

T Cell-Positive Selection Uses Self-Ligand Binding Strength to Optimize Repertoire Recognition of Foreign Antigens

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SUMMARY

Developing T cells express diverse antigen receptors whose specificities are not prematched to the foreign antigens they eventually encounter. Past experiments have revealed that thymocytes must productively signal in response to self antigens to mature and enter the peripheral T cell pool (positive selection), but how this process enhances effective mature T cell responses to foreign antigen is not fully understood. Here we have documented an unsuspected connection between thymic recognition events and foreign antigen-driven T cell responses. We find that the strength of self-reactivity is a clone-specific property unexpectedly directly related to the strength of T cell receptor (TCR) binding to presented foreign antigen. T cells with receptors showing stronger interaction with self dominate in responses to infections and accumulate in aging individuals, revealing that positive selection contributes to effective immunity by skewing the mature TCR repertoire toward highly effective recognition of pathogens that pose a danger to the host.

INTRODUCTION

CD4⁺ and CD8⁺ T cells are crucial effector cells whose response to infection requires recognition of pathogen-derived peptides bound to molecules encoded by the major histocompatibility complex (pMHC). The relevant pMHC antigen receptors are clone-specific heterodimeric molecules ($\alpha\beta$ TCRs) whose chains are derived via quasirandom somatic recombination of gene segments and nontemplated nucleotide addition (Davis and Bjorkman, 1988). This mechanism generates a population of immature T cells collectively capable of recognizing an enormous diversity of pMHC combinations, including clones with potentially harmful self-reactivity. To purge such dangerous cells, immature T cells are tested for self-responsiveness in the thymus. Strong signaling upon self-recognition results in removal of these cells from the mature repertoire (negative selection). Rather surprisingly, thymocyte survival and maturation requires productive signaling in response to self antigens, but

at a lower intensity than that leading to deletion (positive selection) (Morris and Allen, 2012).

Although the necessity of negative selection is easily appreciated, the benefit of positive selection to the immune system is not yet fully understood, although several hypotheses have been proposed. The earliest concept was that positive selection adapts the T cell repertoire to the specific allelic forms of MHC gene products expressed by the host ("MHC restriction") (Bevan, 1977). However, structural data show that contacts between TCR and MHC are mainly mediated by the CDR1 and CDR2 regions of the V α and V β segments of the TCR, which are germline encoded (Garboczi et al., 1996; Reinherz et al., 1999), and, unlike what the MHC restriction hypothesis would predict, concordant variations between V α and V β abundance and MHC haplotype have rarely been observed (Garcia et al., 2009; Marrack et al., 2008; Okada and Weissman, 1989; Rock et al., 1994). Indeed, residues in CDR1 and CDR2 have been described that contact conserved amino acids present in different MHC alleles. Some CDR2 residues are conserved across different species, suggesting that the specificity of the TCR for MHC was evolutionarily selected (Marrack et al., 2008; Scott-Browne et al., 2011). It is the variable region of the TCR, CDR3, that primarily contacts presented peptide and dictates the strength of recognition (Jorgensen et al., 1992), consistent with evidence that the peptides presented in the thymus substantially impact the diversity of the TCR repertoire (Barton and Rudensky, 1999; Germain, 1990; Grubin et al., 1997; Hogquist et al., 1993; Nikolić-Zugčić and Bevan, 1990).

More recently, two other possible roles for self pMHC in the generation of T cell responses to foreign antigen have been described. Interactions of naive T cells with self pMHC result in the partial tyrosine phosphorylation of the TCR ζ chain, polarize components of the signaling apparatus, and sustain T cell sensitivity to foreign antigen (Stefanová et al., 2002). In addition, specific self pMHCs can act as coagonists that augment the activation of T cells by agonist pMHC (Krogsgaard et al., 2005). In some instances, the coagonist self pMHC are the same as those responsible for positive selection in the thymus (Ebert et al., 2009; Lo et al., 2009). However, the *in vivo* relevance of coagonism remains controversial (Krogsgaard et al., 2005; Ma et al., 2008; Spörri and Reis e Sousa, 2002).

One straightforward hypothesis that has not been addressed is simply that TCRs able to bind self pMHC well (but below the negative selection threshold) also bind especially well to foreign

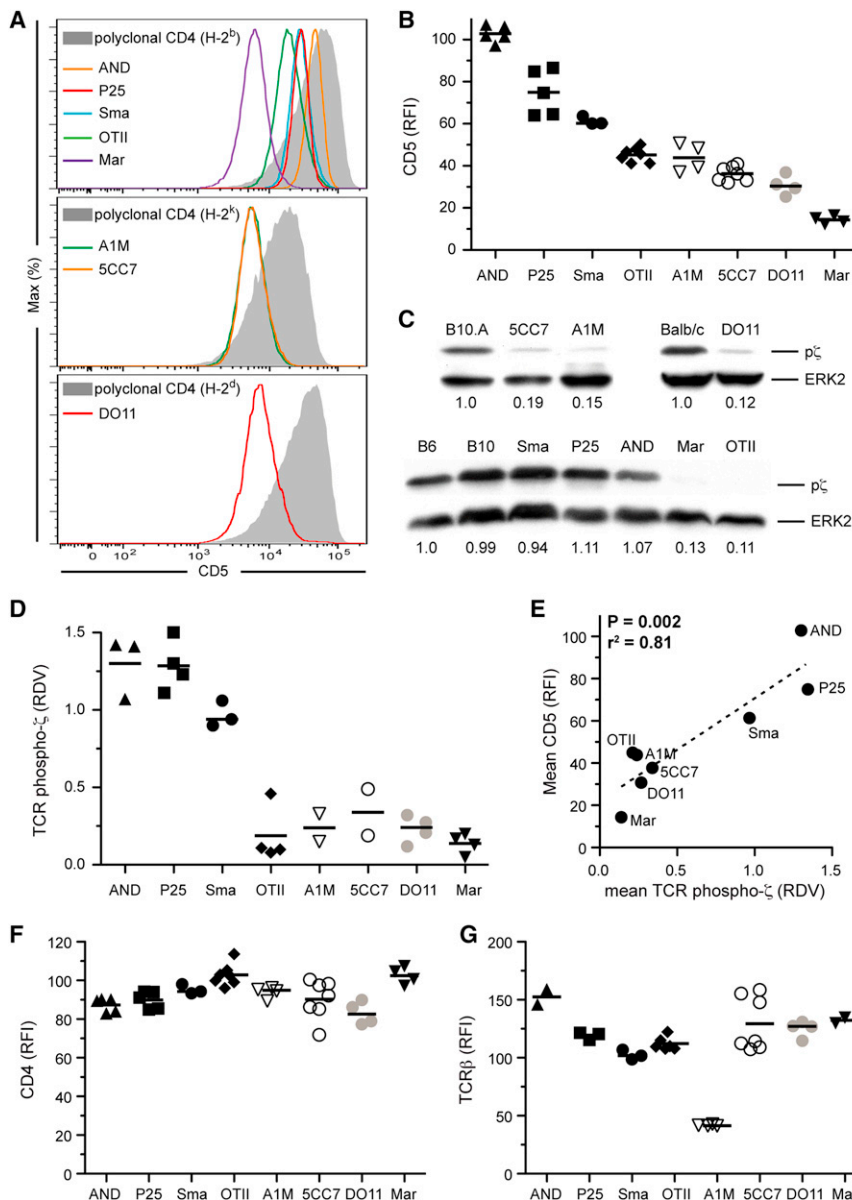


Figure 1. TCR Transgenic CD4⁺ T Cell CD5 Expression Reflects the Clone-Specific Strength of Self-Reactivity

(A and B) CD5 surface expression on naive peripheral CD4⁺ T cells from TCR Tg mice relative to polyclonal CD4⁺ T cells from mice of the respective background. Representative plots (A) and data from two to four independent experiments (B).

(C and D) Immunoblot for tyrosine phosphorylation of the TCR ζ chain and total ERK-2 in purified peripheral CD4⁺ T cells. Representative blots (C) with relative densitometry values (RDV) given below each lane (p-ζ/ERK2 ratio) relative to CD4⁺ polyclonal cells; data from two to four independent experiments (D).

(E) Correlation between mean CD5 relative fluorescent intensity (RFI) and mean pζ chain RDV for each TCR Tg strain.

