mice had enhanced susceptibility to autoimmune diseases, suggesting a predominant inhibitory role in T cells during inflammatory conditions (Watanabe et al., 2003; Wang et al., 2005). BTLA was found to inhibit tumor-antigen specific cytotoxic T cells in melanoma patients (Derre et al., 2010). HVEM may also interact in *cis* with BTLA expressed by the same cell, likely interfering with HVEM activation by other ligands (Ware and Sedy, 2011). Therefore HVEM seems to mediate immune stimulation or inhibition in a switch-like, bi-directional, and context-dependent mode, suggesting that HVEM/LIGHT/CD160/BTLA interactions represent an important regulatory network for controlling immune responses.

Together, combined TCR and CD28/TNFR triggering primes CD8 T cells, followed by positive and negative regulation. The latter involves CTLA-4, PD-1, and BTLA. This highlights the intricate regulatory network that controls the immune system in health and disease (Figure 2). These mechanisms can be exploited therapeutically in patients with infectious or malignant diseases, as well as in autoimmunity and transplantation (Fife and Bluestone, 2008; del Rio et al., 2010).

ACTIVATORY OR INHIBITORY T CELL SIGNALS MAY BE TARGETED FOR THERAPEUTIC IMPROVEMENTS OF CANCER THERAPIES

Since cytotoxic CD8 T cells and T-helper type 1 [Th1] cells have the potential to eliminate cancer cells and to mediate long-term protection from disease (Sallusto et al., 2010), it is important to increase the functions of these anti-cancer T cells in cancer patients. As mentioned above, basic immunology characterized a number of interesting pathways that can be targeted to enhance the performance of tumor-specific CD8 T cells. Some approaches have already reached clinical application, but most still need to be tested in clinical trials. The therapy that seems most efficient for melanoma patients is the adoptive transfer of autologous tumor-antigen specific T cells (Rosenberg, 2011). Molecular modification of T cells before transfer may eventually increase the clinical efficacy, despite that this is currently not the case (Speiser, 2013). Several small-scale clinical studies suggested clinical usefulness of inserting TCRs (Rosenberg, 2011) or chimeric antigen receptors (Porter et al., 2011; Kochenderfer and Rosenberg, 2013). Hopefully, larger patient numbers will soon benefit thanks to steady improvements of these techniques (Thomas et al., 2010; Di Stasi et al., 2011; Linnemann et al., 2011; Ochi et al., 2011).

Not only antigen receptors but also co-receptors can be targeted therapeutically (Figure 2). Receptors that inhibit T cell functions are particularly attractive. Ipilimumab (Yervoy®) is a monoclonal antibody that blocks the inhibitory receptor CTLA-4. It was recently approved for the treatment of metastatic melanoma, as it improves the clinical outcome, likely due to enhanced numbers and functions of tumor-specific T cells (Hodi et al., 2010). More recently, remarkable benefit for patients with advanced kidney cancer, non-small-cell lung cancer, and melanoma (Ribas, 2012) was demonstrated due to treatment with antibodies against PD-1 (Topalian et al., 2012) or its ligand PD-L1 (Brahmer et al., 2012). Likely, these results represent real therapeutic progress, despite significant toxicity linked to autoimmune reactions. Also, antibodies that block LAG-3, TIM-3, B7-H3, or B7-H4 are under development (Pardoll, 2012). Certainly, the clinical oncology landscape will change during the next years due to these novel approaches.

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In addition to the targeting of cell surface receptors, intracellular mechanisms may be considered. In the complex signaling network downstream of the TCR, there are several possibilities. Interventions are for example possible at the level of E3 ligases (Hoyne, 2011) (Figure 2). As therapeutic targets, the SHP protein tyrosine phosphatases have been proposed (Irandoost et al., 2009). A member of a new class of SHP-1 inhibitors is the tyrosine phosphatase inhibitor-1 (TPI-1) that has been shown to inhibit the growth of transplanted tumor cells in mice together with enhanced cytokine production by T cells (Kundu et al., 2010). However, optimal targeting is challenged by the fact that SHP-1 and many other signal transducers are widely expressed. For example, hematopoietic tumors are suppressed by SHP-1 (Lopez-Ruiz et al., 2011), thus precluding this approach for such diseases. Therefore, novel drugs are needed that promote TCR signaling more specifically, suggesting a drug development similar to what is pursued for optimizing the well known tyrosine kinase inhibitors (De Roock et al., 2011; Goldstraw et al., 2011; Cascone and Heymach, 2012). In parallel to approaches targeting TCR pathways, further immune cells and functions can be supported therapeutically, such as e.g., B cells, adhesion- and homing-receptors, or cytokines (Scott et al., 2010; Miller and Rhoades, 2012; Nylander and Hafler, 2012).

