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High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma

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Tumor-specific neo-antigens that arise as a consequence of mutations[1,2] are thought to be important for the therapeutic efficacy of cancer immunotherapies[3–5]. Accumulating evidence suggests that neo-antigens may be commonly recognized by intratumoral CD8+ T cells[3–7], but it is unclear whether neo-antigen–specific CD4+ T cells also frequently reside within human tumors. In view of the accepted role of tumor-specific CD4+ T-cell responses in tumor control[8–10], we addressed whether neo-antigen–specific CD4+ T-cell reactivity is a common property in human melanoma.

Here, we exploit oncogene-immortalized autologous B cells to measure the occurrence of CD4+ T-cell responses against melanoma mutanomes identified by tumor exome sequencing. Using this approach, we detected neo-antigen–reactive CD4+ T cells in four out of five melanoma patients analyzed, including subjects who had a clinical response after adoptive T-cell therapy. These data suggest that recognition of neo-antigens by CD4+ T cells frequently occurs in melanoma. Furthermore, based on the mutational landscape in human tumors[1,2], it is plausible that neo-epitopes that may be recognized by CD4+ T cells are also formed in a substantial fraction of patients with other common cancers. CD4+ T-cell reactivity against such mutated antigens may either be preexisting or be therapeutically induced.

On the basis of (i) the evidence that supports a role for CD4+ T cells in the efficacy of cancer immunotherapies[8–13], (ii) the proposed correlation between mutational load and clinical response to immunotherapy[14] and (iii) the recent observation that neo-antigen–specific CD4+ T cells can mediate tumor regression in a metastatic cholangiocarcinoma[13], it is important to assess whether neo-antigen–specific CD4+ T-cell reactivity is a common phenomenon in human cancers. Specifically, should the formation of neo-antigens that can be recognized by CD4+ T cells prove a frequent event in human cancers, therapeutic manipulation of such reactivity would become a highly attractive strategy[15,16].

Given the high but variable mutational load of melanoma[1,2] and the observation that T-cell products that are generated for adoptive T-cell therapy of melanoma can contain substantial fractions of CD4+ T cells that may contribute to clinical activity[11,17], we chose to examine the occurrence of neo-antigen–specific CD4+ T-cell reactivity in a set of melanoma specimens with varying mutational loads.

To assess the occurrence of intratumoral CD4+ T-cell responses against nonsynonymous somatic mutations within these tumors, we used whole-exome-sequencing and RNA-sequencing data to identify the entire set of tumor-specific, nonsynonymous mutations within expressed genes. Then, to analyze CD4+ T-cell reactivity against any of these mutated peptides, we developed a system in which immortalized autologous B cells are generated by retroviral introduction of BCL-6 and BCL-XL[18,19], serving as an infinite source of antigen-presenting cells (APCs) that can be loaded with mutated peptides (**Supplementary Fig. 1**).

We validated this screening platform by analyzing three melanoma lesions—NKIRTIL018, NKIRTIL034 and NKIRTIL045—from patients who underwent palliative metastasectomy. Total mutational load within these tumors varied considerably (range, 180–464 somatic mutations; **Supplementary Table 1**). As expected, all tumor mutanomes contained a high percentage of UV-associated mutations[1]. On average, 153 mutations (range, 99–188) per tumor were identified as candidate neo-epitopes (defined as tumor-specific, nonsynonymous mutations in a gene with confirmed RNA expression). Peptides (31 amino acids) that covered the individual mutations were then loaded onto the immortalized, autologous B cells, and the resulting targets were incubated with intratumoral CD4+ T cells (routinely ≥97% CD4+) generated using the rapid expansion procedure that is used to create TIL products for therapy[11]. Subsequently, culture

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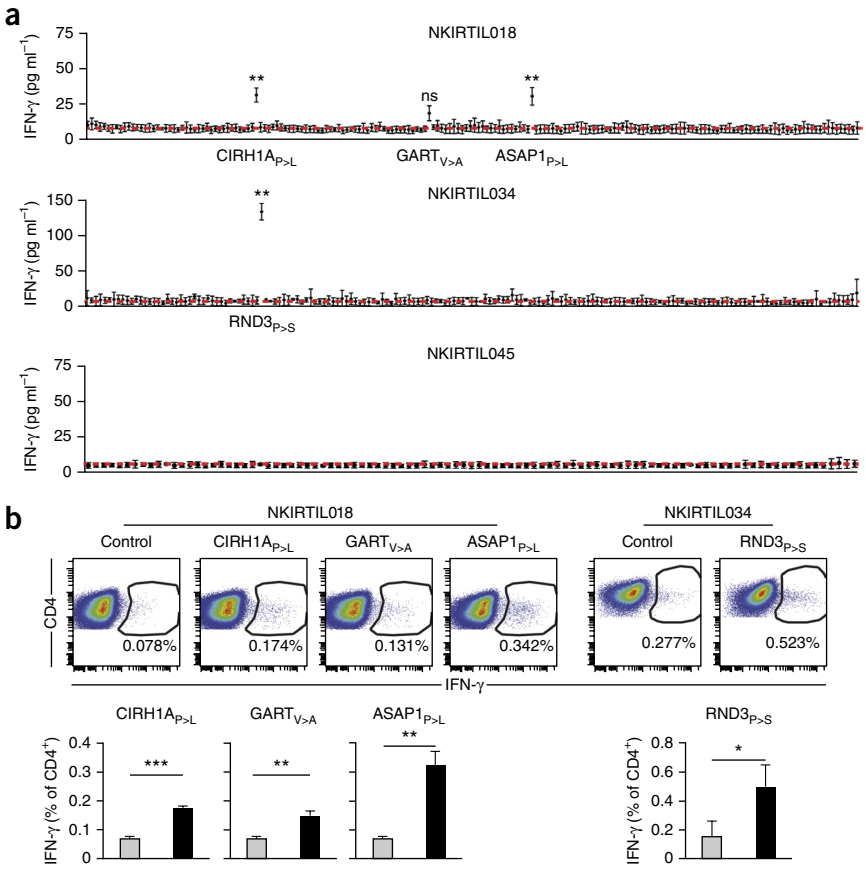