(F and G) CD4 (F) and TCRβ (G) surface expression on naive CD4⁺ T cells from TCR Tg mice relative to polyclonal CD4⁺ T cells from mice of the respective background.

understanding basic T cell biology but suggest ways to probe and manipulate the polyclonal T cell pool to facilitate immune function or limit autoimmunity.

RESULTS

Naive CD4⁺ T Cell CD5 Expression Reflects the Clonotype-Specific Strength of TCR Self-Recognition

To determine whether there is a relationship between the self-reactivity of CD4⁺ T cells and their binding of agonist pMHC, we required methods for assessing the strength of self-recognition on both a clone-specific and population basis. Examining TCR self-binding affinities directly was impractical, given the technical problems of detecting the very low affinity of TCRs for self pMHC and

the difficulty of doing so for all individual naive CD4⁺ T cells. Instead, we took advantage of studies suggesting that the expression of CD5, a negative regulator of TCR signaling, is proportional to TCR signaling strength during immature T cell selection in the thymus (Azzam et al., 1998). The maintenance of CD5 expression on CD4⁺ and CD8⁺ T cells requires contact with self pMHC (Mandl et al., 2012; Smith et al., 2001) and is correlated with the extent of self-pMHC-driven lymphopenia-induced proliferation (Kassiotis et al., 2003; Kieper et al., 2004; Smith et al., 2001). These data suggested the possibility that the steady-state CD5 expression on peripheral naive CD4⁺ T cells might also reflect proximal TCR signal strength in response to self ligands.

To examine this issue, we first quantified CD5 on naive T cells from eight distinct CD4⁺ TCR transgenic (Tg) *Rag1*^{-/-} or *Rag2*^{-/-} mice (Figure 1). Each TCR Tg T cell population had a specific, reproducible surface amount of CD5 that, when

pMHC and hence that positive selection ensures that T cells most useful for host defense against pathogens are selected from a diverse initial repertoire to populate the peripheral T cell pool. A direct relationship between self and foreign antigen binding is not evident in available structural data on pMHC recognition by TCRs, but this would provide such a powerful explanation for the utility of positive selection that we decided to test the possibility experimentally. Here we report that indeed, there is such a direct relationship that can be seen with methods that avoid the complications of possible self-ligand coagonist function or persistent self-recognition by peripheral T cells in vivo. Quantitative studies showed that repertoire binding to self and foreign pMHC is strongly skewed during positive selection toward higher-affinity cells and that the latter predominate in response to diverse pathogens and among memory cells in mice and humans. These findings have implications not only for

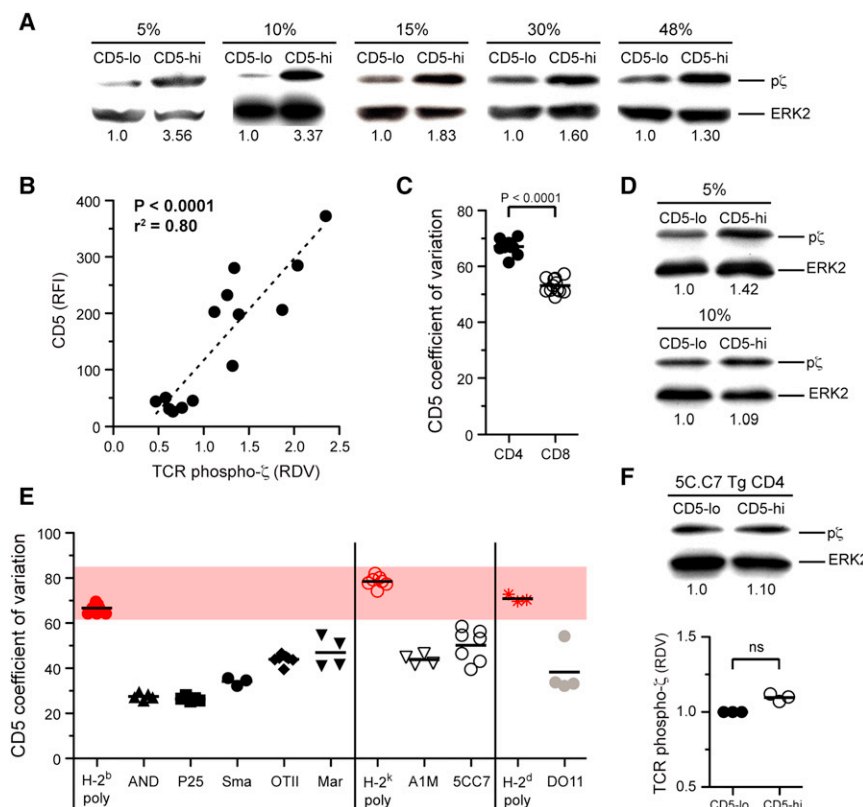


Figure 2. Polyclonal CD4⁺ T Cells Show a Broad Range of Self-Reactivity that Is Correlated with CD5 Expression

(A, D, and F) Immunoblot analysis for tyrosine phosphorylation of the TCR ζ chain and total ERK2 in naive polyclonal CD5^{hi}CD4⁺ and CD5^{lo}CD4⁺ T cells (A), polyclonal CD8⁺ T cells (D), and 5C.C7 TCR Tg CD4⁺ T cells (F). RDV relative to the CD5^{lo} population is given below each lane. Sort stringencies are for the 10% highest or lowest populations unless specified otherwise. Representative data from two to three experiments are shown. (B) Correlation between CD5 RFI and phospho- ζ RDV for CD4⁺ T cells sorted on CD5 expression. RDVs are normalized relative to unsorted CD4⁺ polyclonal cells. Data are from five independent experiments.

(C and E) CD5 coefficient of variation for polyclonal CD4⁺ and CD8⁺ T cells (C) and polyclonal or TCR Tg CD4⁺ T cells (E). ns, not significant.

comparing the various TCR Tg cell populations, covered a wide range from low (Marilyn) to high (AND) (Figures 1A and 1B). The narrow distribution of CD5 expression on cells from a given TCR Tg was in contrast to the broad distribution seen with polyclonal CD4⁺ T cells (Figure 1A). We next quantified steady-state TCR subthreshold signaling due to interaction with self ligands by measuring partial TCR ζ chain phosphorylation (p ζ) by immunoblot, finding that the amount of p ζ varied widely among the distinct clonal populations examined (Figures 1C and 1D). There was a significant correlation between measures of p ζ and CD5 for the different TCR Tg CD4⁺ T cells (Figure 1E), suggesting that CD5 expression reflects a clonotype-specific heterogeneity in TCR self-reactivity that is dependent on the strength of interaction with self pMHC. In contrast, there was no correlation between p ζ and either CD4, TCR β , CD127, CD122, Thy, CD45, CD69, CD25, CD62L, or CD2 (Figures 1F and 1G; Figure S1 available online). Consistent with partial ζ phosphorylation being dependent on interaction with self pMHC, p ζ amounts were unchanged in germ-free mice (Figure S2A).

To determine whether CD5 is a marker for clonotype-specific TCR self-reactivity within a polyclonal CD4⁺ T cell repertoire, we sorted T cells based on high or low CD5 expression and measured p ζ in sorted cells (Figures 2 and S2B). The more separate the sorted CD5^{hi} and CD5^{lo} populations, the greater the difference seen in p ζ amounts (Figure 2A). The difference was maximal (>3-fold) comparing the 5% CD5 brightest and dimmest naive CD4⁺ T cells, but was observed even when the population was divided in two based on CD5 expression (Figure 2A). Binding of CD5 antibody to CD4⁺ T cells did not impact p ζ , nor did it affect the responsiveness of T cells to agonist peptide in vitro

(Figure S2C). As with TCR Tg CD4⁺ T cells, we found a highly significant linear relationship between the proximal TCR signal strength measured by p ζ and the expression of CD5, but not of TCR β or CD4, on sorted polyclonal CD4⁺ T cells (Figures 2B and S2B). The positive correlation between CD5 and p ζ amounts might be unexpected given the reported inhibitory role of CD5 on TCR signaling (Azzam et al., 1998) and a simplistic model in which CD5 mediates perfect feedback control to normalize the functional pMHC signaling response of all T cell clones. These data make clear that any negative regulatory role of CD5, although it may blunt the response of highly reactive T cells, does not equalize TCR subthreshold signal strength among CD4⁺ T cells.