Most likely, we are only at the beginning of understanding the enormous potential that is associated with the therapeutic approaches discussed here. Significant progress is yet to come, despite that immunotherapy has already become standard therapy for some cancer patients. Besides, antibodies blocking CTLA, anti-PD-1, and anti-PD-L1 mAb treatments and adoptive T cell therapy are promising. Novel therapies need to be improved and validated. Furthermore, it is important to learn predicting which therapy is most suitable for which patient. Potentially predictive parameters are the frequencies of tumor-reactive T cells, their ability to migrate to tumor sites, their affinity for antigen recognition, status of effector function, and presence of inhibitory regulatory circuits. More precise knowledge on correlates of protection, and immune monitoring techniques for their characterization in individual patients will support the progress of T cell based therapy against cancer.

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Beyond the antigen receptor: editing the genome of T-cells for cancer adoptive cellular therapies

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Recent early stage clinical trials evaluating the adoptive transfer of patient CD8⁺ T-cells redirected with antigen receptors recognizing tumors have shown very encouraging results. These reports provide strong support for further development of the therapeutic concept as a curative cancer treatment. In this respect combining the adoptive transfer of tumor-specific T-cells with therapies that increase their anti-tumor capacity is viewed as a promising strategy to improve treatment outcome. The *ex vivo* genetic engineering step that underlies T-cell re-direction offers a unique angle to combine antigen receptor delivery with the targeting of cell-intrinsic pathways that restrict T-cell effector functions. Recent progress in genome editing technologies such as protein- and RNA-guided endonucleases raise the possibility of disrupting gene expression in T-cells in order to enhance effector functions or to bypass tumor immune suppression. This approach would avoid the systemic administration of compounds that disrupt immune homeostasis, potentially avoiding autoimmune adverse effects, and could improve the efficacy of T-cell based adoptive therapies.

Keywords: T-cells, genome editing, cancer, cell therapies, immune checkpoints

INTRODUCTION

Although there is still controversy over the role of the immune system in protecting the organism against the development of neoplasms in a natural setting (1) it is well accepted that artificial immunity can efficiently contain and even eradicate established tumors (2). Harnessing the anti-tumor potential of T-cells, and particularly CD8⁺ T-cells, is a promising approach for curative cancer treatment. Because of their relative ease of administration and documented low toxicities therapeutic vaccines that trigger T-cell responses are a very attractive approach. However, even though they efficiently induce antigen-specific immunity, the clinical benefit of cancer vaccines has so far been limited (3). In contrast adoptive cell therapies (ACT), where T-cells are modified *ex vivo* and re-infused in a patient's circulation, are more difficult to implement and require important infrastructural investment. Yet a number of studies have now reported long-term remissions or tumor clearance (4–6), warranting further development of the therapeutic concept.

While conferring the immune system with the ability to recognize tumors through vaccination or ACT is a pre-requisite for the induction of efficient anti-tumor responses it is likely insufficient to achieve long-term clinical benefit in a majority of patients. An increasing body of evidence points to the necessity of combining different therapeutic approaches in order to improve treatment outcome (7, 8). For instance several small-molecule compounds that target oncogenic pathways also enhance tumor destruction by immune mechanisms, e.g., by sensitizing cancer cells to cytolysis (9, 10). The coordinated delivery of these compounds with immunotherapies is expected to improve clinical responses in an additive or even synergistic manner. Similarly the combination of immune-based therapies also holds great potential. Monoclonal

antibodies (mAbs) blocking immune checkpoint receptors have recently emerged as promising therapeutics and many believe that the recent marketing authorization of Ipilimumab, targeting CTLA-4, heralds great strides in this area. Immune checkpoint receptor blocking agents are currently marketed or developed as single therapies but are expected to achieve maximal efficacy in combination with immune stimulatory approaches such as vaccination or ACTs (11, 12). Although generic treatment combinations will undoubtedly provide some degree of clinical benefit it is the prospect of developing personalized therapies tailored to individual needs that holds the greatest potential to improve clinical outcome in cancer therapy. The heterogeneous nature of similar tumor histologies as well as individual genetic variability are believed to account for the varied response levels to generic treatments and the wider availability of prognostic tools should help define adequate treatment options that improve patient response. With respect to cancer vaccines or ACTs information about the nature of the immune checkpoint pathway(s) relevant to a tumor would be particularly useful in order to counteract immune suppression.

