Long-term Persistence of CD4+ but Rapid Disappearance of CD8+ T Cells Expressing an MHC Class I-restricted TCR of Nanomolar Affinity

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Most T cells have T cell receptors (TCR) of micromolar affinity for peptide-major histocompatibility complex (MHC) ligands, but genetic engineering can generate TCRs of nanomolar affinity. The affinity of the TCR used, m33, for its cognate non-self peptide–MHC-I complex (SIYRYYGL-K^b) is 1,000-fold higher than of the wild-type TCR 2C. The affinity of m33 for the self-peptide dEV-8 on K^b is only twofold higher. Mouse CD8$^+$ T cells transduced with an m33-encoding retrovirus showed binding of SIY-K^b and potent function *in vitro*, but *in vivo* these T cells disappeared within hours after transfer into syngeneic hosts without causing graft-versus-host disease (GVHD). Accordingly, in cases where such CD8-dependent self-reactivity might occur in human adoptive T cell therapies, our results show that a peripheral T-cell deletion mechanism could operate to avoid reactions with the host. In contrast to CD8$^+$ T cells, we show that CD4$^+$ T cells expressing m33 survived for months *in vivo*. Furthermore, the m33-transduced CD4$^+$ T cells were able to mediate antigen-specific rejection of 6-day-old tumors. Together, we show that CD8$^+$ T cell expressing a MHC class I-restricted high-affinity TCR were rapidly deleted whereas CD4$^+$ T cells expressing the same TCR survived and provided function while being directed against a class I-restricted antigen.

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INTRODUCTION

T cells recognize target antigens via T cell receptors (TCR) that bind to peptides presented by molecules of the major histocompatibility complex (MHC). TCR diversity is generated by recombination of the TCR locus. Each differentiating T cell needs to survive a positive and a negative selection process in the thymus. For positive selection, T cells must possess reactivity to self-peptide MHC (pMHC) since nonbinding T cells are thought to die of "neglect".[1] Surviving T cells are subsequently eliminated by negative selection if they recognize self-pMHC complexes above a particular affinity threshold. While negative selection is the main mechanism of neonatal or central tolerance (reviewed in ref. 2), additional mechanisms, referred to as peripheral tolerance (reviewed in ref. 3), are needed to prevent the adaptive T-cell immune system from attacking self. Similar to deletion of T cells in the thymus during negative selection, T cells can also undergo apoptosis in the periphery. Analogous to the expression of tissue-restricted antigens by thymic epithelial cells induced by the autoimmune regulator (AIRE),[4] cells in peripheral lymph nodes can also express tissue-restricted antigens and thereby induce apoptosis in peripheral T cells.[5,6] Together, central and peripheral tolerance result in the survival of T cells with TCRs that have relatively low affinities (K_D) for their cognate pMHC, with K_D values ranging from 1 to 100 µmol/l.[7,8] By contrast, antibodies bind their epitopes with high affinities, often in the nanomolar range.[9] (Note that when we use the term high-affinity, we are referring to monovalent interactions with nanomolar binding constants.)

TCRs have been engineered to have affinities in the nanomolar and even picomolar range.[10,11] Libraries of site-directed mutants in the TCR were selected for strong binding to pMHC by phage,[12] yeast,[10] or T cell[13] display. Recently, rationally designed mutagenesis has also been employed to identify TCRs with more modest increases in affinity.[14] *In vitro*, T cell lines or primary T cells expressing high-affinity TCRs showed enhanced antigen-dependent reactivity[10,11,14] and loss of dependence on the CD8 coreceptor.[15,16] In addition, these studies have also revealed that high-affinity TCRs can have reduced specificity and gain functional self-reactivity,[10,15] presumably because of an increase in affinity for the positively selecting self-peptide. The above studies, all done *in vitro*, suggest that the use of T cells expressing high-affinity TCRs *in vivo* may help to study peripheral tolerance by grafting post-thymic T cells with TCRs of different affinities. Furthermore, TCRs with nanomolar affinities may be useful in adoptive T cell transfers, since both CD8$^+$ and CD4$^+$ T may gain desired function and the same specificity when redirected with high-affinity TCRs. CD8$^+$ T cells expressing high-affinity TCRs could compensate for low antigen-expression by cancer cells and thereby prevent the outgrowth of antigen-loss variants after treatment. CD4$^+$ T cells transduced with the same TCR recognizing the same antigen presented on MHC class I may provide help during several phases of the immune response.

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The nanomolar-affinity TCR m33 used in this study was generated from the wild-type TCR 2C using yeast display and selection with SIY-K^b.[16] The 2C T cell clone was derived from a BALB.B (C.B10-*H2*^{b}/LilMcd) mouse immunized with the DBA/2 (H-2^d) mastocytoma line, P815 and restimulated with BALB/c splenocytes *in vitro*.[17] Besides recognizing the allo-MHC L^d,[18] 2C also recognizes the model peptide SIY and the self-peptides dEV-8 and p2Ca presented on K^b.[19–21] dEV-8 is derived from a nuclear-encoded mitochondrial protein, part of complex I of the NADH ubiquinone complex (MLRQ)[20] and p2Ca is derived from α-ketoglutarate dehydrogenase;[22] both are expressed in every cell. The affinity of m33 for SIY-K^b was increased 1,000-fold compared to 2C (K_D = 28 nmol/l versus 32 µmol/l). In contrast, the affinity of m33 for the self-peptide dEV-8 on K^b shows only about a twofold increase (K_D = 38 µmol/l versus 84 µmol/l);[16] the affinity of m33 for p2Ca-K^b is unknown but the affinity of 2C TCR for p2Ca-K^b is very low, about 300 µmol/l.[23]

Presently, the fate *in vivo* of T cells transduced to express nanomolar-affinity TCRs appears to be unknown. In this study, we have therefore transduced primary mouse CD8$^+$ and CD4$^+$ T cells with the high-affinity TCR m33. The m33-transduced CD8$^+$ T cells showed potent function *in vitro*, but *in vivo*, these T cells disappeared within hours after transfer into a syngeneic host without causing graft-versus-host disease (GVHD). In contrast, CD4$^+$ T cells expressing m33 showed CD8-independent antigen-specific anticancer function and survived for more than 3 months *in vivo*.