For polyclonal CD8⁺ T cells, the CD5 distribution was significantly narrower than for CD4⁺ T cells (Figure 2C) and differences in p ζ amounts were less pronounced among CD8⁺ T cells sorted for CD5 expression (Figure 2D). CD8 coreceptor expression is tuned according to self-affinity on CD8⁺ T cells, which may act to reduce the effective breadth in TCR reactivity for self pMHC on CD8⁺ T cells (Park et al., 2007). Consistent with this, we found an inverse relationship between CD8 and CD5 expression on CD8⁺ T cells (Figure S2D). In contrast, on CD4⁺ T cells we saw no such equalizing role for the CD4 coreceptor (Figure 1F). As would be expected if CD5 reflects clonotypic heterogeneity in self-reactivity, for all mouse strains tested, the CD5 coefficient of variation was greater among polyclonal CD4⁺ T cells than for any individual TCR Tg CD4⁺ T cell population (Figure 2E). In contrast to polyclonal cells, we could not detect a significant difference in p ζ between the highest and lowest 10% CD5 expressors among monoclonal 5C.C7 or other TCR Tg cells (Figure 2F and data not shown). Together, these data suggest that CD5 is a surface marker on naive CD4⁺ T cells whose variable expression in a polyclonal T cell population reflects heterogeneity in the strength of individual antigen receptor reactivity to nonagonist self ligands.

An Unexpected Direct Relationship between TCR Self-Reactivity and Strength of Binding to Agonist Peptide-MHC

Given the diversity among TCR Tg T cells in self-reactivity and our ability to track this parameter by using CD5 expression, we next asked whether there were also differences in the binding strength of these TCRs to agonist foreign pMHC antigen, which contributes to clonal dominance during foreign antigen responses (Busch and Pamer, 1999; Malherbe et al., 2004; Price et al., 2005; Savage et al., 1999). To this end, we used pMHC-II tetramers, which are antigen-based reagents able to stain only cells with TCRs whose binding strength is above a particular threshold (Sabatino et al., 2011). TCRs with a low binding strength often dissociate faster from pMHC tetramers and therefore binding can be partly or completely absent at the time of post-wash analysis (Busch and Pamer, 1999; Crawford et al., 1998; Laugel et al., 2007; Yee et al., 1999). This method to evaluate foreign antigen recognition rather than functional response assays avoids the possible confounding effects of self ligands contributing as coagonists to the measured response. OT-II, 5C.C7, and DO11.10 TCR Tg T cells, all low in CD5 surface expression, did not show staining with their specific tetramer at 13 μ g/ml or less. In contrast, SMARTA, AND, and P25 TCR Tg T cells, which are high CD5 expressors, had detectable pMHC-II tetramer staining under these conditions (Figure 3A). These data are consistent with results in the literature: 2D2 TCR Tg T cells do not stain with specific MOG:I-A^b pMHC-II tetramers (Sabatino et al., 2011) and have low CD5 and p ζ (data not shown).

At much higher concentrations, tetramer binding was observed with OT-II, DO11.10, and 5C.C7 Tg cells (Figure S3A), indicating that the absence of tetramer binding at lower concentrations was not due to nonfunctional reagents. Furthermore, omitting the wash step, or acquiring the fluorescent signal of cells immediately after washing, enhanced the staining of the CD5^{lo} TCR Tg 5C.C7 and OT-II cells (Figure S3B), suggesting that the absence of tetramer binding for CD5^{lo} TCR Tg cells was attributable to a faster off-rate. The tetramer staining conditions used did not lead to T cell activation as measured by CD69 expression or to changes in CD5 expression (data not shown). The PCC:I-E^k tetramer that failed to stain 5C.C7 Tg T cells was identical to the tetramer used to successfully stain AND TCR Tg T cells at the same concentrations (Figure 3A). An MCC:I-E^k tetramer does stain 5C.C7 Tg T cells (Savage et al., 1999), but the staining was nonetheless still of lower intensity than AND cells exposed to the same tetramer concentration (Figure S3C). A tetramer dissociation assay showed that CD5^{lo} 5C.C7 cells had a faster dissociation rate from MCC:I-E^k than CD5^{hi} AND cells (Figure S3D). Together, these data allowed us to separate our panel of TCR Tg mice into two groups: CD5^{hi} clones that showed good tetramer binding and CD5^{lo} clones that showed poor tetramer binding, suggesting a possible relationship between strength of TCR self-pMHC reactivity and their specific foreign-pMHC binding strength.

To ask whether there is indeed a direct relationship between the strength of self- and foreign-antigen recognition, we assessed the relationship between CD5 expression and tetramer staining in a pool of naive polyclonal CD4⁺ T cells. In this

case, all the antigen-reactive cells above a certain affinity threshold for the pMHC employed would be stained by the same reagent. By using a magnetic bead enrichment method, we examined a population of 2W1S:I-A^b-specific CD4⁺ T cells, for which there are ~200 precursor cells in a preimmune mouse (Moon et al., 2007). Concatenation of staining data from five to seven mice yielded ~1,000 tetramer⁺ events, allowing us to divide these into equal event gates according to tetramer staining intensity and examine both CD5 and CD3 expression on cells within each gate (Figure S3E). We found a significant positive correlation between CD5 and 2W1S tetramer staining intensity but not between CD3 and 2W1S tetramer staining intensity (Figure 3B). Furthermore, inverting the fluorophores used for CD3 and CD5 staining gave the same result (Figure 3B). Because only the 2W1S-specific CD4⁺ T cell precursor frequency was large enough to perform such a correlation analysis with available MHC-II tetramer reagents, we investigated whether the relationship between agonist pMHC binding and CD5 expression was present with other specific preimmune CD4⁺ T cell cohorts by a modified methodology. We sorted polyclonal naive CD4⁺ T cells from 20 mice for the 15% CD5 highest and 15% dimmest and then stained the sorted populations with one of two distinct foreign antigen tetramers, LCMV GP₆₆₋₇₇:I-A^b or Flg_{n456-475}:I-A^b. As for 2W1S-specific CD4⁺ T cells, the more self-reactive CD5^{hi}CD4⁺ T cells had a greater tetramer staining intensity with each reagent compared with the less self-reactive CD5^{lo}CD4⁺ T cells (Figure 3C). This difference could not be attributed to differences in CD4 or TCR β expression (Figure 3C).

TCR polarization results from active recognition of self ligands by CD4⁺ T cells in the lymphoid microenvironment (Stefanová et al., 2002). TCR clustering in response to tonic self-engagement could facilitate effective multivalent tetramer binding and explain the differential staining of CD5^{lo} and CD5^{hi} cells, rather than such differences arising from the biophysical ligand-binding properties of the TCR recognition domain. To examine this possibility, we tested whether T cells maintained in the absence of self ligands show the differences in tetramer binding seen for freshly isolated naive T cells. We generated hybridomas bearing the 5C.C7 (CD5^{lo}) and the AND (CD5^{hi}) TCRs and tested them for the ability to bind MCC:I-E^k tetramer. Despite growing autonomously in the absence of self pMHC, 5C.C7 and AND hybridomas showed the same difference in tetramer binding as fresh TCR Tg cells, demonstrating that TCR-intrinsic biophysical properties determine the tetramer staining differences observed (Figure 4A). This difference was not a result of lower TCR expression by the 5C.C7 hybridoma, as shown by the fact that CD3 ϵ expression was similar to that of the AND hybridoma (Figure 4A).

We also considered the possibility that CD5 was directly involved in pMHC binding and assessed tetramer binding of Cd5^{-/-} TCR Tg CD4⁺ T cells. Both TCR β and tetramer staining was diminished on Cd5^{-/-} compared to wild-type AND and 5C.C7 cells (Figure 4B). However, reduced TCR β expression in Cd5^{-/-} cells entirely explained the reduction in tetramer staining. Therefore, consistent with studies that showed that the activity of CD5 as a negative regulator of TCR signaling does not require its extracellular domain (Bhandoola et al., 2002), CD5 played no direct role in agonist pMHC binding (Figure 4B).