T-cell-based ACTs rely on the infusion in a patient's circulation of *ex vivo* expanded tumor-infiltrating lymphocytes (TILs) or peripheral blood T-cells transduced with viral vectors expressing a tumor-specific antigen receptor. This engineering step offers the opportunity to transfer additional genetic material conferring T-cells with enhanced anti-tumor activity. Targeted genome editing relying on viral gene transfer could readily be combined with the delivery of antigen receptors at little additional cost in one unique therapeutic entity. This approach would avoid the drawbacks associated with combining treatment modalities of different nature requiring distinct administration regimens, e.g., cellular therapy and mAb injection. In addition cell-intrinsic disruption

of immune checkpoints in tumor-specific T-cells is likely to display a better safety profile than the systemic administration of blocking agents. Recently developed gene targeting technologies such as zinc-finger proteins (ZFPs), transcription activator-like proteins (TALs), and RNA-guided endonucleases (RGENs) could thus be harnessed in order to silence the expression of T-cell-intrinsic genes that restrain their anti-tumor potential.

MAIN TEXT

TECHNICAL ASPECTS AND CHALLENGES TO THE MODULATION OF GENE EXPRESSION IN T-CELLS

RNA interference (RNAi) often is the technique of choice to silence gene expression in somatic cells and lentivirus-mediated RNAi is a good option for sustained and efficient silencing. Most lentiviral RNAi systems express short-hairpin RNAs (shRNAs) from RNApolIII promoters, which drive high levels of transcription using precise initiation and termination sites. A recurrent problem of lentivirus-mediated RNAi, which is particularly salient in T-cells (13), is that the constant generation of shRNAs interferes with endogenous miRNA biogenesis and can result in the deregulation of gene expression (14, 15). This issue has prompted investigators to seek alternative methods to silence gene expression (16).

Recently developed genome editing technologies based on DNA-targeting proteins have the potential to revolutionize ACTs by offering convenient tools to alter gene expression. TAL effector-nucleases (TALENs) and ZFP-nucleases (ZFNs) effect complete gene knockout (Figure 1A) and are promising alternatives to RNAi for therapeutic applications (17–19). TALs are bacterial DNA-binding proteins consisting of near identical 34 amino-acid modules that bind one nucleotide with high affinity. The variable 12th and 13th amino-acids of TALs, called repeat-variable di-nucleotide confers base specificity (NN → G/A, NI → A, NG → T, NK → G, HD → C, and NS → A/T/C/G) and TAL arrays that target a nucleotide sequence can be generated by assembling individual modules (17, 20). ZFPs are eukaryotic DNA-binding proteins. Cys2-His2 fingers, which are used for genome editing, are the most common ZFP motif (21) and are each specific for a nucleotide triplet. Artificial ZFP domains that target specific DNA sequences, usually 9–18 nt long, can be constructed by assembling individual fingers (18). ZFPs and TALs have similar modular configurations but TALs can in theory target any stretch of nucleotides beginning with a thymidine whereas some structural incompatibilities between individual ZFP modules due to overlapping DNA-binding domains make the assembly