Figure 1 Detection of neo-antigen–specific CD4+ T cells in human melanoma lesions. (**a**) Mean IFN-γ concentration in culture supernatants after co-culture of peptide-loaded autologous B cells with *in vitro*–expanded intratumoral CD4+ T cells (NKIRTIL018: $n = 3$; NKIRTIL034 and NKIRTIL045: $n = 2$). Red dashed lines indicate mean IFN-γ production of CD4+ T cells after co-culture with unloaded B cells. Error bars, s.d. $CIRH1A_{P>L}$ $P = 0.0026$, $GART_{V>A}$ $P = 0.0645$, $ASAP1_{P>L}$ $P = 0.0063$, $RND3_{P>S}$ $P = 0.0061$. (**b**) Detection of intracellular IFN-γ levels after co-culture of peptide loaded or unloaded ('Control') autologous B cells with *in vitro*–expanded intratumoral CD4+ T cells for NKIRTIL018 and NKIRTIL034. Representative flow cytometry plots depict single, live CD4+ IFN-γ+ T cells. Bar graphs depict mean percentage of IFN-γ–producing CD4+ T cells in response to unloaded (gray) and peptide-loaded (black) autologous B cells ($n = 3$). Error bars, s.d. $CIRH1_{P>L}$ $P < 0.0001$, $GART_{V>A}$ $P = 0.0028$, $ASAP1_{P>L}$ $P = 0.0012$, $RND3_{P>S}$ $P = 0.037$. (Here and throughout, ns indicates $P > 0.05$, $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

supernatants were assessed for the presence of the T_{H1}, T_{H2} and T_{H17} cytokines IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10 and IL-17a.

For the first subject (NKIRTIL018), we observed IFN-γ production by tumor-derived CD4+ T cells in response to three putative neo-epitopes, CIRH1A P333L ($CIRH1A_{P>L}$), GART V551A ($GART_{V>A}$) and ASAP1 P941L ($ASAP1_{P>L}$) (**Fig. 1a**). Notably, these neo-antigen–specific CD4+ T-cell responses were easily detected above background when using autologous BCL-6/BCL-XL–immortalized B cells, but were obscured by background when autologous Epstein-Barr virus (EBV)-immortalized B cells of the same individual were used (**Supplementary Fig. 2**). Melanoma-derived CD4+ T cells of subject NKIRTIL034 also showed neo-antigen reactivity, in this case against the mutated gene product RND3 P49S ($RND3_{P>S}$). Only in the subject with the lowest mutational load, NKIRTIL045, reactivity against neo-antigens as measured by production of IFN-γ was not observed within the intratumoral CD4+ T-cell compartment (**Fig. 1a**). For all four epitopes, the presence of CD4+ T cells within the melanoma lesions of NKIRTIL018 and NKIRTIL034 that responded to the identified putative mutant epitopes was confirmed by analysis of intracellular IFN-γ levels upon antigen stimulation (**Fig. 1b**). Activity of these cells was restricted to IFN-γ production, with no evidence of production of TNF-α, IL-10, IL-2, IL-4, IL-6 or IL-17a in response to any of the mutant peptides that covered the autologous mutanome (**Supplementary Fig. 3**).

T-cell receptors are known to be able to cross-react with large numbers of epitopes[20], and in the above screens, a diverse pool of T cells was tested for recognition of a sizable set of peptides. This made it important to assess whether the T-cell reactivities observed represented true neo-antigen–driven T-cell responses, rather than haphazard cross-reactivity with any of the experimental peptides. In the former case, two predictions can be made: first, reactivity should only be observed when CD4+ T cells are confronted with the autologous mutanome; and second, reactivity of these CD4+ T cells should be biased toward the mutant peptide, relative to its wild-type counterpart.

To address the first issue, we tested the ability of CD4+ T cells from NKIRTIL018 and NKIRTIL034 to react against any of the non-autologous neo-epitopes. Incubation of immortalized B cells loaded with any of the peptides from the 'mismatched' epitope set did not lead to IFN-γ production by tumor-derived CD4+ T cells from either subject (**Supplementary Fig. 4**). To also test whether these CD4+ T-cell populations displayed the expected preferential reactivity toward the mutant peptide, we generated a panel of neo-epitope–reactive CD4+ T-cell clones for three of the identified epitopes ($ASAP1_{P>L}$, $CIRH1A_{P>L}$, $GART_{V>A}$, **Supplementary Fig. 5**). Stimulation of the resulting CD4+ T-cell clones with either the mutant peptide or its parental sequence revealed preferential (≥100-fold more sensitive) or exclusive recognition of the mutant sequence for the vast majority of all T-cell clones tested (**Fig. 2a**; **Supplementary Fig. 6**). Thus, by their restriction toward the autologous mutanome set, and their preferential recognition of the mutant peptide over its wild-type counterpart, these tumor-resident CD4+ T-cell responses are defined as true neo-antigen–driven T-cell reactivities. In further support of this, these neo-antigen–specific T-cell responses are oligoclonal to polyclonal, as revealed by sequencing of TCRαβ genes for a small set (8–11) of T-cell clones for each antigen (2–7 clonotypes per epitope, **Fig. 2b**), resembling the diversity generally seen in antigen-specific T-cell responses. Truncation of two of these neo-epitopes revealed that peptides of 13 amino acids (RKITFLHRCLISC; $CIRH1A_{P>L}$) and 19 amino acids (KPPPGDLPLKPTELAPKPQ; $ASAP1_{P>L}$) were still recognized with high efficiency (**Fig. 2c**).