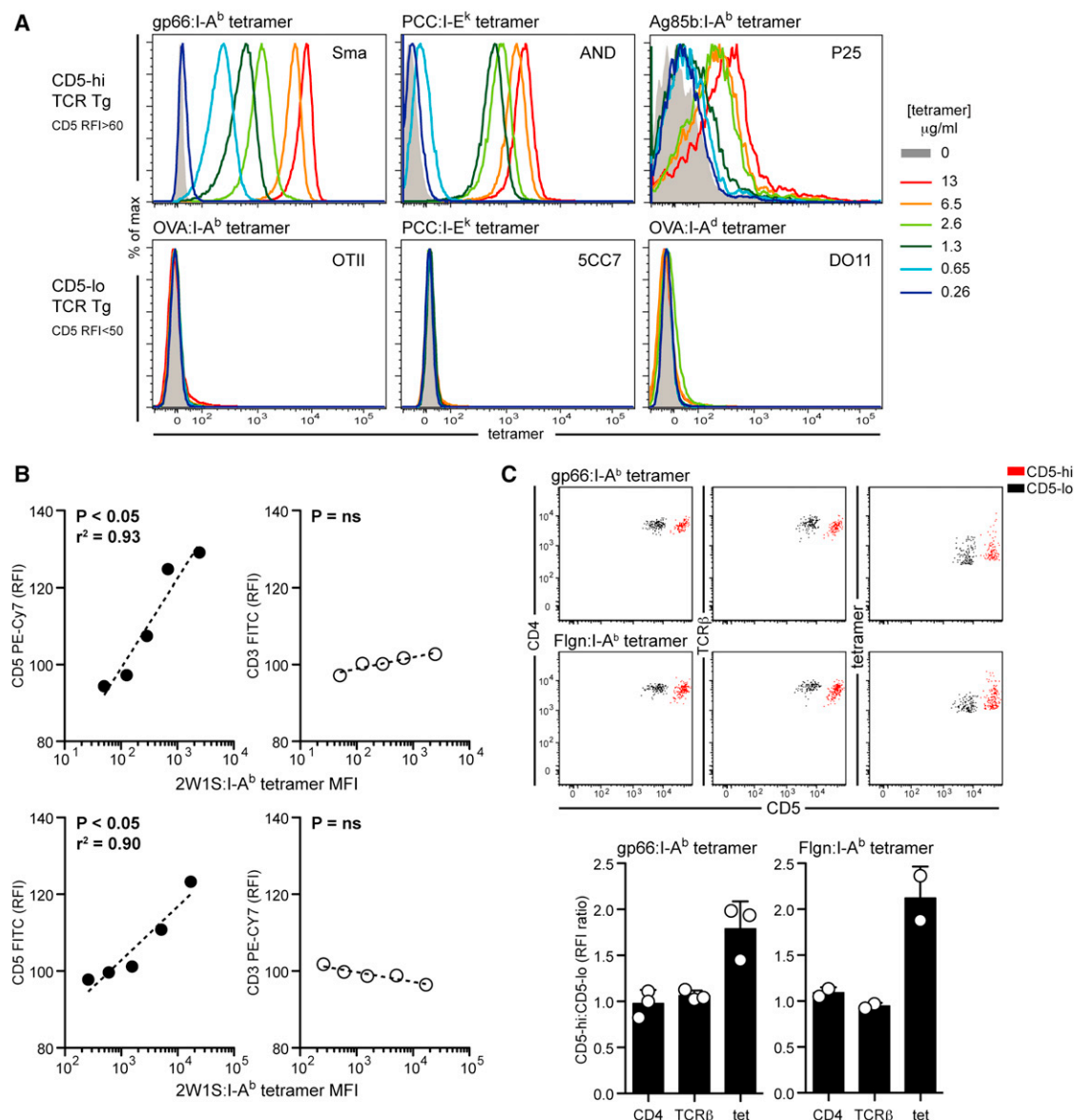


Figure 3. CD4⁺ T Cell Self-Reactivity Is Positively Correlated with TCR Strength of Binding to Foreign Antigen

(A) Dose response of binding of specific pMHC tetramers to TCR Tg CD4⁺ T cells. Data are representative of three to four independent experiments.

(B) Naive CD4⁺ T cells sorted from preimmune mice with 2W1S:I-A^b tetramer binding were gated by tetramer staining intensity after concatenation of data from five to seven mice and analyzed for CD3 and CD5 expression by flow cytometry. Correlation between tetramer MFI and CD3 or CD5 RFI is shown (top). Results were unchanged when fluorochromes were inverted (bottom). Data are representative of three independent experiments.

(C) CD4, TCRβ, and pMHC-tetramer staining are shown as representative dot plots (top) or the RFI ratio between the brightest and dimmest 15% CD5^{hi} and CD5^{lo} cells sorted from naive polyclonal CD4⁺ T cells pooled from 20 mice (bottom). Bars show mean ± SD; ns, not significant.

Heterogeneity in TCR Self-Reactivity Results in the Differential Contribution of CD4⁺ T Cells to Foreign Antigen Responses

If this relationship between self and foreign antigen recognition represents a key aspect of functional T cell biology, we would expect variation in self-reactivity to influence the response of polyclonal T cell responses to infectious agents. We predicted that naive CD4⁺ T cells on the high end of the scale of self-reactivity would contribute more to foreign antigen responses *in vivo*, although the difference would not be expected to be large

because less useful cells have already been filtered out by thymic “neglect.” Previous studies describing the outgrowth of T cells with greater affinity for agonist pMHC have relied on identifying specific cells via tetramers (Busch and Pamer, 1999; Malherbe et al., 2004; Price et al., 2005; Savage et al., 1999), which may miss a substantial number of T cells contributing to the response (Sabatino et al., 2011). Therefore, we sorted the 15%–20% CD5 brightest and dimmest cells from two naive polyclonal CD4⁺ T cell populations distinguishable by a congenic marker, mixed the sorted cells in a ~1:1 ratio,

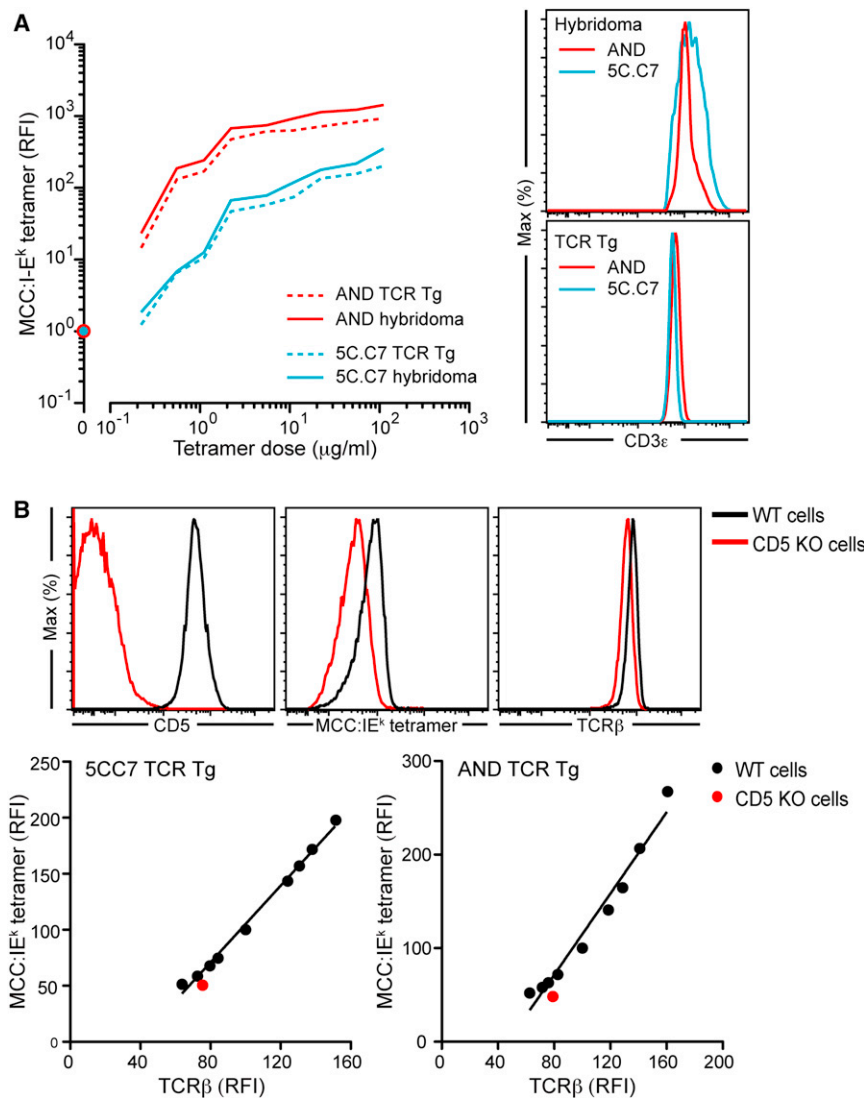


Figure 4. Relationship between CD5 and TCR Agonist Binding Strength Is Not Explained by Factors aside from TCR Specificity

(A) T cell hybridomas derived from 5C.C7 and AND TCR Tg T cells were expanded in culture 2 weeks after hybrid selection, and their ability to bind to MCC:IE^K was determined. Left: binding curves for fresh TCR transgenic cells (solid lines) versus hybridomas (dashed lines) bearing the AND or 5C.C7 TCR, calculated relative to a no-tetramer control (circles). Right: CD3ε expression of these cells.