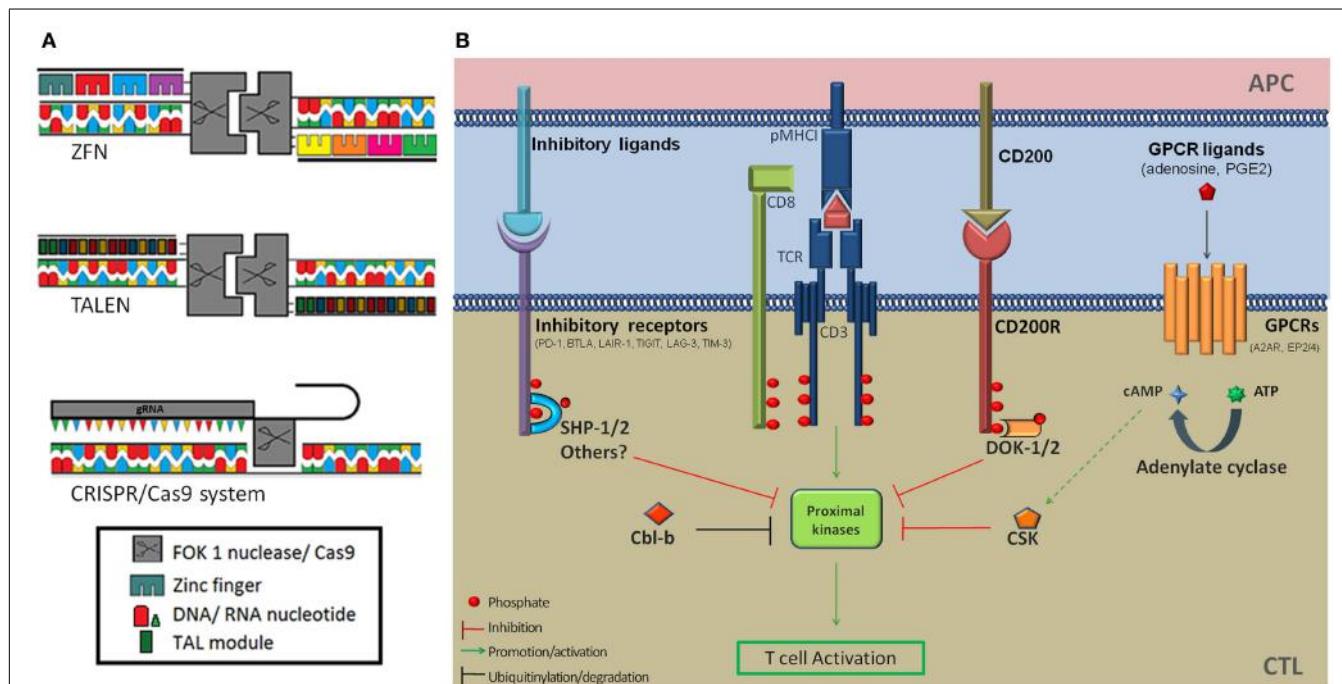


FIGURE 1 | (A) Schematic diagram of the ZFN, TALEN, and CRISPR/Cas9 genome editing tools. **(B)** Inhibition of T-cell activation by immune checkpoint receptors and downstream signaling proteins. Several co-inhibitory receptors (PD-1, BTLA, and LAIR-1) inhibit T-cell signaling by recruiting the SHP-1 and/or SHP-2 tyrosine phosphatases at proximity of the TCR signaling complex via ITIMs and ITSMs. This results in the dephosphorylation of proximal kinases downstream of TCR triggering. In addition PD-1 ligation was shown to induce increased expression of the Cbl-b E3 ubiquitin ligase, which targets signaling molecules for degradation. Activation of the CD200R leads to the recruitment of DOK2 and RasGAP to its intra-cellular domain, resulting in the inhibition of downstream MAP kinases. The adenosine receptor 2A and PGE2 receptors EP2 and EP4 modulate T-cell activation through mobilization of the cAMP-PKA-CSK pathway. CSK phosphorylates the inhibitory C-terminal

tyrosine residue of Src kinases and negatively regulates TCR signaling. A2AR, adenosine A2a receptor; APC, antigen-presenting cell; BTLA-4, B- and T-lymphocyte attenuator; Cbl-b, casitas B-cell lymphoma; CTL, cytotoxic T-lymphocyte; DOK-1/2, docking protein 1/2; EP2/4, prostaglandin E receptor 2/4; Erk, extra-cellular signal regulated kinases; ITIM, immunoreceptor tyrosine-based inhibition motif; ITSM, immunoreceptor tyrosine-based switch motif; GPCR, G-protein coupled receptor; LAG-3, lymphocyte-activation gene 3; LAIR-1, leukocyte-associated immunoglobulin-like receptor 1; Lck, lymphocyte-specific protein tyrosine kinase; MHC1, major histocompatibility complex class 1; PD-1, programmed death receptor 1; PD1-L1, programmed death receptor 1-ligand 1; SHP-1, Src homology 2 domain containing protein tyrosine phosphatase; TCR, T-cell receptor; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TIM-3, T-cell immunoglobulin domain and mucin domain 3.