RESULTS

Optimized nanomolar-affinity TCRs can be functionally expressed on primary CD8$^+$ T cells

We used retroviral vectors to transfer the nanomolar-affinity TCR m33 and its wild-type counterpart 2C into peripheral T cells, resulting in high levels of TCR expression (**Figure 1**). The β-chain of both TCRs (Vβ8) was expressed on the surface of 2C-transduced and m33-transduced OT-I T cells at similar levels. Heterodimerization of 2C α and β TCR chains was confirmed using the anti-clonotypic antibody 1B2. Since this antibody does not bind to the TCR αβ dimer that contains the mutated α-chain of m33, we used SIY peptide-loaded K^b-IgG fusion protein dimers to verify proper expression of the m33 αβ TCR, and to prove antigen-specific binding of transduced receptors. No difference between 2C and m33 in TCR surface levels was detected. The peptide sensitivity of both transduced T cell populations revealed no differences, as 2C- or m33-expressing CD8$^+$ T cells responded to SIY-loaded T2-K^b cells with a similar SD_{50} of 1×10^{-11} to 1×10^{-12} mol/l (**Figure 2a**, upper panel). Together, the results show functional expression of the wild-type and high-affinity TCRs on mouse CD8$^+$ T cells.

Nanomolar-affinity TCR-transduced CD8$^+$ T lymphocytes are reactive against high self-peptide levels

Analysis of the peptide sensitivity of 2C- or m33-transduced T cells for the self-peptides dEV-8 (EQYKFYSV) and p2Ca (LSPFPFDL) confirmed a higher sensitivity of m33-expressing T cells for dEV-8-K^b (**Supplementary Figure S1**; SD_{50} of 1×10^{-8} to 1×10^{-9} mol/l versus only slight stimulation of 2C at 1×10^{-6} mol/l). Interestingly, stimulation by p2Ca-K^b was slightly reduced for the m33 TCR-transduced T cells population, compared to 2C, suggesting that the affinity of m33 for p2Ca-K^b is, if anything, lower than of 2C.[23] These results are consistent with the very high degree of structural similarity between SIY (the original selecting peptide for m33) and dEV-8.[24] While the tertiary structure of the p2Ca-K^b complex is unknown, the two peptides SIY and p2Ca exhibit less primary structural similarity (SIYRYYGL and LSPFPFDL) than SIY and dEV-8.

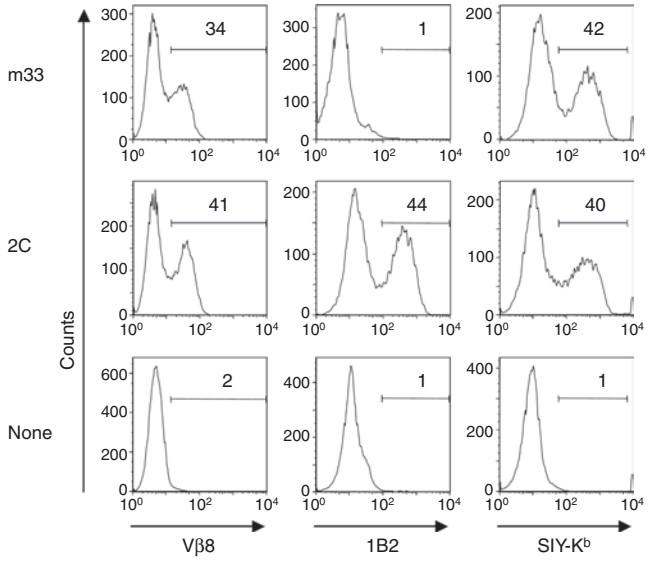

Figure 1 m33 T cell receptor (TCR)-transduced CD8$^+$ T cells bind SIY-K^b. OT-I cells were transduced with retrovirus encoding the 2C or m33 TCRs or not transduced (None). Two days after transduction, cells were stained with anti-CD8 and anti-Vβ8, 1B2 or (SIY-K^b)$_2$/IgG, and gated on CD8$^+$ for analysis. Numbers within each panel indicate percentage of positive cells among CD8$^+$ cells. The data are representative for more than five transductions, resulting in a mean of 30% of CD8$^+$ cells expressing 2C (raging from 10 to 52%) and 27% expressing m33 (ranging from 12 to 46%).

To evaluate the cytolytic capabilities of the transduced T cells *in vitro*, we determined how effectively these cells killed MC57 cancer cells that over expressed the "foreign" SIY peptide or the "self" dEV-8 peptide. While both the 2C- and m33-transduced OT-I cells killed MC57-SIY cancer cells, only the high-affinity m33-expressing T cells were able to lyse MC57-dEV-8 cells (**Figure 2b**). 2C- or m33-transduced OT-I cells did not lyse non-transfected MC57 cells. This suggests that physiological levels of dEV-8, as found in nontransfected MC57, do not suffice to sensitize these cells to lysis by the m33-expressing OT-I T cells.

Nanomolar-affinity TCR-expressing CD8$^+$ T cells are deleted *in vivo*

Having established that m33-transduced CD8$^+$ T cells are functional *in vitro*, we next analyzed their behavior *in vivo*. Thy1.1$^+$ OT-I host animals received an adoptive transfer of 2C- or m33-transduced Thy1.1$^-$ OT-I T cells. Before transfer, T cell populations revealed similar percentages of Vβ8$^+$ cells (41% and 34%, respectively; **Figure 1**). Three days after transfer, we analyzed the transferred Thy1.1$^-$ T cells in the blood of Thy1.1$^+$ recipients. To our surprise, we could not detect any Vβ8$^+$ transferred T cells