On the basis of the observation that neo-antigen–specific CD4+ T-cell reactivity can readily be detected through the combination of cancer-exome data and oncogene-immortalized autologous B cells (**Fig. 1**), and the fact that a T-cell product containing a high frequency of neo-antigen–reactive

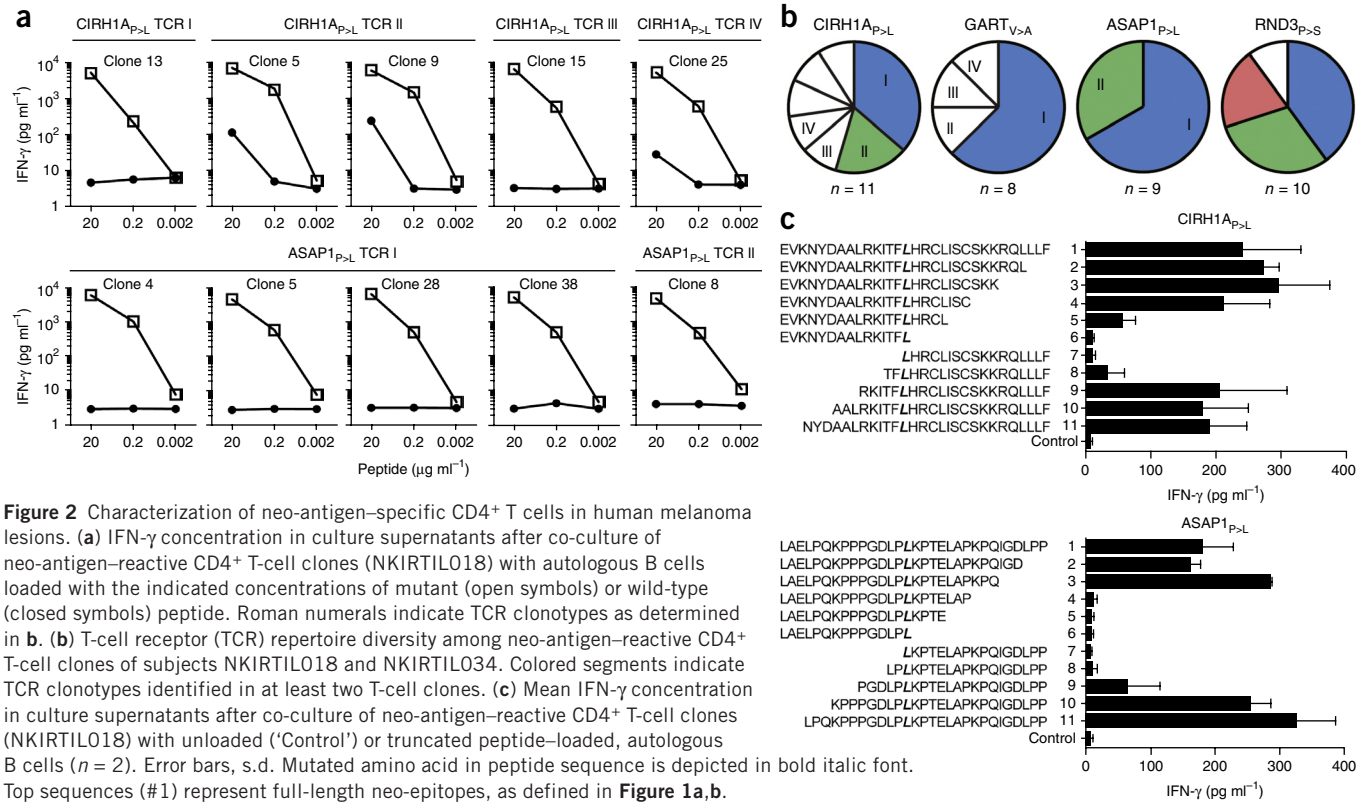

Figure 2 Characterization of neo-antigen–specific CD4$^+$ T cells in human melanoma lesions. (**a**) IFN-γ concentration in culture supernatants after co-culture of neo-antigen–reactive CD4$^+$ T-cell clones (NKIRTIL018) with autologous B cells loaded with the indicated concentrations of mutant (open symbols) or wild-type (closed symbols) peptide. Roman numerals indicate TCR clonotypes as determined in **b**. (**b**) T-cell receptor (TCR) repertoire diversity among neo-antigen–reactive CD4$^+$ T-cell clones of subjects NKIRTIL018 and NKIRTIL034. Colored segments indicate TCR clonotypes identified in at least two T-cell clones. (**c**) Mean IFN-γ concentration in culture supernatants after co-culture of neo-antigen–reactive CD4$^+$ T-cell clones (NKIRTIL018) with unloaded ('Control') or truncated peptide–loaded, autologous B cells ($n = 2$). Error bars, s.d. Mutated amino acid in peptide sequence is depicted in bold italic font. Top sequences (#1) represent full-length neo-epitopes, as defined in **Figure 1a**,**b**.