of oligomeric ZFPs error-prone and narrow down the diversity of possible target DNA sequences. A successful and popular application of these technologies is the fusion of customized ZFPs or TALs to the catalytic domain of the restriction nuclease Fok1 (ZFNs and TALENs). Fok1 nucleases catalyze DNA double strand breaks (DSBs) when they dimerize (22). TALENs and ZFNs are therefore designed in pairs that target adjacent sequences on opposite DNA strands, thereby promoting Fok1 dimerization, separated by a spacer region where DNA cleavage occurs. Non-homologous end joining (NHE) repair of DSBs results in insertions or deletions at the DNA cleavage site (17, 23) and bi-allelic frameshift mutations that result in complete knockout occur at low frequencies (18, 24). The overall efficiency of the approach is sufficient to generate knockout cells following appropriate selection procedures. Of note TALEN design is more flexible as they can accommodate spacers of different lengths (25) whereas ZFNs strictly require 5–7 nt (26). Taking this into account, as well as structural constraints mentioned above, it is estimated that the frequency of target sequences is 1 in 500 bp for ZFNs and 1 in 35 bp for TALENs (20).

RNA-guided endonucleases provide a distinct and attractive alternative to genome editing compared with protein-guided nucleases. The functions of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins as a system providing adaptive immunity to bacteria against bacteriophages (27, 28) was recently harnessed for genome engineering (29, 30). The Cas9 nuclease binds to a short complementary RNA (crRNA) providing DNA-targeting specificity and to a trans-activating crRNA (tracrRNA), required for crRNA processing, expressed individually or combined as a chimeric guide RNA (gRNA) (Figure 1A). CRISPR-Cas9 systems displayed a cleavage efficiency comparable (31) or superior (32) to TALENs in human cells. The clear advantage of RGEN is that it can be easily adapted to target different genomic sequences by customizing the synthetic crRNA/gRNA delivered in combination with Cas9 (33). In comparison ZFNs and TALENs require extensive engineering and validation steps.

The delivery of genome editing agents to T-cells is a crucial aspect of their successful application to ACTs. Because nuclease-based genome editing relies on generating transmissible mutations, protein- or RNA-guided nucleases only need to be transiently expressed. In fact transient expression probably minimizes off-target DNA cleavage (34). Provasi et al. have used integration-deficient lentiviruses as well as adenoviruses in order to modify the genome of T-cells with ZFNs (35). Of note it was recently shown that, due to their very repetitive nature, TAL arrays were incompatible with efficient reverse transcription required for the delivery of genetic material using lentiviruses (36), thereby limiting the range of delivery methods for TALENs.

APPLICATION OF THERAPEUTIC GENOME EDITING TO T-CELLS

Crucially therapies based on T-cell genome editing have already entered clinical development. A phase II clinical trial based on preventing the expression of the CCR5 gene, acting as a co-receptor for HIV in CD4⁺ T-cells, using ZFNs (37) was recently initiated for the treatment of HIV/AIDS (NCT01252641). The safety results will be of huge importance for ZFN-based therapies and for

genome editing in T-cells in general. Moreover a recent study provided proof of concept for the combination of TCR gene delivery with genome editing by using ZFNs specific for the endogenous constant TCR gene segments in order to prevent mispairing with ectopic TCR chains (35). The success of this approach provides a good rationale for wider applications of ZFN genome editing to T-cells.