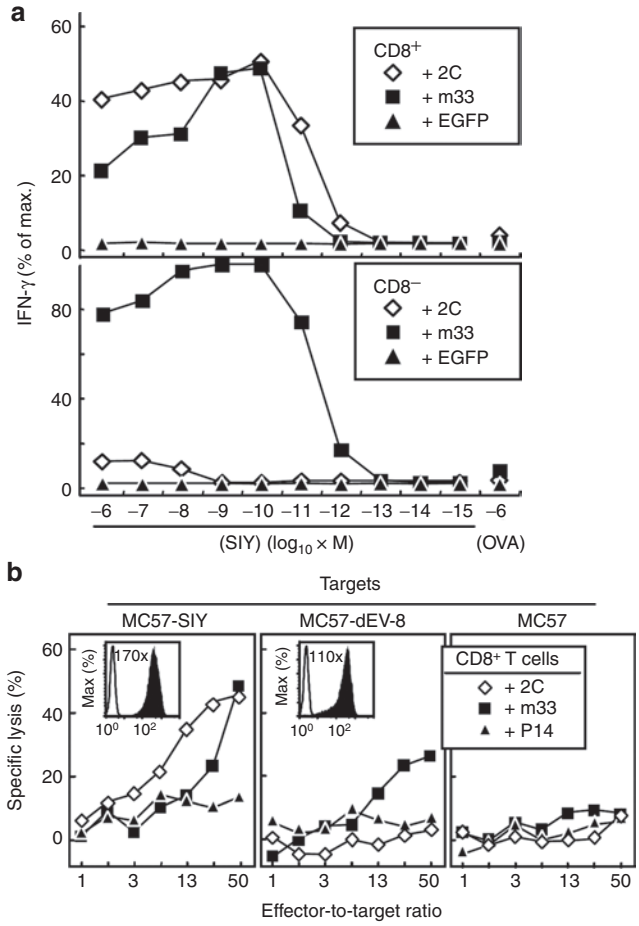

Figure 2 m33 or 2C-TCR-expressing CD8$^+$ T cells have similar peptide sensitivity. Only m33-transduced T cells kill cancer cells over expressing the self-peptide dEV-8, and CD4$^+$ T cells only respond to SIY when expressing m33. (**a**) CD4$^{-/-}$ (CD8$^+$) and CD8$^{-/-}$ (CD8$^-$) T cells were transduced to express m33, 2C, or enhanced green fluorescent protein (EGFP). Rested cells were subsequently cultured with serially diluted SIY or $OVA_{257–264}$-pulsed T2-K^b cells for 24 hours. Coculture supernatants were analyzed for secreted interferon-γ (IFN-γ). Data are depicted as percentage of maximal stimulation (anti-CD3 and anti-CD28 antibody) and are representative for four independent experiments. (**b**) 4.5 hours ^{51}Cr-release assay with the fibrosarcoma lines MC57, MC57-SIY, and MC57-dEV-8 as targets and m33, 2C or P14 T cell receptor (TCR)-transduced OT-I T cells as effectors. Insets demonstrate similar levels of antigen expression by the cancer cells, as determined by fluorescence of the triple-peptide/GFP fusion protein. Results are representative for five independent experiments.

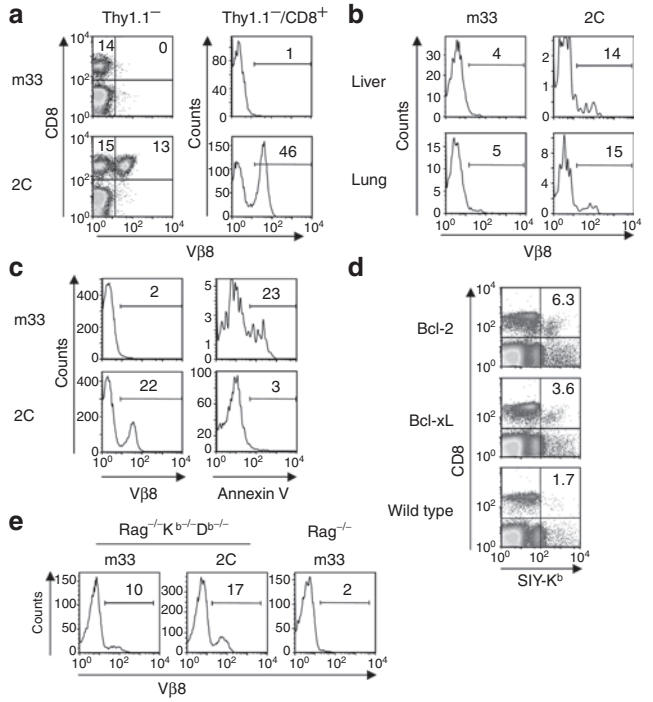

Figure 3 m33 TCR-expressing CD8$^+$ T cells are rapidly deleted *in vivo*. (**a**) Blood of OT-I/Thy1.1 recipient mice were analyzed 3 days after adoptive transfer of 2C or m33-transduced OT-I T cells. Leukocytes were stained with anti-Thy1.1, anti-CD8, and anti-Vβ8. Left panels show cells gated on Thy1.1$^-$ lymphocytes, numbers indicate the percentage of cells of all Thy1.1$^-$ cells. The right panels show Thy1.1$^-$, CD8$^+$ lymphocytes and the numbers indicate the percentage of Vβ8$^+$ cells among Thy1.1$^-$ CD8$^+$ cells. Similar results were obtained in three experiments, showing a mean of 21% of CD8$^+$ T cells expressing 2C (ranging from 10 to 46%) and only 0.8% expressing m33 (ranging from 0 to 1%). (**b**) Analysis of liver and lung of OT-I/Thy1.1 mice that received 2C or m33-transduced OT-I T cells 6 hours earlier. Leukocytes were stained with anti-Thy1.1, anti-CD8, anti-Vβ8. Cells shown were gated on Thy1.1$^-$ CD8$^+$ lymphocytes, the numbers in each panel indicate percentage of Vβ8$^+$ cells among Thy1.1$^-$ CD8$^+$ cells. Data shown are representative for three independent experiments. (**c**) Analysis of OT-I/Thy1.1 mice described in (b) on day 3 after transfer. The left graphs show Thy1.1$^-$ CD8$^+$ lymphocytes; the numbers in each panel indicate the percentage of Vβ8$^+$ cells among Thy1.1$^-$ CD8$^+$ cells. The right graphs are additionally gated on Vβ8$^+$ cells and numbers indicate the percentage of Annexin V-positive Thy1.1$^-$ CD8$^+$ Vβ8$^+$ cells. (**d**) Blood of OT-I/Thy1.1 recipient mice was analyzed 2 days after adoptive transfer of m33-transduced Bcl-2-, Bcl-xL-transgenic or wild-type T cells (m33 expression pretransfer was 19, 12, and 33%, respectively). Leukocytes were stained for CD8, Thy1.1, and with $(SIY\text{-}K^b)_2$/IgG. Cells shown in the dot plots were gated on Thy1.1$^-$ cells. Numbers in each panel indicate percentage of SIY-K^b-binding T cells among Thy1.1$^-$ CD8$^+$ T cells. (**e**) Blood of *Rag*$^{-/-}$*K*$^{b-/-}$*D*$^{b-/-}$ and *Rag*$^{-/-}$ recipient mice was analyzed 3 days after adoptive transfer of m33- or 2C-transduced OT-I T cells. Leukocytes were stained for CD8 and Vβ8. Cells shown in the histograms were gated on CD8$^+$ cells. Numbers in each panel indicate percentage of Vb8$^+$ cells among CD8$^+$ T cells.

in mice that had received the m33-transduced T cells (**Figure 3a**). In contrast, 46% of the transferred 2C-transduced CD8$^+$ T cells were Vβ8$^+$, a percentage similar to that seen before transfer.