(B) TCRβ and MCC:IE^K staining was performed on wild-type or *Cd5*^{-/-} 5C.C7 or AND TCR Tg lymph node cells. Top: example staining for CD5, TCRβ, and MCC:IE^K in one 5C.C7 *Cd5*^{+/+} or 5C.C7 *Cd5*^{-/-} mouse. Both tetramer staining and TCRβ expression was lower in *Cd5*^{-/-} CD4⁺ T cells. Bottom: lymph node CD4⁺CD3⁺ populations from 5C.C7 or AND mice were electronically divided into subpopulations based on TCRβ expression with FlowJo and the MCC:IE^K MFI was calculated for each subpopulation. Graphs show the correlation between these two parameters in the subpopulations of wild-type cells (black) and the bulk transgenic population obtained from the *Cd5*^{-/-} mice (red). The lower tetramer binding of *Cd5*^{-/-} CD4⁺ T cells is completely explained by lower TCRβ expression and is not due to the absence of CD5 expression. Results are representative of two to three independent experiments.

be attributed to differences in responsiveness to IL-7 or survival rates. In the absence of any pathogen challenge, we detected no differences in the in vivo survival or homeostatic proliferation of CD5^{hi} versus CD5^{lo} naive CD4⁺ T cells on day 4 or day 43 after adoptive transfer to lymphoreplete recipients (Figures

and adoptively transferred them to recipient mice. Recipients were infected 1–3 days later with either lymphocytic choriomeningitis virus Armstrong (LCMV), Δ ActA *Listeria monocytogenes*-expressing OVA-2W1S (LM), or an OVA_{323–339}-expressing influenza A virus (Figures 5A and 5B). Transferred sorted cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) to track responding cell division. In all three infections, the recovery of cells that underwent extensive pathogen-induced cell division and survived at the time of assay was greater for CD5^{hi}CD4⁺ T cells than for CD5^{lo}CD4⁺ T cells (Figure 5C). This distinct in vivo responsiveness could not be attributed to intrinsic differences in the proliferative capacity of CD5^{lo} and CD5^{hi} cells. First, the response of these cells to polyclonal stimulation in vitro was similar (Figure S4A). Second, this differential response was seen only upon TCR stimulation with cognate pMHC, as indicated by the fact that in vivo administration of anti-CD3 and anti-CD28 after adoptive transfer of both CD5^{hi}CD4⁺ and CD5^{lo}CD4⁺ cells into the same recipients resulted in equivalent expansion and recovery (Figure 5D). Nor can the greater antigen responsiveness of the CD5^{hi} population

S4B–S4D). These findings are consistent with our conclusion that CD5^{hi}CD4⁺ T cells have a greater predictable reactivity with foreign ligands than CD5^{lo}CD4⁺ T cells and with published data documenting that mature peripheral T cells with stronger TCR binding of foreign pMHC are enriched after infection or immunization (Busch and Pamer, 1999; Malherbe et al., 2004; Price et al., 2005; Savage et al., 1999).

As an additional test of whether CD5^{hi} cells predominate in a polyclonal immune response to a pathogen and persist after its clearance, we infected mice with LM and followed CD5 expression of CD4⁺ T cells specific for 2W1S:I-A^b or for a peptide derived from listeriolysin O (LLO). We found that in both antigen-specific T cell populations, mean CD5 expression was increased at the peak of the CD4⁺ T cell response to LM (d5) and remained higher among specific memory CD4⁺ T cells after pathogen clearance (d21) (Figure 5E). Given that CD5 expression of CD4⁺ T cells for a given TCR clonotype was unchanged by the transition from naive to memory (Figure 5F), our data suggest that of the antigen-specific cells originally present in the naive pool, CD5^{hi} cells are indeed contributing more to an antigen

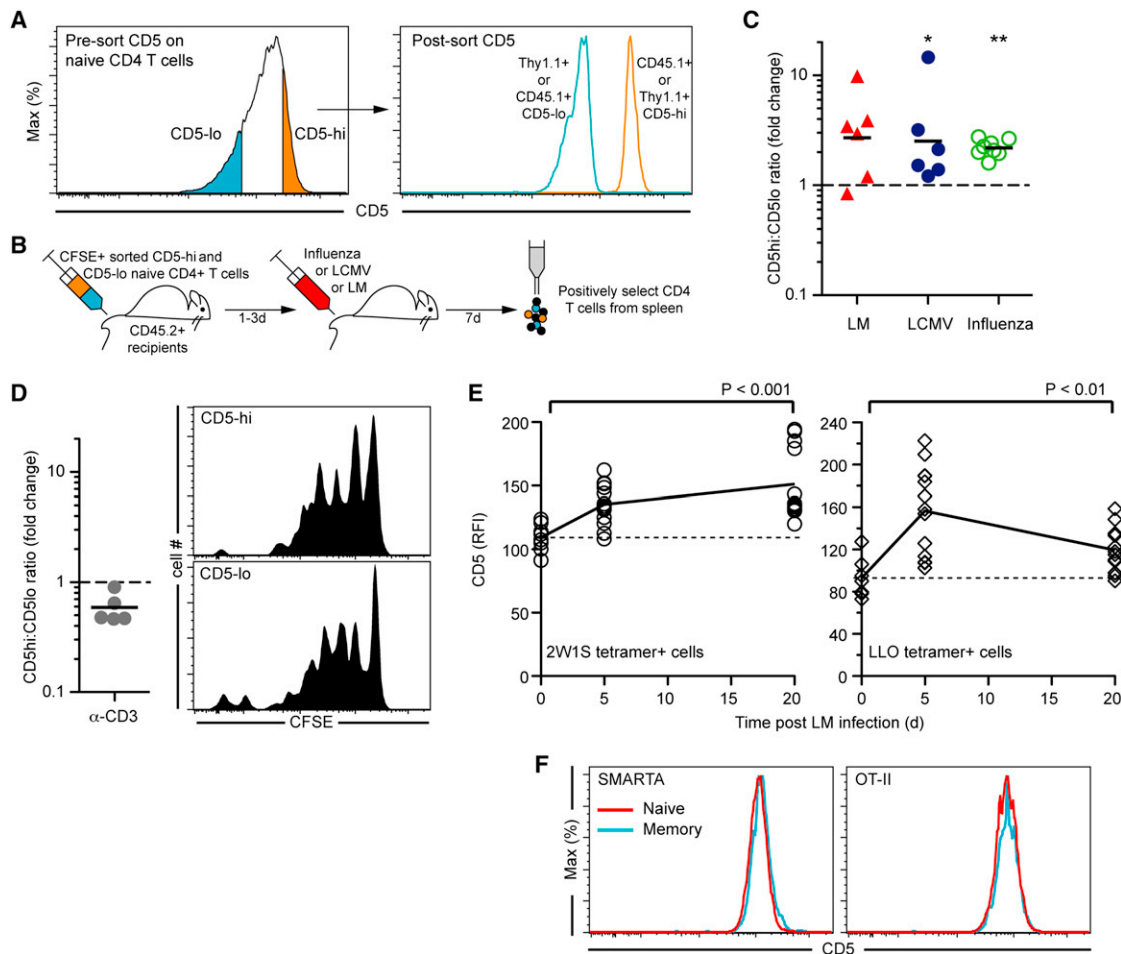


Figure 5. CD5^{hi} T Cells with the Greatest Reactivity for Self Antigens Dominate the Response to Diverse Foreign Antigens

(A and B) Experimental setup: congenically marked 15%–20% CD5^{lo} or CD5^{hi} naive CD4⁺ T cells were sorted, labeled with CFSE, mixed in a ~1:1 ratio, and adoptively transferred to CD45.2⁺ recipients that were then infected with *Listeria monocytogenes* (LM), influenza A virus, or LCMV.

(C) Fold change in CD5^{hi}:CD5^{lo} ratio in divided (CFSE^{lo}) versus undivided (CFSE^{hi}) transferred cells 7 days after infection. Each triangle or circle represents an individual mouse, lines denote means. Data summarized from two to three independent experiments.