Enhancing the anti-tumor potential of CD8⁺ T-cells through genome editing can be done in many ways. Here we will focus on disrupting the expression of genes that inhibit T-cell functions as a result of the suppressive activity of the tumor micro-environment. T-cell inhibitory pathways targeted by genome editing in the context cancer ACTs should meet several criteria. First, their mechanism of action should be strictly cell-intrinsic. Second, they should be relevant to effector T-cells as opposed to naive T-cells. For instance CTLA-4 does not meet these two criteria since it works at least partly by reducing the availability of co-stimulatory molecules on the surface of antigen-presenting cells during the priming of naive T-cells (38). Finally, since only anti-tumor T-cells are modified, one of the advantages of this approach is that it allows targeting ubiquitous suppressive pathways whose systemic blockade or inhibition might result in serious adverse effects. Because it is clinically validated the most obvious target is probably PDCD1: the gene encoding the co-inhibitory receptor PD-1. PD-1 is expressed on activated T-cells and its engagement by its two known ligands PD-L1 and PD-L2 inhibits proximal signaling events triggered by TCR stimulation through recruitment of the phosphatase SHP-2 (39) and increased expression of the E3 ubiquitin ligase Cbl-b (40), which impair key components of the TCR signaling cascade through dephosphorylation and proteasomal degradation (Figure 1B). High cellular expression levels of PD-1 are characteristic of exhausted CD8⁺ T-cells in chronic viral infections as well as TILs and correlate with impaired effector functions (41). Histological analyses have revealed that numerous tumor types express one or both PD-1 ligands (42, 43), prompting the targeting of this pathway in order to augment anti-tumor immunity. PD-1 blockade has shown promising objective response rates in a range of cancer indications and it is anticipated that PD-1 blocking agents will be approved for marketing authorization as mono-therapies. In addition these therapeutics are evaluated in combination with cancer vaccines, small-molecule signaling inhibitors, tumor-targeting mAbs, and cytokine therapy (<http://clinicaltrials.gov/ct2/results?term=pd1&Search=Search>). The combination of PD-1 blockade with these treatments, as well as with cancer ACTs, is expected to further enhance anti-tumor activity (11). Several other co-inhibitory receptors expressed by T-cells qualify as targets for gene editing coupled with antigen receptor delivery (Table 1; Figure 1B). *In vivo* and *in vitro* pre-clinical data strongly support the development of reagents targeting TIM-3 and LAG-3. Dual targeting of PD-1 and TIM-3 or LAG-3 with mAbs synergistically enhanced anti-tumor responses (44) and pre-clinical evaluations of a soluble Fc-LAG3 complex, which has now entered clinical development, were promising (45). Other targets are currently under similar evaluation procedures and might expand the list of druggable co-inhibitory receptors for cancer immunotherapy (Table 1; Figure 1B).

Table 1 | Potential immune checkpoint receptor targets for genome editing in the context of cancer adoptive cellular therapies.

	Name (gene)	Function	Ligand	Intra-cellular signaling/second messengers	Recognition motif	References
Co-inhibitory receptors	PD-1 (CD279)	Inhibition of T-cell activation and promotion of tolerance	PD-L1 (B7-H1) PD-L2 (B7-DC)	SHP-1 SHP-2	ITIM ITSM	Keir et al. (56), Parry et al. (57)
	LAG-3 (CD223)	Down regulation of T-cell cytokine secretion and proliferation	MHCII	–	–	Pardoll (12), Turnis et al. (58)
	BTLA (CD272)	Suppression of T-cell response	HVEM	SHP-2	ITIM	Murphy et al. (59), Watanabe et al. (60)
	OX2R (CD200R)	Inhibits T-cell function	CD200	DOK2	NPxY	Kretz-Rommel et al. (61), Moreaux et al. (62), Pallasch et al. (63)
	TIM-3	Down regulation of T-cell cytokine secretion and proliferation	Galectin 9 Phosphatidylserine	–	–	Pardoll (12), Zhu et al. (64)
	TIGIT	Inhibition of T-cell activation	VR, PVRL2, and PVRL3	–	ITIM	Joller et al. (65)
	LAIR-1	Inhibits cytotoxic activity	Collagen	SHP-1 SHP-2	ITIM	Lebbink et al. (66), Meyaard (67)
	PGE2 receptors EP2/4	Inhibition of T-cell activity	PGE2	Adenylyl cyclase cAMP	–	Mahic et al. (68), Oberprieler et al. (53)
Receptors for soluble regulatory mediators	Adenosine receptor 2A (A2AR)	Blocks T-cell activity	Adenosine	Adenylyl cyclase cAMP	–	Pardoll (12), Ohta et al. (50), Raskovalova et al. (69)

Non-extensive list of immune checkpoint receptors known to impair anti-tumor T-cell immunity in a cell-intrinsic manner. The relevant intra-cellular signaling and second messenger pathways, when known, are indicated.