In order to understand the fate of m33-expressing CD8$^+$ T cells *in vivo*, we analyzed the distribution of the Vβ8$^+$ T cells at earlier time points and in various organs. As early as 3 hours post-transfer, there were more 2C- than m33-expressing T cells in blood, spleen, lung, and liver. **Figure 3b** shows that 6 hours post-transfer, m33-expressing cells were hardly detectable in lung and liver while a distinct population of 2C-expressing cells could be seen. The few transferred Vβ8$^+$ CD8$^+$ T cells found in mice that received m33-transduced cells, showed a higher rate of apoptosis, as detected by Annexin V staining, compared to mice that received 2C-transduced cells (**Figure 3c**). When we transduced T cells from Bcl-2- or Bcl-xL-transgenic mice with m33 and transferred these into Thy1.1$^+$ recipients; the survival of m33-expressing CD8$^+$ T cells was partially restored (**Figure 3d**).

To confirm that the T cells died of apoptosis and did not escape detection in the bloodstream of recipient mice by down-modulating m33 TCR expression, we transduced OT-I CD8$^+$

T cells to coexpress green fluorescent protein (GFP) and 2C or m33. TCR/GFP double-positive T cells were sorted (>85% purity) and transferred into *Rag1*$^{-/-}$ mice. One week later, we analyzed the blood of the host mice for circulating T cells expressing both Vβ8 and GFP. While 50% of all circulating CD8$^+$ T cells were TCR/GFP double-positive when transduced with 2C, only 4% were TCR/GFP double-positive when transduced with m33 (**Supplementary Figure S2a**). Importantly, the percentage of GFP single-positive T cells did not increase in the population of circulating CD8$^+$ T cells isolated from recipients of the m33/GFP-transduced cells compared to the percentage of GFP single-positive T cells found in recipients of the 2C-transduced population. Thus, m33/GFP-transduced T cells did not remain in circulation by down-modulating the m33 TCR.

Although the binding affinity of m33 for the self-peptide dEV-8-K^b complex is only about twofold higher than of 2C, m33-, and not 2C-transduced T cells recognized and killed targets that overexpressed dEV-8-K^b (**Figure 2b**), and disappearance of transduced T cells *in vivo* was clearly dependent on m33 TCR expression (**Figure 3** and **Supplementary Figure S2**). To analyze whether disappearance was caused by binding to self-ligands in H-2^b mice, we observed the survival of m33-expressing T cells in an environment that does not express H-2^b. Transduction and transfer experiments were performed using *Rag*$^{-/-}$*K*$^{b-/-}$*D*$^{b-/-}$ triple knockout mice, which received 2C or m33-transduced OT-1 T cells. Three days after transfer, m33-expressing CD8$^+$ T cells were still detected in these mice lacking the MHC class I molecules K^b and D^b (**Figure 3e**). A similar experiment was performed in C3H mice, whose H-2^k haplotype is not known to be recognized by 2C or m33. Wild-type C3H T cells transduced to express m33 or 2C TCRs were transferred into C3H *Rag2*$^{-/-}$ mice and analyzed for survival 6 days later. Although the percentage of cells that expressed the transferred TCR was lower for m33 compared to 2C (6% versus 10%) (**Supplementary Figure S2b**, left panel), more m33-transduced CD8$^+$ cells survived in mice of the H-2^k background than in mice of the H-2^b haplotype. Together our results indicate that m33 expression on CD8$^+$ T cells and expression of MHC class I of the b haplotype in the host lead to apoptosis of these T cells.

m33- but not 2C-expressing CD4$^+$ cells bind detectably to SIY presented on the MHC class I molecule K^b

We transduced CD4$^+$ T splenocytes of a CD8-deficient mouse with 2C or m33. Both populations showed a large fraction of cells expressing Vβ8 (63% and 66%, respectively; **Figure 4a**). 21% of untransduced T cells stained for Vβ8 as expected for T cells with a wild-type TCR repertoire. As for CD8$^+$ T cells, most CD4$^+$ cells that expressed Vβ8 did express the correctly paired α- and β-chains of 2C (1B2 staining) and m33 (SIY-K^b dimer). In contrast to CD8$^+$ T cells, CD4$^+$ cells could only bind the SIY-K^b dimer if they expressed m33; inability to detect binding of the 2C TCR to SIY-K^b in the absence of CD8 has been shown previously.[25] Similar data were also obtained for CD4$^+$ T cells from OT-II TCR transgenic mice (**Supplementary Figure S3**). Consistent with this finding, only m33-expressing CD4$^+$ T cells responded to SIY-loaded T2-K^b cells by interferon-γ (IFN-γ) secretion (**Figure 2a**, lower panel).

We next set up a 24 hours cocultivation of 2C-, m33-, or P14-transduced CD4$^+$ T cells, the latter recognizing an irrelevant peptide of the lymphocytic choriomeningitis (LCMV)