(D) Setup as in (A), but recipients were given anti-CD3 and anti-CD28 i.v. The fold change in CD5^{hi}:CD5^{lo} ratio in divided versus undivided transferred cells and representative CFSE profiles 4 days after antibody administration are shown.

(E) CD5 expression on antigen-specific CD4⁺ T cells identified with tetramers (2W1S:I A^b, left; LLO:I A^b, right) on days 0, 5, or 21 after LM infection. Each symbol represents an individual mouse and lines denote means.

(F) CD5 expression on naive (CD44^{lo}) and memory (CD44^{hi}) SMARTA and OT-II cells from TCR Tg mice.

*p < 0.05, **p < 0.01.

response than CD5^{lo} cells after LM infection (Figure 5E). Thus, these in vivo results further validate our striking finding that there is a direct relationship between the strength of self-recognition of a TCR and its strength of binding to foreign antigen.

Positive Selection in the Thymus Skews the TCR Repertoire toward the Higher End of Self-Reactivity

Given these findings, we asked whether thymic-positive selection has a preference for TCRs binding self more avidly by examining the distribution of CD5 expression on naive CD4⁺ T cells. Most cellular proteins are log-normally distributed within a cell population because of stochasticity in gene expression (Niepel et al., 2009). We therefore asked whether the distribution of CD4, TCR β , and CD5 followed this pattern, log-transforming

fluorescent intensity data measured on naive CD4⁺ T cell populations from seven individual mice and fitting Gaussian curves to the data. As expected, the distribution of CD4 and TCR β were well approximated by normal curves with no significant difference between the mode of the best normal fit and that of the data (Figures 6A and 6B). In contrast, the distribution of CD5 deviated significantly from being log-normal, showing an enrichment for CD5^{hi} cells (Figures 6A and 6B). This skew was apparent only among polyclonal CD4⁺ T cell populations and not among monoclonal TCR Tg CD4⁺ T cells and thus was not a property of how CD5 expression is regulated in a population of cells expressing the same TCR (Figure S5).

If positive selection is operating to place the most foreign-antigen-reactive cells in the peripheral repertoire, we would

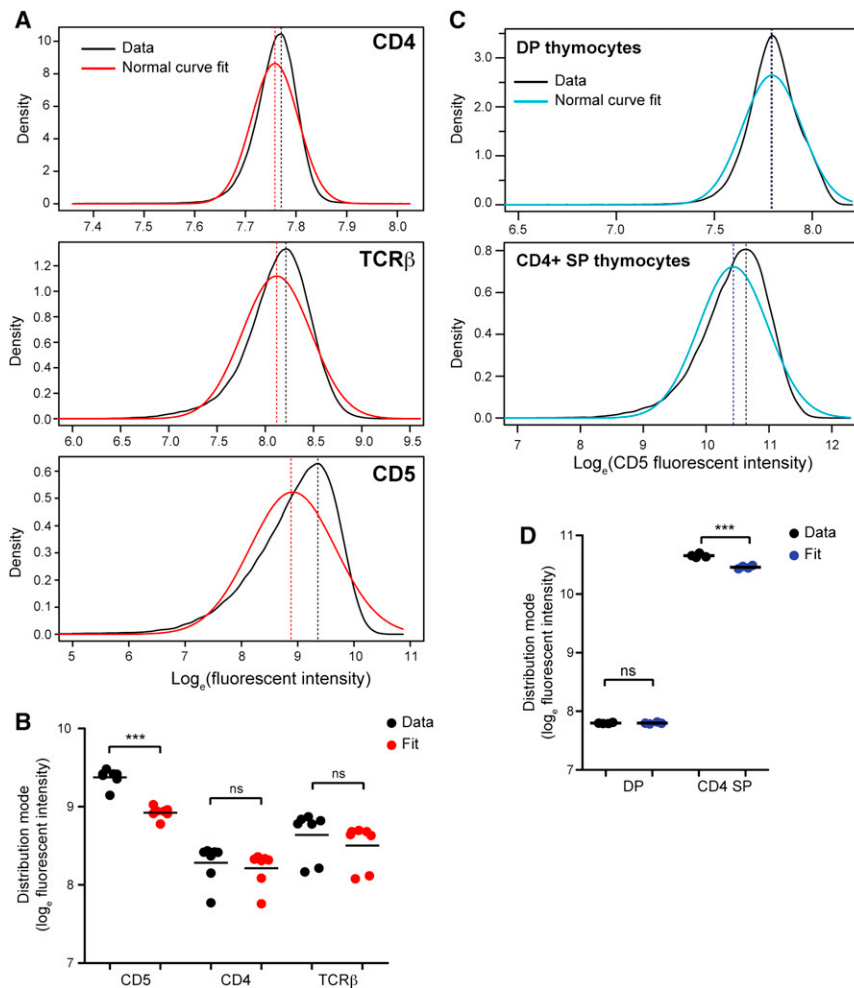


Figure 6. Thymic Selection Skews the Naive CD4⁺ T Cell Repertoire toward Cells with Greater Self-Sensitivity

A normal distribution was fit to the natural log-transformed distribution of CD4, TCRβ, or CD5 fluorescent intensity measured in individual mice. The mode of the best normal fit and of the experimental data were compared to assess for a skew in the distribution of the fluorescent intensity data.

(A and C) Representative data with the best normal fit are shown for peripheral CD4⁺ T cells (A) and double-positive, CD3^{lo} CD4⁺CD8⁺ (DP) or single-positive (SP) thymocytes (C). Dashed lines indicate distribution mode.

(B and D) Mode of fluorescent intensity data or best normal fit to the data is shown for peripheral CD4⁺ T cells (B) and DP or SP thymocytes (D). Circles represent modes from data or fit of individual animals, lines denote means. ***p < 0.01, ***p < 0.001; ns, not significant.

mean CD5 expression was significantly lower in adult CD4⁺ naive T cells as compared to those of neonates. Furthermore, among adults, memory CD4⁺ T cells had a greater mean CD5 expression as compared to naive CD4⁺ T cells (Figure 7A). Strikingly, we observed the same pattern for CD5 expression on naive CD4⁺ T cells from cord blood versus adult humans (Figure 7B), thus indicating that CD5 may also be associated with strength of self-pMHC reactivity on human T cells. In addition, as in mice, we found that in human adults, CD5

expect this skew in the distribution of self-reactivity among naive CD4⁺ T cells to arise after positive selection in the thymus. We therefore examined CD5 expression by double-positive (DP) thymocytes that have not yet undergone selection and by single-positive CD4⁺ thymocytes that have been successfully selected and found that the right-skewed CD5 distribution became apparent only among SP thymocytes (Figures 6C and 6D). Thus, positive selection exhibits a preference for the maturation of TCRs that have a greater reactivity for self (but are within the permissible range of TCR affinities constrained by negative selection on the high end).

Antigen Selection Further Skews the Postimmune TCR Repertoire and Impacts the Naive TCR Repertoire in Adults

Given our data showing that upon infection, CD5^{hi} cells outperform CD5^{lo} cells, we anticipated that with age, more CD5^{hi} than CD5^{lo} naive T cells would be recruited into the memory pool. As the thymic supply of naive T cells declines, the mean CD5 expression of the naive T cell population should decrease while that of memory T cells should be higher. To investigate this hypothesis, we compared CD5 expression by naive CD4⁺ T cells from neonatal and adult mice. Indeed, we found that

expression was significantly greater on memory as compared to naive CD4⁺ T cells (Figure 7B).