CD4$^+$ T cells was recently shown to mediate partial regression of a cholangiocarcinoma[13], we analyzed whether a neo-antigen–reactive CD4$^+$ T-cell compartment is observed in melanoma patients who experience a clinical response upon adoptive T-cell therapy.

The presence of neo-antigen–specific CD4$^+$ T-cell reactivity was assessed for two patients with stage IV melanoma who had been treated with T-cell therapy: patient NKIRTIL027 experienced a partial clinical response (6 months) upon TIL therapy, and patient

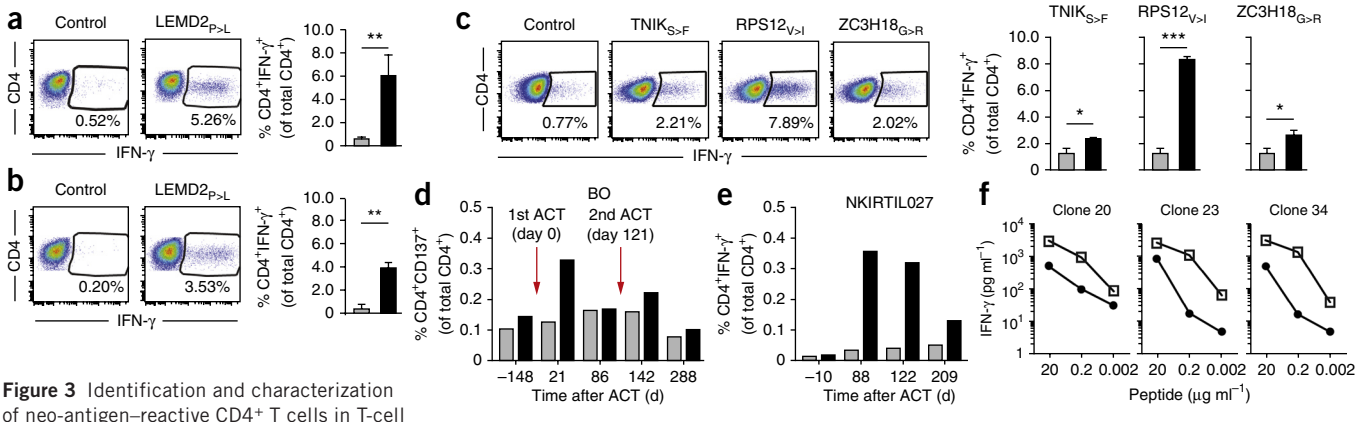

Figure 3 Identification and characterization of neo-antigen–reactive CD4$^+$ T cells in T-cell products used for adoptive T-cell therapy. (**a**–**c**) Intracellular IFN-γ production by *in vitro*–expanded intratumoral CD4$^+$ T cells (NKIRTIL027; **a**), the TIL infusion product (NKIRTIL027; **b**) or *in vitro*–expanded, intratumoral CD4$^+$ T cells derived from infusion product after co-culture with peptide-loaded or unloaded ('Control') autologous B cells (BO; **c**). Representative flow cytometry plots depict single, live CD4$^+$ IFN-γ$^+$ T cells. Bar graphs depict mean percentage of IFN-γ producing CD4$^+$ T cells in response to unloaded (gray) and peptide-loaded (black) autologous B cells ($n = 3$). LEMD2$_{P>L}$ $P = 0.0061$ (**a**), $P = 0.0011$ (**b**), TNIK$_{S>F}$ $P = 0.0136$, RPS12$_{V>I}$ $P = 0.0001$, ZC3H18$_{G>R}$ $P = 0.0226$ (**c**). (**d**) Expression of CD137 on PBMC (BO) after co-culture with unloaded (gray) or RPS12$_{V>I}$ peptide–loaded (black) autologous B cells. (**e**) Detection of intracellular IFN-γ production by PBMC (NKIRTIL027) after co-culture with unloaded (gray) or LEMD2$_{P>L}$ peptide–loaded (black) autologous B cells. (**f**) IFN-γ concentration in supernatant after co-culture of LEMD2$_{P>L}$-specific CD4$^+$ T-cell clones with autologous B cells loaded with the indicated concentrations of mutant (open) or wild-type peptide (black). (**g**) Intracellular IFN-γ production of LEMD2$_{P>L}$-specific CD4$^+$ T-cell clones after culture without autologous tumor cells (light gray) or with untreated (dark gray) or IFN-γ–pretreated autologous tumor cells (black). Bar graphs depict mean percentage of IFN-γ–producing CD4$^+$ T cells ($n = 2$). Clone 20 $P = 0.0354$, clone 23 $P = 0.0025$, clone 34 $P = 0.0267$. Error bars, s.d.

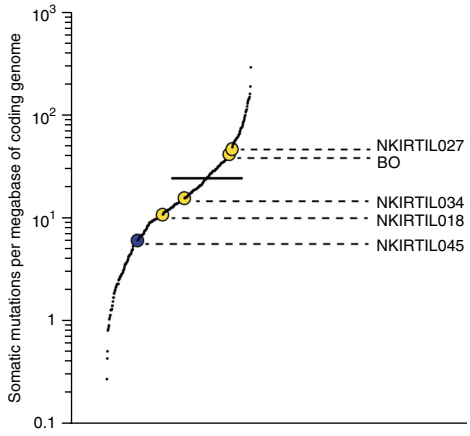

Figure 4 Mutational load and neo-antigen–specific CD4+ T-cell reactivity in melanoma patients. The number of somatic mutations is depicted for the melanoma samples described in Alexandrov *et al.*[1] (which includes NKIRTIL018) and for melanomas NKIRTIL045, NKIRTIL034, NKIRTIL027 and BO. Black circles represent individual samples; bar indicates the mean number of somatic coding mutations in the data set. Yellow circles indicate screened melanoma samples in which neo-antigen–specific CD4+ T-cell responses were detected; blue circle depicts the one screened melanoma sample in which no neo-antigen–specific CD4+ T-cell response was detected.