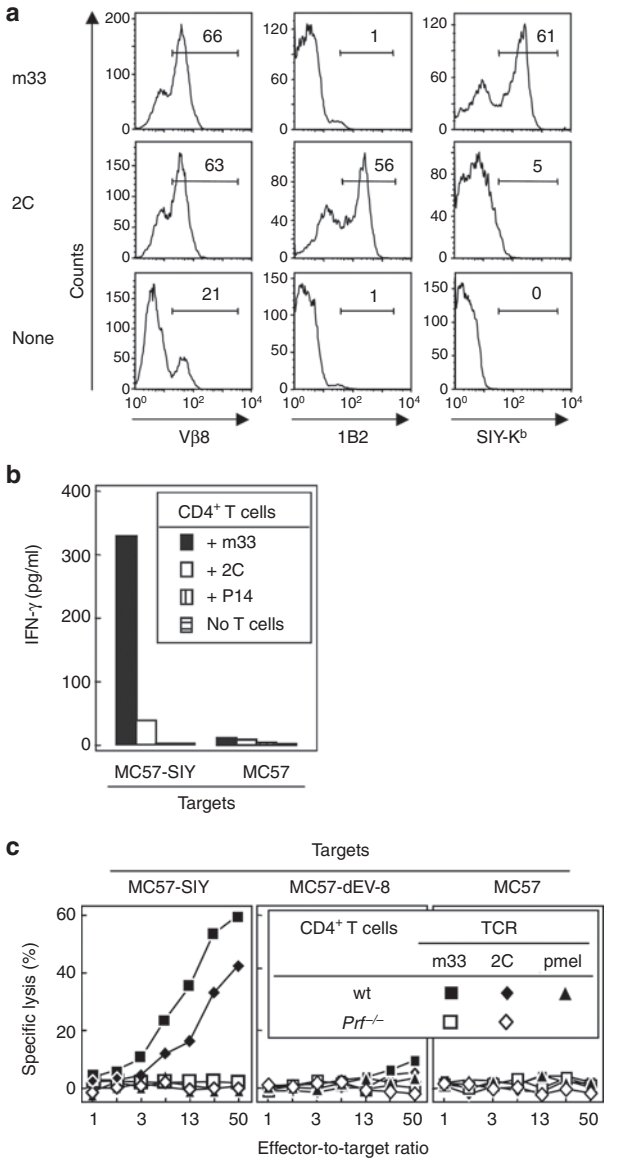

Figure 4 m33, but not 2C-expressing CD4$^+$ cells, bind SIY presented on the histocompatibility complex (MHC) class I molecule K^b. (**a**) CD4$^+$ T cells from CD8$^{-/-}$ mice were transduced with 2C, m33 or not transduced (None). Two days after transduction, cells were stained with anti-CD4 and anti-Vβ8, 1B2, or (SIY-K^b)$_2$/IgG, and gated on CD4$^+$ for analysis. Numbers in each panel indicate percentage of positive cells among CD4$^+$ cells. The data are representative for more than five transductions, resulting in a mean of 75% of CD4$^+$ T cells expressing 2C (raging from 63 to 88%) and 76% expressing m33 (ranging from 66 to 87%). (**b**,**c**) 2C and m33 T cell receptor (TCR)-transduced CD4$^+$ T cells were tested *in vitro* for functionality. (**b**) TCR-transduced responder T cells from CD8$^{-/-}$ mice were cultured with the cancer cell lines MC57 or MC57-SIY for 24 hours and secreted interferon-γ (IFN-γ) was quantified by ELISA. Data are representative for five independent experiments. (**c**) 4.5 hours ^{51}Cr-release assay with MC57 and MC57-SIY as targets and m33-, 2C-, or pmel TCR-transduced CD4$^+$ T cells as effectors. T cells from wild-type and *Prf*$^{-/-}$ mice were sorted for CD4$^+$ T cells before transduction. Similar results were obtained in five independent experiments, only two of which included *Prf*$^{-/-}$ T cell responders and MC57-dEV-8 targets.

glycoprotein gp33. Transduced cells were cultured with the cancer cell lines MC57 or MC57-SIY. $CD4^+$ T cells that expressed m33 released large amounts of IFN-γ when cultured with the SIY-expressing cancer cells (**Figure 4b**). In contrast, 2C-transduced $CD4^+$ T cells produced ninefold less IFN-γ. P14-transduced cells were not stimulated by any of the three cancer cell lines.

Although it is known that $CD4^+$ T cells are inefficient killers,[26] several studies have reported killing by $CD4^+$ T cells that express high-affinity TCRs.[14] In ^{51}Cr-release assays, m33-expressing $CD4^+$ T cells killed MC57-SIY somewhat more effectively than 2C-expressing $CD4^+$ T cells (**Figure 4c**). Nevertheless, parental MC57 cancer cells expressing natural levels of dEV-8 were not killed and even overexpression of dEV-8 was inefficient in sensitizing MC57 cells. pmel-transduced $CD4^+$ T cells, which recognize an irrelevant peptide of murine gp100, did not kill any of the cancer cells. The killing by m33 or 2C-transduced $CD4^+$ T cells was perforin-dependent as shown by transduction of sorted $CD4^+$ T cells from perforin-deficient mice (**Figure 4c**, empty symbols). Killing and release of IFN-γ by $CD4^+$ T cell expressing m33 shows that these cells recognize the cognate peptide SIY presented on the class I molecule K^b.

Nanomolar-affinity TCR-expressing $CD4^+$ cells survive *in vivo* long term

Having shown the function of m33-expressing $CD4^+$ T cells *in vitro*, we were interested to see whether these cells could survive *in vivo*. 2C or m33-transduced $CD4^+$ T cells of $CD8^{-/-}$ mice (shown in **Figure 4a** before transfer) were adoptively transferred into $Thy1.1^+$ OT-I mice. In sharp contrast to m33-transduced $CD8^+$ T cells, $CD4^+$ T cells expressing the nanomolar-affinity TCR survived *in vivo*. Three days after transfer, both 2C and m33-expressing $CD4^+$ T cells were detected in the blood of host animals (**Figure 5a**). The ratio of transduced to nontransduced cells before and after transfer remained the same for both populations of redirected T cells (63 and 66% pretransfer; 67 and 65% post-transfer for 2C and m33, respectively). Furthermore, 48% of the transferred m33-transduced T cell population bound the SIY-K^b dimer when analyzed *ex vivo*. Long-term survival of both T cell populations was similar: 80 days after adoptive transfer comparable numbers of $CD4^+$ T cells still expressed the transferred TCR 2C or m33 (32% and 34%, respectively).

To verify the importance of the lack of the CD8 coreceptor for survival of m33-expressing $CD4^+$ T cells, we cotransduced OT-II $CD4^+$ T cells with either the 2C or the m33 TCR and the CD8αβ heterodimer. 28–31% of the T cells expressed TCR and CD8 pretransfer (Pre, **Figure 5b**). Upon transfer, $CD4^+$ cells expressing m33 and CD8αβ were again deleted, while cells expressing m33 alone and cells expressing 2C and CD8αβ survived (Day 4, **Figure 5b**). Transductions of C57BL/6 wild-type T cells with either the 2C or the m33 TCR retrovirus showed, consistent with our earlier observations, that $CD8^+$ T cells expressing the m33 TCR were deleted, while m33-expressing $CD4^+$ T cells survived (**Supplementary Figure S4**). In summary, high-affinity TCR-transduced $CD4^+$ T cells survived *in vivo,* for which they needed to lack the CD8 coreceptor, and could be detected in animals for >80 days after transfer.