DISCUSSION

Although the process of positive selection occupies a central place in immunological thinking and was described 35 years ago (Bevan, 1977; Zinkernagel et al., 1978), why selection on self pMHC is an effective way to construct a T cell repertoire able to anticipate the unpredictable foreign antigens that will be encountered has remained incompletely understood. The findings we present here provide compelling evidence that there is a direct relationship between the strength of binding of a TCR to self pMHC and to agonist pMHC on a per T cell basis, thus providing an unsuspected connection between thymic developmental events and foreign-antigen-driven responses. We reached this conclusion by first establishing in TCR Tg and polyclonal naive CD4⁺ T cell populations that CD5 expression reflected the clone-specific strength of TCR reactivity with self pMHC, based on the correlation of CD5 with partial pζ in these T cells. Using CD5 as a measure of self-reactivity, we then documented a direct relationship between self-ligand binding strength and foreign-pMHC tetramer binding in the absence of

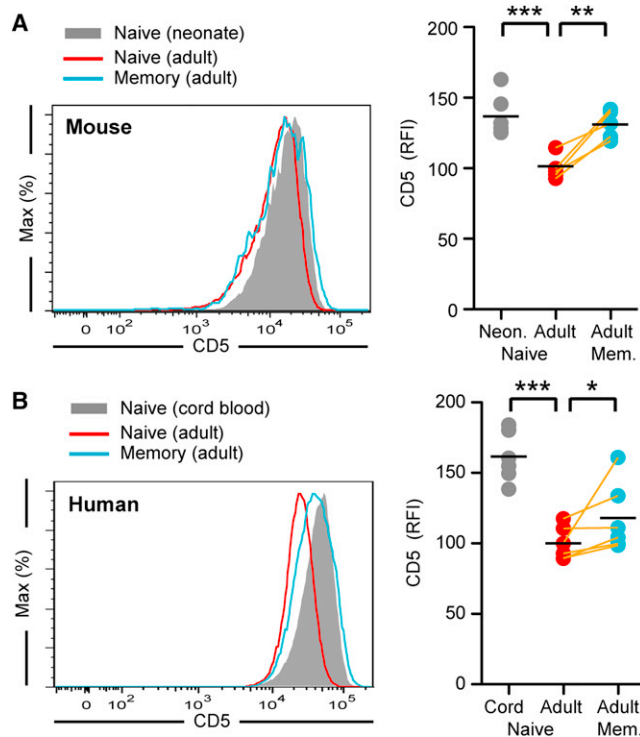


Figure 7. Antigen Selection Further Skews the Postimmune TCR Repertoire and Impacts the Naive TCR Repertoire in Adults

(A) CD5 expression on naive cells of day 5 neonates ($n = 6$) and naive or memory cells of day 40 adult mice ($n = 5$).
(B) CD5 expression on naive CD4⁺ T cells (CD45RO⁺CD27⁺) from human cord blood ($n = 10$) and naive or memory (CD45RO⁺CD27⁺) CD4⁺ T cells from peripheral blood of healthy donors ($n = 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

any confounding effects of self ligands in the assay. The establishment of a CD5-p ζ correlation also allowed us to assess the heterogeneity in self-reactivity present within a polyclonal pool of T cells, to examine the distribution of this heterogeneity, and to assess the function of cells with distinct self-reactivities with respect to pathogen responsiveness. We found that positive selection biases the peripheral CD4⁺ TCR repertoire toward cells with greater self-reactivity, yielding a larger fraction of mature cells better able to respond to foreign antigen.

Because the key conclusion of this study relies on use of tetramer binding and its relationship with self-ligand-induced p ζ amounts, it was critical to assess whether explanations other than a direct TCR-based relationship between self- and foreign-antigen reactivity could explain these data. We and others have found no evidence that CD5 directly affects pMHC binding (Bhandoola et al., 2002). T cell hybridomas cultured in the absence of interaction with self pMHC retained the differences in tetramer binding seen for primary monoclonal T cells with the same TCRs, arguing against the notion that in vivo self-ligand-induced TCR clustering could explain the connection between CD5 and tetramer staining intensity. Finally, by assessing TCR affinity for agonist pMHC via tetramers, we could also exclude that self pMHC was contributing to agonist-pMHC binding through a feedback process dependent on signaling. It

has previously been shown by some groups (Ebert et al., 2009; Krogsgaard et al., 2005; Lo et al., 2009), although not by others (Spörri and Reis e Sousa, 2002), that endogenous pMHC can augment signaling in T cells in response to agonist pMHC. However, because we measured agonist-pMHC binding strength independently of self pMHC with tetramers, the co-agonist action of self pMHC cannot explain the relationship between self and agonist TCR binding that we detect.

In terms of the better response of CD5^{hi} cells in vivo to infectious agents, neither we nor others (Cho et al., 2010) have found any relationship between CD5 and expression of the cytokine receptors CD25, CD127, or CD122 on naive CD4⁺ T cells (unlike for CD8⁺ T cells) and thus there is no evidence that would suggest that the differential outgrowth of CD5^{hi} cells during infection is a consequence of greater sensitivity to cytokines. Nor is this behavior explained by differences in survival between CD5^{lo} and CD5^{hi} cells. Our results also cannot be explained by intrinsic differences in the proliferative capacity of CD5^{hi} and CD5^{lo} cells, given equivalent responses to anti-CD3 and anti-CD28 stimulation in vivo.

It is thus the TCR itself that appears to lie at the heart of the relationship between self and agonist binding strength as well as the variation in pathogen response. Yet neither available structural information nor previous studies examining MHC restriction or the role of self in responses of T cells to foreign antigen predicts a direct relationship between self-reactivity and affinity for agonist (Garcia et al., 2009; Germain, 1990; Krogsgaard et al., 2005; Nikolić-Zugič and Bevan, 1990; Stefanová et al., 2002), making the existence of such a relationship a surprising finding. The lack of information on the specific self ligands that positively select the majority of T cell clones makes experiments to investigate the molecular basis for this relationship challenging. For the few positively selecting self peptides that have been described for particular monoclonal TCRs, there is no evidence that sequence resemblance to agonist peptides beyond key residues anchoring the peptide to the MHC binding groove is a necessary feature of selecting peptides (Berg et al., 1999; Ebert et al., 2009; Lo et al., 2009; Santori et al., 2002; Sasada et al., 2001). It may be that the positioning of the epitopic residues recognized by the TCR CDR3 regions with respect to these anchor sites plays an important role in the self-foreign binding relationship we report here (Garcia et al., 2009; Germain, 1990) or the ability of TCRs to specifically interact with structurally different ligands in the same general orientation but with slightly different strategies (Colf et al., 2007; Felix et al., 2007) account for these observations. In either case, our findings argue for revisiting the issue of how pMHC recognition occurs and what structural elements of the TCR and pMHC might support the unanticipated ability of T cells selected on self to bind well to foreign peptides bound to the same MHC molecule.

Given that all naive CD4⁺ T cells in an individual have passed the extensive filtering process in the thymus (Scollay et al., 1980), it is remarkable that within the permissible range of self-reactivity present after thymic selection, we could nonetheless detect a greater contribution of CD5^{hi}CD4⁺ T cells to an antipathogen response. The modest (~2- to 10-fold) difference in contribution between CD5^{hi} and CD5^{lo} cells is expected if positive selection indeed acts to optimize the TCR repertoire in the way

that we propose. It would not be doing so very effectively if CD5^{lo} cells were largely useless in responding to foreign antigen. In fact, consistent with recent studies with assays that are able to detect antigen-specific but tetramer-staining-negative CD4⁺ T cells (Sabatino et al., 2011), our data similarly indicate that CD5^{lo} cells do contribute to responses against infectious agents. Yet our data are also in agreement with studies that have described an increase in the frequency of stronger agonist binding TCRs in the course of immune responses (Busch and Pamer, 1999; Malherbe et al., 2004; Price et al., 2005; Savage et al., 1999).

Given this dominance of CD5^{hi} TCRs in in vivo immune responses, it might have been expected that most TCR Tg CD4⁺ T cells derived from actively immunized mice would have greater CD5 expression than the mean of a naive polyclonal population. However, the TCR α and β genes used for the generation of these transgenic animals primarily come from T cells repeatedly stimulated in vitro with high amounts of agonist ligand, which leads to the preferential activation-induced cell death of the most avid T cells in the population (Kruisbeek, 2001; Lenardo et al., 1999; Ryan et al., 2005). Therefore, this method of obtaining monoclonal TCRs may have biased the available population of Tg TCRs toward those with low-intermediate agonist-pMHC binding strength. Indeed, the use of CD5 expression as a means of identifying CD4⁺ T cells that dominate an antigen-specific response could be a useful tool for the selection of more avid monoclonal TCR populations.

Compensation for lower TCR avidity by elevated CD8 coreceptor expression prevented us from conducting an adequate analysis of the CD8⁺ T cell population for the relationship we document here for CD4⁺ T cells. Our results nonetheless provide an intuitive explanation for the effectiveness of thymic-positive selection in predicting the unknown and generating a useful T cell repertoire with which to combat infection in the absence of any “knowledge” of which foreign antigens will be encountered. If the capacity to bind self pMHC is a direct predictor of affinity for foreign agonist, it is easy to see why the TCR testing strategy of positive selection is successful, because it ensures the preferential maturation of TCR clonotypes that have the greatest capacity to respond to foreign antigen. Although this may also result in the T cell repertoire being closer to the threshold at which overt responses to self pMHC are possible, having a system that is poised at this fine balance may be beneficial if this will provide a sufficient pool of diverse T cells capable of effectively responding to pathogenic invasion when antigen presentation is low. This poising at the overt self-reactivity border may explain the many additional factors needed to ensure that peripheral T cells do not cross the activation threshold in response to self. It will be important to investigate further whether CD5 expression provides sufficient information on the strength of self- and foreign-antigen reactivity of particular T cells to make use of this approach for clinical purposes. The availability of a surface marker to predict which cells will be better effectors during polyclonal immune responses could prove of substantial value in guiding development of immunomodulatory therapeutics, either to reduce pathogenic autoimmune and allograft rejection responses or to improve immunity against tumors or infectious agents.