BO received multiple infusions of autologous *in vitro*–expanded T cells obtained by stimulation of peripheral blood mononuclear cells (PBMCs) with autologous tumor[17], leading to a complete tumor remission (ongoing for 7 years). Exome sequencing revealed a very high mutational burden in the tumors of both subjects (**Supplementary Table 1**), leading to collections of 582 and 501 candidate neo-epitopes, respectively. Subsequently, peptide-loaded BCL-6/BCL-XL–immortalized B cells were used as targets for CD4+ T cells expanded from an autologous melanoma lesion (NKIRTIL027) or from the infusion product (BO). This analysis identified strong CD4+ T-cell reactivity against the mutated gene product LEMD2 P495L ($LEMD2_{P>L}$) in NKIRTIL027 (**Supplementary Fig. 7**; **Fig. 3a**), which was also observed in the cell product used for therapy (**Fig. 3b**, 3.79% of CD4+ T cells). In subject BO, neo-antigen–specific CD4+ T-cell responses were identified for three mutant epitopes, TNIK S502F ($TNIK_{S>F}$), RPS12 V104I ($RPS12_{V>I}$) and ZC3H18 G269R ($ZC3H18_{G>R}$) (**Fig. 3c**, combined >13% of CD4+ T cells in the infusion product). In both individuals, these neo-epitope–specific CD4+ T cells were undetectable in peripheral blood before therapy (**Fig. 3d**,**e**). However, after infusion of the first two cell products, $RPS12_{V>I}$-specific CD4+ T cells were transiently identified in blood samples from BO (**Fig. 3d**; note that treatment did not entail lymphodepleting therapy before infusion in this case). In NKIRTIL027, T-cell infusion likewise resulted in the induction of an $LEMD2_{P>L}$-specific CD4+ T-cell response, in this case detectable for more than 200 d (**Fig. 3e**). As observed for the neo-epitope–specific CD4+ T-cell clones in other subjects (**Fig. 2a**; **Supplementary Fig. 5**), the vast majority of $LEMD2_{P>L}$-, $RPS12_{V>I}$-, $TNIK_{S>F}$- and $ZC3H18_{G>R}$-specific CD4+ T-cell clones showed a preferential or exclusive recognition of the mutated gene product as compared to the wild-type counterpart (**Fig. 3f**; data not shown).

Constitutive or IFN-γ–induced expression of major histocompatibility (MHC) class II by melanoma cells has been previously described[21]. To test whether identified neo-antigen–specific CD4+ T cells might directly recognize antigen displayed on tumor cells, we incubated $LEMD2_{P>L}$-specific CD4+ T-cell clones with NKIRTIL027 tumor cells (37% of tumor cells show constitutive MHC class II expression; 91% after 48 h IFN-γ exposure). For all T-cell clones tested (n=10), tumor-cell exposure induced IFN-γ production by $LEMD2_{P>L}$-specific CD4+ T cells (**Fig. 3g** and data not shown).

In this study, we investigated the extent of neo-antigen recognition by intratumoral CD4+ T cells in a group of five melanoma patients. High-throughput screens have previously established that CD8+ T cells in human melanoma frequently recognize both shared and mutated antigens[3,4,22–24]. Although a number of studies have reported that CD4+ T cells can also recognize different tumor antigen classes[13,25–28], the lack of high-throughput tools has precluded a similar systematic assessment of tumor-specific CD4+ T-cell reactivity. Platforms using autologous antigen-presenting cells as described here make it possible to comprehensively profile CD4+ T-cell reactivity against both shared and private tumor antigens, and may thereby allow one to determine whether CD4+ T-cell reactivity against certain (types of) antigens has prognostic or predictive value.

The main biological conclusion of this work is that CD4+ T-cell reactivity against mutated gene products is a frequent event in melanoma patients. Although such T-cell reactivity only involves a small fraction of the total mutanome (around 0.5% of mutated peptides) in individual subjects, CD4+ T-cell reactivity against the autologous mutanome was nevertheless observed in four out of five cases, in two instances involving multiple epitopes.

The formation of mutant peptides that have the potential to be the target of either naturally developing or therapeutically induced CD4+ T-cell responses can be considered a probabilistic process, in which each additional mutation raises the odds that a T-cell neo-epitope will be formed. The melanomas analyzed here carried a mutational load around or above ten somatic mutations per megabase of coding genome (**Fig. 4**). Apparently, this mutational load is sufficient to frequently lead to the formation of neo-epitopes that can be recognized by autologous CD4+ T cells. It therefore seems reasonable to speculate that neo-antigens that can be recognized by CD4+ T cells may also frequently be formed in other cancer types with similar mutational loads. Notably, in addition to ~60% of human melanomas, ~40% of lung cancers exhibit a mutational load of above ten somatic mutations per megabase. By the same token, 35% of uterine cancers and 23% of colorectal cancers have mutational loads that exceed ten somatic mutations per megabase of coding genome[1].