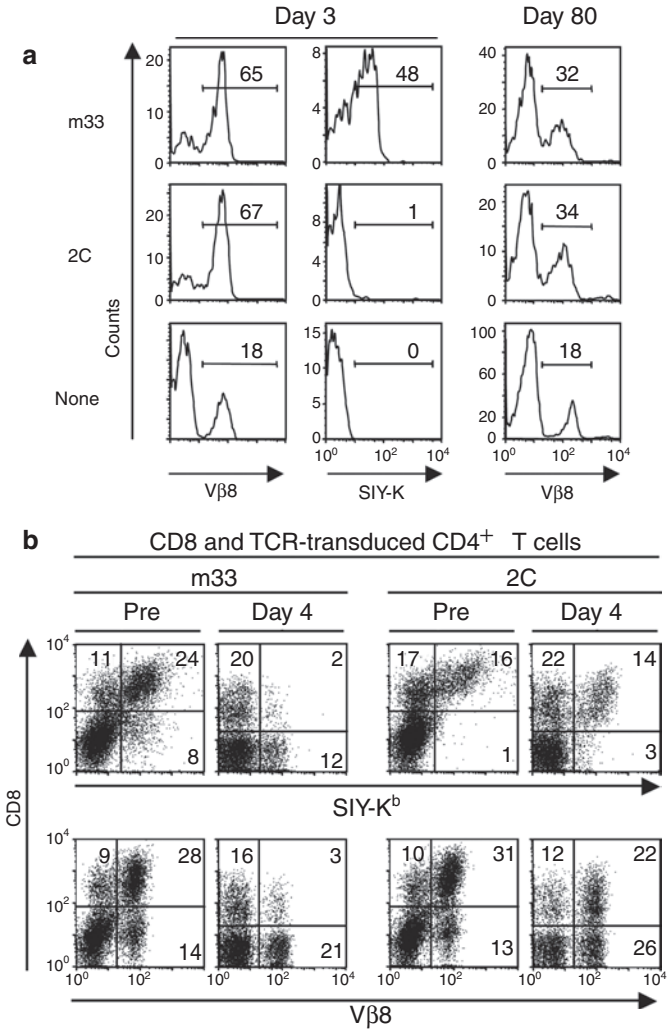

Figure 5 m33 T cell receptor (TCR)-expressing $CD4^+$ cells survive *in vivo* due to the lack of CD8 expression. (**a**) Blood of OT-I/Thy1.1 recipient mice were analyzed 3 and 80 days after adoptive transfer of 2C- or m33-transduced $CD8^{-/-}$ T cells. Leukocytes were stained with anti-CD4 and anti-Vβ8 or $(SIY\text{-}K^b)_2$/IgG, and gated on $Thy1.1^-$ $CD4^+$ cells. Numbers in each panel indicate percentage of positive cells among $Thy1.1^-$ $CD4^+$ cells. Data is representative for three independent experiments with two mice per group, showing a mean of 33% of $CD8^+$ T cells expressing 2C (ranging from 25 to 42%) and 25% expressing m33 (ranging from 19 to 32%). (**b**) OT-II/$CD8^{-/-}$ T cells were cotransduced with the 2C or m33 TCR and CD8αβ heterodimer. T cells were transferred in OT-I/$Thy1.1^+$ mice and their survival analyzed 4 days later. Cells pretransfer and cells from blood of mice on day 4 post-transfer were stained with anti-CD4 and anti-CD8 and anti-Vβ8 or $(SIY\text{-}K^b)_2$/IgG and dot plots show cells gated on $Thy1.1^-$ and $CD4^+$. Numbers indicate the percentage of cells within the respective quadrant of $Thy1.1^-$ $CD4^+$ cells. The results are representative for two independent experiments with two mice per group.

Nanomolar-affinity TCR expressing $CD4^+$ T cells retain their reactivity for SIY-K^b and reject cancer cells

Since nanomolar-affinity TCR expressing $CD4^+$ T cells were functional *in vitro* and survived long-term *in vivo*, we next examined whether they also retained several of their functional activities *in vivo*. Thus, we adoptively transferred m33 or 2C-transduced $CD4^+$ T cells of an OT-II mouse into OT-I $Thy1.1^+$ hosts. We immunized the recipient mice with $OVA_{323–339}$ peptide. Ten days later,