EXPERIMENTAL PROCEDURES

Mice

Animal housing, care, and research were in accordance with the Guide for the Care and Use of Laboratory animals and all procedures performed were approved by the NIAID Animal Care and Use Committee. Mouse strains and sources are listed in [Supplemental Experimental Procedures](#).

Cell Surface and Tetramer Staining

Lymphoid cells from lymph nodes and/or spleen were collected and washed in PBS 2 mM EDTA + 5% FCS, Fc-receptors were blocked with 2.4G2 (BD Biosciences), and antibodies were used for surface staining. Cells were acquired on a LSRII (BD Biosciences). Tetramer staining was performed as previously described (Moon et al., 2007; Savage et al., 1999) at 37°C for 1 hr (except for 2W1S:I-A^b, which was done at 25°C) in buffer containing 0.1% sodium azide to reduce the effects of TCR-MHC internalization. Flow cytometric data were analyzed with FlowJo (Tree Star). Reagents used are listed in [Supplemental Experimental Procedures](#).

Hybridoma Preparation

T cell hybridomas were rederived from 5C.C7 or AND TCR transgenic cells as previously described (Kruisbeek, 2001). Two weeks after drug selection, viable cells were expanded and used in tetramer binding assays at room temperature, as described above.

Cell Sorting

Spleen and lymph node cells were pooled and pre-enriched for CD4⁺ T cells by negative selection with EasySep (StemCell Technologies). The enriched fraction was stained with antibodies for CD8 (53-6.7), CD5 (53-7.3), B220 (RA3-6B2), and CD44 (IM7) (eBiosciences). CD8⁺ B220⁺ CD44^{lo} cells were sorted into CD5^{hi} and CD5^{lo} populations with the required stringency indicated in each experiment. Sorts were performed with a FACSAria (BD Bioscience).

Immunoblot Analysis

To quantify partial tyrosine phosphorylation of the TCR ζ chain and ERK-2, fresh CD4⁺ T cells were obtained by negative selection (MACS, Miltenyi) or by sorting CD4⁺ T cell fractions as described. Cells were lysed in buffer containing 1% NP-40 (Pierce) with protease and phosphatase inhibitors and immunoblotting performed as described (Dorfman et al., 2000). Densitometry was done with ImageJ software (NIH).

Tetramer-Based Enrichment and Analysis

Lymph node and spleen cells from individual mice were collected and a 2W1S:I-A^b tetramer pull-down was done as described (Moon et al., 2007). For the CD5 versus tetramer staining intensity correlation analysis, bound fractions obtained from individual mice were electronically concatenated in FlowJo. Tetramer stainings were done with the following panel, unless otherwise described in the figure legend: B220, CD11b, CD11c, Ly6-G, F4/80, NK1.1 (dump channel) eFluor450; CD4 BD Horizon V500; TCR β or CD3 FITC; CD8 PercP-eFluor710; CD44 Alexa Fluor 700; CD5 PE-Cy5. Naive tetramer⁺ cells were defined as Dump⁺ TCR β ⁺ CD4⁺ CD8⁺ CD44^{lo} Tetramer⁺ cells (Figure S3E).

Adoptive Transfers and In Vivo Stimulations or Infections

Congenically marked Thy1.1⁺ CD5^{hi} and CD45.1⁺ CD5^{lo} sorted cells (or sorted cells with the congenic markers inverted relative to CD5 expression) were CFSE labeled and mixed at an approximately 1:1 ratio, and 4–6 $\times 10^6$ cells were injected into 2- to 4-month-old C57BL/6 recipients. Recipients were infected 1–3 days later with either 2 $\times 10^5$ PFU LCMV Armstrong, 1 $\times 10^7$ CFU Δ ActA 2W1S-OVA *Listeria monocytogenes*, or 0.6 LD₅₀ influenza H1N1 PR8 virus expressing OVA₃₂₃. Alternatively, on the same day as the adoptive transfer, recipients were given 10 μ g anti-CD3 and 5 μ g anti-CD28 i.v. (BD Bioscience). Day 7 after infection or day 4 after antibody administration, spleens were harvested, CD4⁺ T cell positively selected by MACS, and CFSE dilution in the transferred cells was analyzed by FACS. To assess whether there were differences in division of CD5^{hi} versus CD5^{lo} T cells after infection for each individual mouse, results were expressed as the fold

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change in CD5^{hi}:CD5^{lo} ratio in the population that diluted CFSE^{lo} (divided) versus the CFSE^{hi} (undivided) cells.

Analysis of CD5 Changes during Aging and Infection

C57BL/6 mice were infected with 1×10^7 CFU Δ ActA 2W1S-OVA LM. Mice were euthanized at the peak of CD4⁺ T cell expansion (day 5) or after pathogen clearance (day 21). Noninfected mice were used as controls to obtain naive antigen-specific T cells. In all cases, lymph nodes and spleens were harvested and 2W1S or LLO-specific CD4⁺ T cells were enriched as described. All samples (naive, d5, and d21) were prepared and acquired on the same day. CD5 expression was also determined on CD4⁺ T cells from the spleen and lymph nodes of neonate (5-day-old) and adult (8-week-old) sex-matched mice.

Analysis of CD5 Distribution Skew

Fluorescent intensity data for CD4, TCR β , and CD5 on individual peripheral naive polyclonal CD4⁺ T cells from a total of seven mice (from two independent experiments) and for CD5 on individual CD3^{lo}, CD4⁺CD8⁺ double-positive, and CD3⁺CD4⁺ single-positive polyclonal thymocytes from a total of four mice (from two independent experiments) or from AND or 5C.C7 TCR Tg mice were exported from FlowJo. Because of the large number of events, even small deviations from normality would be significant when using a normality test. Instead, the data sets were aggregated and the skew of the distribution of the data was assessed by (1) fitting a Gaussian distribution to the histograms of either TCR, CD4, or CD5 expression after log-transforming this data, via the *fitdist* routine in the *fitdistrplus* package in the statistical software R Project, version 2.10.1 (Ihaka and Gentleman, 1996), (2) taking the mode of the best Gaussian fit and the mode of the actual data, and (3) performing an ANOVA to assess whether the mode of the best Gaussian fit is significantly different from the mode of the data across multiple mice for TCR, CD4, and CD5 fluorescent intensity. A significant difference between the mode of the data and of the best normal fit indicated a skew in the actual data and a significant deviation from normality.

Human Cell Analyses

Human adult peripheral blood mononuclear cells (PBMCs) and cord blood samples were kindly provided by D. van Baarle (University Medical Center Utrecht, the Netherlands). Cord blood was obtained from healthy full-term neonates directly after delivery and adult blood was obtained by vena-puncture from blood bank donors visiting the University Medical Center Utrecht, the Netherlands. Mononuclear cells were obtained by Ficoll-Paque density gradient centrifugation and cryopreserved until further use. Written informed consent was obtained from all participants or their legal guardians in agreement with the Helsinki Declaration of 1975, revised in 1983, and the acquisition of these samples was approved by the medical ethical committee of the University Medical Center Utrecht (the Netherlands). Cryopreserved PBMC were thawed and 1×10^6 cells incubated with anti-CD5 (UCHT2) and anti-CD4 (RPA-T4) (Biolegend), CD27 (CLB-CD27/1, 9F4) (Sanquin Reagents), anti-CD8 (RPA-T8) and anti-CD45RO (UCHL1) (BD Biosciences), and CD3 (OKT3) (eBioscience). Cells were stained and acquired as described.

Statistical Analyses

We analyzed data with GraphPad, Prism or R Project version 2.10.1 (Ihaka and Gentleman, 1996). A criterion of $p < 0.05$ was used in all statistical analyses. Coefficient of variation analyses (robust CV) of flow cytometric data was performed with FlowJo.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2012.09.011>.

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