The presence of cognate ligands within the tumor micro-environment is a crucial aspect for targeting co-inhibitory receptors and other immune checkpoint receptors. In the case of PD-1 retrospective analysis of patient biopsies in the phase 1b clinical trial assessing the blocking mAb BMS-936558 showed that the objective response rate in patients whose tumors expressed PD-L1 reached 36% compared with 18% in the entire cohort and 0% among patients with PD-L1-negative tumors (46). These striking results highlight the importance of prognosis and patient stratification for the design of appropriate cancer immunotherapies based on PD-1 inhibition. Such a strong correlation is still to be established for other immune checkpoint receptors but it is tempting to speculate that similar principles are applicable. However, even though their relevance in tumor immunity is established, it is not entirely clear what the actual ligands for several co-inhibitory receptors are in the context of anti-tumor immunity. More fundamental and clinical investigations are required in order to unambiguously identify relevant inhibitory ligands and assess their presence in the tumor environment.

Several soluble regulatory mediators also act as immune checkpoints in anti-tumor immunity. For instance high levels of extracellular adenosine are found in the vicinity of many solid tumors because of the hypoxic environment, a well-known environmental factor promoting adenosine release. Suppressive adenosine is also generated through direct dephosphorylation of extracellular adenosine nucleotides by the cell-surface nucleotidases CD39 and CD73 expressed by regulatory T-cells (T_{regs}) and some tumors, e.g., ovarian carcinomas (47, 48). The adenosine receptor

2A (A2AR), belonging to the G-protein coupled receptor family (GPCRs), is expressed on T-cells and has been identified as a target for immunotherapy for over a decade (12, 49–51). The A2AR inhibits T-cell activation through the cAMP-PKAi-CSK pathway (Figure 1B) and pre-clinical *in vivo* models have shown that A2AR knock-down or antagonism in adoptively transferred T-cells dramatically increased anti-tumor immunity (50). Inhibiting adenosine-mediated immune suppression is therefore believed to be an efficient strategy for cancer immunotherapies. Yet because adenosine receptors are ubiquitously expressed and involved in many physiological processes, especially in neurotransmission, classical antagonistic approaches are likely to result in a number of side effects. Such systemic adverse effects could be avoided in the context of adoptive T-cell therapies through T-cell-intrinsic gene editing or the *in vitro* selection of desensitized and irresponsible T-cells (52). Similarly, prostaglandin E2 (PGE2) directly suppresses T-cell activation through the cAMP second messenger pathway in effector/memory CD8⁺ T-cells (53). Tumor-associated T_{regs} as well as colorectal cancer cells express high levels of immunosuppressive PGE2 (54). Interfering with EP2/EP4 receptors expression in T-cells may therefore enhance their anti-tumor potential.

A broader, possibly riskier, alternative to targeting individual immune checkpoint receptors would be interfering with the expression of downstream molecules conveying intra-cellular inhibitory signals. For instance several co-inhibitory receptors use the tyrosine phosphatases SHP-1 and/or SHP-2 to inhibit T-cell activation (Figure 1B). Inhibition of SHP-1/2 expression may therefore confer resistance to several checkpoint pathways used by

tumors. Stromnes and colleagues reported that conditional knockout of SHP-1 in mature murine CD8⁺ T-cells improved effector cell functions and tumor clearance in an adoptive transfer setting similar to cancer ACTs without resulting in autoimmune toxicity, thereby providing a good rationale for such an approach (55).

CONCLUDING REMARKS

T-cell based ACTs that rely on the re-infusion of patient T-cells expressing an artificial antigen receptor is an epitome of personalized medicine. These therapies require the identification of specific tumor antigens and/or patient HLA-type and would undoubtedly benefit from further prognostic analysis and subsequent treatment customization. Based on recent successes in cancer immunotherapy, immune checkpoint receptors that suppress T-cells represent a particularly attractive class of targets for such an approach. We believe that enhancing the anti-tumor potential of re-directed T-cells by targeting inhibitory pathways through genome editing can further improve the efficacy of cancer ACTs. Moreover cell-intrinsic inhibition of these pathways may display an advantageous safety profile compared with immune checkpoint blockade relying on the systemic administration of mAbs, recombinant proteins, or small molecules.

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Expression of concern: Co-receptor CD8-mediated modulation of T-cell receptor functional sensitivity and epitope recognition degeneracy

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EXPRESSION OF CONCERN

The Editorial Board has been informed by the authors of “Co-receptor CD8-mediated modulation of T-cell receptor functional sensitivity and epitope

recognition degeneracy” that the parameter values as reported in the legends are mutually inconsistent and therefore the results of the simulations have to be reconfirmed. This is to notify readers that the data as published in the original article are not validated and that the authors have stated to the Editorial Board that a Corrigendum is in preparation.

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