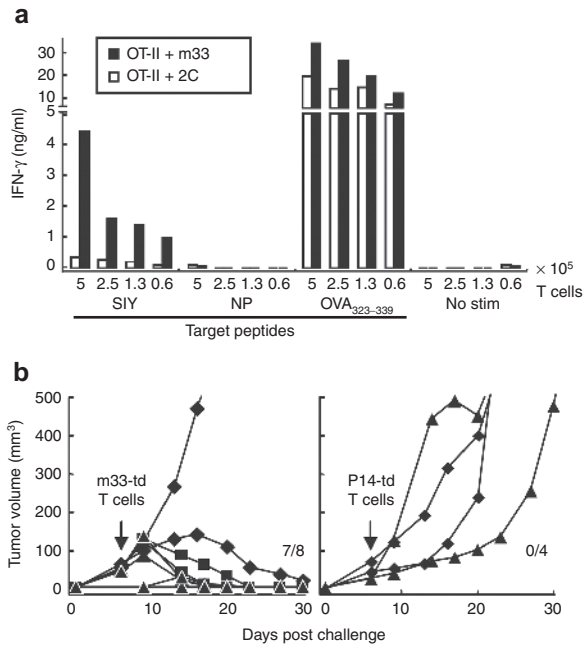

Figure 6 Nanomolar-affinity T cell receptor (TCR) expressing CD4$^+$ T cells specific for peptide-histocompatibility complex (MHC) class I secrete cytokine *ex vivo* and cause rejection of tumor cell inocula. (**a**) Secretion of interferon-γ (IFN-γ) by T cells stimulated with peptide-pulsed splenocytes for 24 hours *ex vivo*. T cells were derived from two mice that had received 2C or m33-expressing OT-II TCR-transgenic CD4$^+$ T cells. More than 1 month after T cell transfer, mice were injected with $OVA_{323–339}$ in complete Freund's adjuvant (CFA) in both foot pads. Popliteal lymph nodes of each mouse were isolated 10 days after immunization. 5, 2.5, 1.3, and 0.6 × 10^5 T cells were stimulated with SIY, NP, or $OVA_{323–339}$ peptide-pulsed splenocytes or not (no stim). Secretion of IFN-γ was analyzed 24 hours later. The results are representative for three independent experiments using $OVA_{323–339}$ or NP peptides for stimulation *in vivo* (see **Supplementary Figure S5**). (**b**) *Rag*$^{-/-}$ mice growing tumors for 6 days after inoculation with MC57-SIY cancer cells were treated with m33 (left panel) or P14 (right panel) TCR-transduced CD4$^+$ T cells. T cells were derived from OT-II (squares) or wild-type mice (triangles and diamonds) and were activated using beads (squares and triangles) or plate-bound antibody (diamonds). Numbers indicate number of mice with rejected tumors per number of treated mice. Results from three independent experiments are shown, $P = 0.01$ comparing m33 to P14.

draining popliteal lymph nodes were isolated and the T cells were restimulated with different peptides. Only cells from the mouse that had received m33-transduced CD4$^+$ T cells showed secretion of IFN-γ upon restimulation with SIY peptide (**Figure 6**), while T cells expressing 2C did not respond to SIY. Cells from both mice responded to restimulation with $OVA_{323–339}$, the antigen for the OT-II TCR. Similar results were obtained in experiments, where we transferred transduced T cells from CD8$^{-/-}$ mice (**Supplementary Figure S5**).

The reactivity and SIY-K^b-specificity of m33-trasduced CD4$^+$ T cells, and their long-term persistence *in vivo*, prompted us to examine if this population could function in the rejection of a tumor that expresses SIY. m33-transduced CD4$^+$ T cells, transferred on day 6 after cancer cell inoculation, rejected the MC57-SIY tumors of 50 mm^3 (7 of 8 mice) while P14-TCR transduced T cells did not reject the same tumors (0 of 4 mice, $P = 0.01$ for three experiments; **Figure 6b**). Together, these experiments *ex vivo* and *in vivo* demonstrate the ability of CD4$^+$ T cells that express a nanomolar-affinity TCR to survive long term, to retain their specificity toward a MHC class I presented peptide, and to reject cancer cell inocula that expresses the SIY peptide.

DISCUSSION

In this study, we show that CD8$^+$ T cells expressing the nanomolar-affinity MHC class I-restricted TCR m33 were deleted within hours in H-2^b mice, without causing GVHD. CD4$^+$ T cells expressing the same receptor survived for months and rejected cancer cell inocula. *In vitro*, we showed that m33-expressing CD4$^+$ T cells were able to lyse target cells through recognition of the cognate class I-restricted antigen. These CD4$^+$ T cells were also capable of CD8-independent cancer rejection when targeting the same class I-restricted antigen. Using CD4$^+$ T cells expressing nanomolar-affinity TCRs for adoptive transfers is attractive because, in addition to the direct anticancer effects described here, many studies have shown the importance of the interplay of CD8$^+$ and CD4$^+$ T cell subsets for proper anticancer immune responses (reviewed in ref. 27). Accordingly, use of a high-affinity class I-restricted TCR could abolish the need for separate helper epitopes presented on MHC class II, which are less frequently expressed and/or difficult to find on cancer and other target cells.

CD4$^+$ T cells are important in affinity maturation[28] and class switching (reviewed in ref. 29) of antibodies but also in memory,[30–32] persistence,[33,34] and the effector phase[35–37] of CD8$^+$ T cells. Furthermore, CD4$^+$ T cells are known to have suppressive and regulatory functions.[38–40] Some studies suggest that Foxp3$^+$ TCR-transduced CD4$^+$ regulatory T cells could be used to suppress specific immune responses.[41] Other studies indicate that double-negative T cells with high-affinity TCRs for alloantigens can suppress CD8$^+$ T cells with the same allospecificity.[42] Thus, it will be important in future studies to examine whether nanomolar-affinity TCRs as described here can drive the CD4$^+$ T cell populations toward particular lineages *in vivo*, in addition to functions such as the direct anticancer effects described here.

In vitro studies by other laboratories and us have shown that T cells expressing high-affinity TCRs can exhibit higher peptide sensitivity[10,11,14] and coreceptor (CD8) independence.[15,16] Using the high-affinity TCR m33 and its wild-type counterpart 2C, we could recapitulate the findings. Primary mouse CD8$^+$ T cells expressing m33 recognized and lysed cancer cells overexpressing the self-peptide dEV-8, while 2C-transduced T cells only lysed SIY-overexpressing cells. Interestingly, cocultures with diluted SIY peptide on APC did not show an increased sensitivity of CD8$^+$ T cells expressing m33. This is consistent with an earlier publication using a T cell hybridoma expressing these TCRs[43] and is most likely due to the strong impact of the CD8 coreceptor on increasing avidity and signaling capabilities by recruiting Lck. CD4$^+$ T cells expressing m33 were not stimulated by dEV-8-K^b while they were stimulated efficiently by SIY-K^b, resulting in IFN-γ secretion and lysis of target cells. CD4$^+$ cells expressing 2C showed only low reactivity for SIY. We believe that the difference in function between m33 and 2C becomes more evident when cytokine secretion is measured rather than killing because the threshold of required TCR–pMHC interactions is likely higher for induction of cytokine release than for killing.[44] The affinity of 2C

may be high enough to bind to sufficient SIY-K^b to trigger killing, but not to secrete IFN-γ.

Importantly, once $CD8^+$ T cells transduced with the m33 TCR were transferred *in vivo*, they were rapidly deleted. In contrast, wild-type 2C TCR-transduced $CD8^+$ T cells survived long-term. Since K^b-self-reactivity of m33-transduced $CD8^+$ T cell hybridomas had been shown previously,[10] deletion of these cells *in vivo* without causing severe or any detectable GVHD could not have been predicted. In fact, a recent study showed GVHD induced by TCR-transduced T cells expressing mispaired TCR α- and β-chains.[45] Our results suggest that another consequence of TCR gene transfer is peripheral T cell tolerance through deletion, presumably because of self-reactivity of nanomolar-affinity TCRs. The fact that $CD8^+$ T cells expressing the high-affinity TCR were deleted may mitigate some of the potential risks associated with overt self-reactivity. Why rapid deletion was the outcome in our experiments, rather than T cell-induced GVHD is not clear. It is possible that the mispaired αβ TCRs had lower affinities for self-pMHC than the m33 TCR and T cells were therefore not deleted and could cause GVHD, or the target antigens could have had a more restricted tissue distribution than the m33 self-antigens. Another difference could have been the mode of priming prior to transduction of the T cells. We used peptide or anti-CD3 and anti-CD28 antibodies with low-dose IL-2, while Bendle and colleagues used concanavalin A and IL-7.[45] Also, TCR gene transfer-induced GVHD was dependent on strategies promoting T cell function *in vivo*, such as IL-2 injection or cotransduction of T cells with dominant-negative TGF-β receptor. These differences show that the precise protocol of adoptive T cells transfer is important and may alter the outcome and success of T cell therapies.