It is important to realize that the potential formation of such CD4+ neo-epitopes in these tumors does not necessarily imply the induction of autologous CD4+ T-cell reactivity. Specifically, differences within the tumor microenvironment between tumor types are likely to influence the ability of the T-cell repertoire to react to any neo-antigens that are formed. In view of this, the development of therapeutic interventions that induce or boost CD4+ T-cell responses against cancer-exome–defined potential MHC class II–presented neo-epitopes would be attractive. With respect to such interventions, all eight mutations for which neo-antigen–reactive CD4+ T-cell populations were here identified were unique to their tumor of origin within a panel of 403 melanomas (reported in this work and in ref. 1) Thus, CD4+ neo-antigen reactivity in this cancer is primarily directed toward private mutations. As such, the exploration of strategies to target such neo-epitopes by personalized immunotherapies, in melanoma and other tumor types with overlapping mutational loads, appears warranted.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. DNA and RNA sequencing data for the reported melanoma specimens have been deposited in the European Genome-Phenome Archive with accession codes EGAD00001000243 and EGAD00001000325.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.L., M.M.v.B. and L.B. designed, performed, analyzed and interpreted experiments and wrote the paper. E.M.E.V. and M.V. designed, performed, analyzed, and interpreted the analysis of subject BO. R.S. generated BCL-6/BCL-XL–immortalized B-cell lines and provided essential culture reagents and advice for the development of the B-cell screening platform. J.J.A.C. developed bioinformatics scripts for TCR sequence identification in NGS data. H.H. and D.e.A. synthesized peptide libraries. S.B., M.R.S. and A.V. performed and analyzed exome and RNA sequencing and developed the pipeline for identification of somatic mutations. J.B.A.G.H. supervised the clinical treatment of NKI TIL patients, supplied patient material and provided clinical interpretation of results. H.S. developed the BCL-6/BCL-XL immortalization technology and provided essential culture reagents and advice for the development of the B-cell screening platform. S.H.v.d.B. supervised, analyzed and interpreted the analysis of subject BO. T.N.M.S. supervised the project, designed and interpreted all experiments, and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

1. Alexandrov, L.B. *et al.* Signatures of mutational processes in human cancer. *Nature* **500**, 415–421 (2013).
2. Vogelstein, B. *et al.* Cancer genome landscapes. *Science* **339**, 1546–1558 (2013).
3. Robbins, P.F. *et al.* Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat. Med.* **19**, 747–752 (2013).
4. van Rooij, N. *et al.* Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J. Clin. Oncol.* **31**, e439–e442 (2013).
5. Lu, Y.C. *et al.* Mutated PPP1R3B is recognized by T cells used to treat a melanoma patient who experienced a durable complete tumor regression. *J. Immunol.* **190**, 6034–6042 (2013).
6. Wick, D.A. *et al.* Surveillance of the tumor mutanome by T cells during progression from primary to recurrent ovarian cancer. *Clin. Cancer Res.* **20**, 1125–1134 (2014).
7. Rajasagi, M. *et al.* Systematic identification of personal tumor-specific neoantigens in chronic lymphocytic leukemia. *Blood* **124**, 453–462 (2014).
8. Kenter, G.G. *et al.* Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N. Engl. J. Med.* **361**, 1838–1847 (2009).
9. Quezada, S.A. *et al.* Tumor-reactive CD4+ T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J. Exp. Med.* **207**, 637–650 (2010).
10. Ossendorp, F., Mengede, E., Camps, M., Filius, R. & Melief, C.J. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J. Exp. Med.* **187**, 693–702 (1998).
11. Friedman, K.M. *et al.* Tumor-specific CD4+ melanoma tumor-infiltrating lymphocytes. *J. Immunother.* **35**, 400–408 (2012).
12. Kitano, S. *et al.* Enhancement of tumor-reactive cytotoxic CD4+ T cell responses after ipilimumab treatment in four advanced melanoma patients. *Cancer Immunol. Res.* **1**, 235–244 (2013).
13. Tran, E. *et al.* Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science* **344**, 641–645 (2014).
14. Champiat, S., Ferte, C., Lebel-Binay, S., Eggermont, A. & Soria, J.C. Exomics and immunogenics: bridging mutational load and immune checkpoints efficacy. *OncoImmunology* **3**, e27817 (2014).
15. Britten, C.M. *et al.* The regulatory landscape for actively personalized cancer immunotherapies. *Nat. Biotechnol.* **31**, 880–882 (2013).
16. Overwijk, W.W., Wang, E., Marincola, F.M., Rammensee, H.G. & Restifo, N.P. Mining the mutanome: developing highly personalized Immunotherapies based on mutational analysis of tumors. *J. Immunother. Cancer* **1**, 11 (2013).
17. Verdegaal, E.M. *et al.* Successful treatment of metastatic melanoma by adoptive transfer of blood-derived polyclonal tumor-specific CD4+ and CD8+ T cells in combination with low-dose interferon-alpha. *Cancer Immunol. Immunother.* **60**, 953–963 (2011).
18. Kwakkenbos, M.J. *et al.* Genetic manipulation of B cells for the isolation of rare therapeutic antibodies from the human repertoire. *Methods* **65**, 38–43 (2014).
19. Kwakkenbos, M.J. *et al.* Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. *Nat. Med.* **16**, 123–128 (2010).
20. Mason, D. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol. Today* **19**, 395–404 (1998).
21. Deffrennes, V. *et al.* Constitutive expression of MHC class II genes in melanoma cell lines results from the transcription of class II transactivator abnormally initiated from its B cell-specific promoter. *J. Immunol.* **167**, 98–106 (2001).
22. Kvistborg, P. *et al.* TIL therapy broadens the tumor-reactive CD8(+) T cell compartment in melanoma patients. *OncoImmunology* **1**, 409–418 (2012).
23. Kvistborg, P. *et al.* Anti-CTLA-4 therapy broadens the melanoma-reactive CD8+ T cell response. *Sci. Transl. Med.* **6**, 254ra128 (2014).
24. Lu, Y.C. *et al.* Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions. *Clin. Cancer Res.* **20**, 3401–3410 (2014).
25. Jandus, C. *et al.* Tumor antigen-specific FOXP3+ CD4 T cells identified in human metastatic melanoma: peptide vaccination results in selective expansion of Th1-like counterparts. *Cancer Res.* **69**, 8085–8093 (2009).
26. Mautner, J., Jaffee, E.M. & Pardoll, D.M. Tumor-specific CD4+ T cells from a patient with renal cell carcinoma recognize diverse shared antigens. *Int. J. Cancer* **115**, 752–759 (2005).
27. Wang, R.F., Wang, X., Atwood, A.C., Topalian, S.L. & Rosenberg, S.A. Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. *Science* **284**, 1351–1354 (1999).
28. Hunder, N.N. *et al.* Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N. Engl. J. Med.* **358**, 2698–2703 (2008).

ONLINE METHODS

Generation of TIL material, tumor cell lines and BCL-6/BCL-XL–immortalized B cells. PBMC, tumor tissue and TIL material was obtained from individuals with stage IV melanoma in accordance with national guidelines, where applicable following signed informed consent and after approval of the local medical ethical committee (Medisch Ethische Toetsingscommissie) at NKI-AVL and LUMC. NKIRTIL018, NKIRTIL034, NKIRTIL045 and NKIRTIL027 received ipilimumab treatment prior to $CD4^+$ T-cell analysis. Patients were treated in the expanded access program in the Netherlands (NL EAP) (NCT00495066). PBMC material was prepared by Ficoll-Isopaque density centrifugation. TIL material and short-term tumor lines of NKIRTIL018, NKIRTIL045, NKIRTIL034 and NKIRTIL027 were obtained from resected melanoma lesions. Fresh tumor material was minced and digested overnight in RPMI 1640 (Life Technologies) supplemented with penicillin-streptomycin (Roche), 0.01 mg ml^{-1} pulmozyme (Roche) and 1 mg ml^{-1} collagenase type IV (BD Biosciences). Tumor lines were obtained by culture of the resulting cell suspension in RPMI 1640 supplemented with penicillin-streptomycin (Roche) and 10% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich). TIL were obtained by culturing the suspension cells in RPMI 1640 supplemented with penicillin-streptomycin, 10% (v/v) AB serum (Sanquin Blood Supply and Life Technologies), L-glutamine (Life Technologies) and 6,000 IU ml^{-1} rhIL-2 (Novartis) for 2–5 weeks. Tumor and TIL material of subject BO were obtained as previously described[17]. Autologous B cells from PBMC material were immortalized by BCL-6/BCL-XL gene transfer, as previously described[18,19]. BCL-6/BCL-XL–transduced B cells were cultured in IMDM (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Hyclone), penicillin-streptomycin (Roche) and 50 ng ml^{-1} rm-IL21 (produced in-house), and were stimulated every 3–5 d by addition of irradiated (50 Gy) mouse L cell fibroblasts expressing CD40L (2:1 B cell/L cell ratio).

Generation of $CD4^+$ T-cell material from TIL and infusion product. Cell sorting was performed on a FACSAria I (BD Biosciences) or MoFlo Astrios (Beckman Coulter). Bulk $CD4^+$ T-cell populations were generated from cryopreserved TIL material by staining with anti-CD8 (BD Biosciences; SK1; 1:50) and anti-CD4 (BD Biosciences; SK3; 1:50), and subsequent sorting of live single $CD4^+CD8^-$ T cells using 0.5 µg ml^{-1} DAPI (Sigma-Aldrich). Isolated cells were expanded using the rapid expansion procedure, using 30 ng ml^{-1} anti-CD3 (OKT-3; Janssen-Cilag) and 3,000 IU ml^{-1} rh-IL-2 (Novartis) in a 1:1 (v/v) medium mixture of RPMI 1640 and AIM-V (Life Technologies), supplemented with 10% AB serum (Life Technologies), Glutamax (Life Technologies) in the presence of feeder cells (1:200 T cell/feeder cell ratio) for 14 d. Obtained $CD4^+$ T-cell populations (routinely >97% $CD4^+$) were used for determination of T-cell reactivity against neo-epitopes.

Detection of neo-antigen–reactive $CD4^+$ T cells. BCL-6/BCL-XL immortalized B cells (1×10^5 per well in 96-well round-bottom plates) were loaded with peptide (20 µg ml^{-1}, unless indicated otherwise) for 18–24 h in 200 µl IMDM medium (Life Technologies), supplemented with 10% (v/v) fetal bovine serum (Hyclone), penicillin-streptomycin (Roche) and 50 ng ml^{-1} rm-IL21. Subsequently, medium was removed and $CD4^+$ T cells (1×10^5 per well) were added in 200 µl RPMI 1640 (Life Technologies), supplemented with 10% (v/v) AB serum (Life Technologies), penicillin-streptomycin (Roche) and 50 ng ml^{-1} rm-IL21. After 48 h, culture supernatants were harvested and analyzed using the Human $T_{H1}/T_{H2}/T_{H17}$ cytometric bead array (BD Biosciences) or IFN-γ Flex bead E7 cytometric bead array (BD Biosciences), according to manufacturer's guidelines. For flow cytometric detection of intracellular levels of IFN-γ, $CD4^+$ T cells were stimulated with peptide-loaded B cells for 24 h in the presence of Golgi-Plug (BD Biosciences; 1:1,000). Subsequently, cells were stained using surface antibodies and IR-Dye (Life Technologies; 1:100) to exclude dead cells, and intracellular IFN-γ was detected using the Cytofix/Cytoperm kit (BD Biosciences) and anti-IFN-γ (BD Biosciences; 25723; 1:50) staining, according to the manufacturer's guidelines. Expression of CD137 was analyzed as previously described[17].

Generation of neo-antigen–reactive $CD4^+$ T-cell clones. For isolation of live, antigen-specific $CD4^+$ T cells, T cells were stimulated with peptide-loaded B cells for 6 h. Subsequently, cells were stained using the IFN-γ capture kit (Miltenyi Biotec) according to manufacturer's guidelines plus anti-CD4 antibody (BD Biosciences; SK3; 1:50). Single, live IFN-γ–producing $CD4^+$ T cells were sorted by flow cytometry and collected in 96-well round-bottom culture plates containing 2×10^5 irradiated PBMCs, 30 ng ml^{-1} anti-CD3 and 3,000 IU ml^{-1} recombinant human IL-2 (Novartis) in 200 µl of a 1:1 (v/v) medium mixture of RPMI 1640 and AIM-V (Life Technologies), supplemented with 10% AB serum (Life Technologies), penicillin-streptomycin (Roche) and Glutamax (Life Technologies). After 7 d, 100 µl was replaced with fresh medium supplemented with rh-IL-2 (3,000 IU ml^{-1} final) and T-cell specificity was confirmed after 14 d, by assessment of IFN-γ production in response to the relevant neo-antigen.

Tumor reactivity of neo-antigen–reactive $CD4^+$ T-cell clones. NKIRTIL027 tumor line was maintained in RPMI 1640 (Life Technologies) supplemented with penicillin-streptomycin (Roche) and 10% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich). Prior to the assay, tumor cells were either left untreated or incubated with 200 IU ml^{-1} IFN-γ for 48 h.

$CD4^+$ T cells (1×10^5 per well) and tumor cells (5×10^4 per well) were co-cultured in 200 µl RPMI 1640 (Life Technologies), supplemented with 10% (v/v) AB serum (Life Technologies), penicillin-streptomycin (Roche) in the presence of Golgi-Plug (BD Biosciences; 1:1,000) and then analyzed as described for the detection of neo-antigen–reactive $CD4^+$ T cells.

Exome sequencing. Genomic DNA was extracted from cell pellets using the QIAGEN DNeasy purification kit. Libraries of genomic DNA were prepared using the Illumina Paired-End Sample Prep kit, following the manufacturer's guidelines. Resulting gDNA libraries were enriched for exonic sequences as described previously, using the Agilent Sure Select Human All Exon 50 Mb target enrichment system[29]. Sequencing was performed on an Illumina HiSeq DNA Analyzer (75 bp paired-end reads), according to the manufacturer's instructions. Reads were aligned to the human reference genome (NCBI Build 37) using the Burrows-Wheeler Aligner algorithm[30]. Unmapped reads, read mapping outside the targeted region and PCR-derived duplicates were excluded from further analyses. Procedures used for calling of substitutions and indels are available upon request.

RNA sequencing. RNA was isolated using the QIAGEN RNeasy purification kit. Poly(A)-selected RNA libraries were prepared, using the TruSeq RNA library protocol (Illumina, San Diego, CA, USA), and resulting libraries were sequenced on an Illumina HiSeq2000 using 75-bp paired-end reads. Reads were aligned using Tophat (version 1.3.3)[31]; expression values were calculated as FPKM using Cufflinks (version 1.0.2)[32]. DNA and RNA sequencing data for reported melanoma specimens are deposited: EGAD00001000243; EGAD00001000325.

Peptide synthesis. Peptides were synthesized on a SYRO II synthesizer, using preloaded Wang resin and standard Fmoc Solid Phase Peptide Chemistry, with PyBop and Dipea as activator and base.

Identification of TCR sequences. cDNA of T-cell clones was generated and used to prepare DNA libraries with the Illumina TruSeq DNA library preparation kit. The resulting DNA libraries were sequenced on an Illumina MiSeq sequencer using paired-end 150-bp chemistry.

Sequencing reads in FASTQ files were mapped to the human genome, build NCBI36/hg18, using BWA[33] and SAMtools[34]. PCR duplicates in resulting BAM files were filtered using Picard (http://broadinstitute.github.io/picard/). CDR3 TCR sequences were identified as previously reported[35]. TCRα and TCRβ sequences were inferred using an in-house–developed Python script (this script will be made available upon request).

Statistical analysis. Differences in cytokine concentrations and frequencies of cytokine-producing T cells were analyzed using a two-tailed Student's *t*-test.

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P values <0.05 were considered significant; significance values are indicated as * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$).

29. Varela, I. *et al.* Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. *Nature* **469**, 539–542 (2011).
30. Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**, 589–595 (2010).
31. Trapnell, C., Pachter, L. & Salzberg, S.L. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**, 1105–1111 (2009).
32. Trapnell, C. *et al.* Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **28**, 511–515 (2010).
33. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754–1760 (2009).
34. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).
35. Bolotin, D.A. *et al.* MiTCR: software for T-cell receptor sequencing data analysis. *Nat. Methods* **10**, 813–814 (2013).