Analysis of the disappearance of m33-expressing $CD8^+$ T cells showed that the cells do not down-modulate their TCR and thereby become undetectable, but rather disappear from circulation and organs analyzed. The disappearance of nanomolar-affinity TCR-transduced $CD8^+$ T cells was dependent on the interaction of m33, CD8 and H-2^b, as omitting any of the three led to survival of the transferred cells. Furthermore, when we introduced the CD8αβ coreceptor into $CD4^+$ T cells expressing m33, these cells were also deleted, indicating the important role of CD8 in the deletion, likely by enhancing the T cell avidity, and perhaps driving signaling through an apoptotic pathway. Earlier studies describe the large increase (10^6-fold) in peptide sensitivity *in vitro* of a T cell hybridoma line by the additional MHC-I–CD8 interaction.[16]

As we have detected apoptosing Annexin V-positive cells in the tissues shortly after transfer, and could demonstrate partial restoration of survival of m33-expressing $CD8^+$ T cells when transducing cells from Bcl-2 or Bcl-xL-transgenic mice, we conclude that $CD8^+$ T cells expressing m33 die in the periphery due to activation-induced cell death. The induction of apoptosis either through strong, persistent binding of the high-affinity TCR to pMHC or through high abundance of the recognized self-peptide, leading to the integration of many weak signals, may be similar to the mechanism of hysteresis observed for negative selection.[46] Considering the two known self-peptides recognized by m33, we suggest that the most likely antigen that led to activation-induced cell death is dEV-8. The affinity of m33 for p2Ca-K^b is not known, but our peptide sensitivity results suggest that the affinity of this interaction is, if anything, less than that of the 2C:p2Ca-K^b interaction.[23] Furthermore, the m33 TCR was selected on SIY-K^b, and it is well known that SIY and dEV-8 are structurally very similar.[24] Depletion *in vivo* of m33-expressing T cells may therefore not be surprising, provided that the self-antigen is expressed at sufficient level. It is important to note that these T cells were not stimulated by naturally expressed dEV-8 *in vitro*, probably because the level of expression of endogenous dEV-8-K^b is below the threshold required for induction of T cell activity, but apparently at a level that can induce activation-induced cell death *in vivo*. Therefore, the outcome of the transfer of m33-expressing $CD8^+$ T cells could not have been predicted based on the studies *in vitro*. It remains to be demonstrated whether the affinity of some TCRs for specific non-self antigens can be augmented to nanomolar-range without enhancing the affinity for the self-peptides recognized by the same TCR due to molecular mimicry. Several studies have shown that an increase in affinity does lead to an increase in self-reactivity,[10] or a loss of specificity, which could also have been increase in self-reactivity, as the wide reactivity observed remained MHC-restricted.[47]

m33-expressing $CD8^+$ T cells also disappeared in Perforin ($Prf^{-/-}$) and *Rag2* and IL-2 receptor (common) gamma chain double nanomolar-knockout mice ($Rag2^{-/-}Il2rg^{-/-}$), further supporting a cell intrinsic process rather than an involvement of the host killing activated T cells (**Supplementary Figure S6**). It is important to note that the survival *in vivo* of T cells expressing chimeric antibody receptors[48,49] with similar affinities to m33 does not contradict our findings. Binding of chimeric antibody receptors to their cognate epitope does not involve MHC nor is it thought to involve coreceptor recognition (*i.e.*, reflecting the situation where $CD4^+$ T cells express m33, where no deletion occurred).

Our results showing the rapid deletion of m33-transduced T cells suggest that some higher affinity TCRs will not be useful for adoptive transfer of TCR-transduced $CD8^+$ T cells. They also show however, that the risk of overt GVHD induced by TCR-transduced T cells may be in some cases alleviated due to rapid deletion of the self-reactive T cells. In order to circumvent the deletion of putative effective $CD8^+$ T cells, TCRs with affinities above a threshold for CD8-dependence, but below a threshold resulting in loss of $CD8^+$ T cells through activation-induced cell death, could be chosen. In addition, TCRs will have to be selected for high-affinity to the peptide of interest and at the same time selected against affinity increases for self-peptides. Whether this is possible remains to be shown.

Our findings also show that $CD4^+$ T cells transduced with MHC class I-restricted TCRs of nanomolar affinity reject cancer cell inocula and persist long-term. The anticancer effect was achieved by targeting tumors growing in mice for 6 days. It remains to be investigated what the effect on larger tumors will be. Apart from the strong direct lytic effects of CD4 cells described here (perforin and IFN-γ), it is also conceivable that these high-affinity, CD8-independent TCR-expressing $CD4^+$ T cells could provide help to $CD8^+$ T cells. Our recent findings show that effective elimination of cancer cells as bystanders may require that $CD4^+$ as well as $CD8^+$ T cells recognize epitopes presented in

very close proximity in the local tumor environment.[35] With this in mind, being able to target the same antigen on MHC class I by $CD4^+$ and $CD8^+$ T cells is particularly interesting.

MATERIALS AND METHODS

Retroviral vectors. 2C and m33 TCR, and CD8α and β-chain expression cassettes were cloned into the retroviral vector pMP71. T cells were activated for 24 hours and transductions were performed using ecotropic TCR retroviruses. See **Supplementary Materials and Methods** online for details.

Cell lines. See **Supplementary Materials and Methods** online for cell lines used.

Peptides. The peptides dEV-8 (EQYKFYSV), mp68 (SNFVFAGI), Influenza A virus nucleoprotein peptides 4445 (RSALILRGSVAHKS), and nucleoprotein 5253 (SQVYSLIRPNENPAHK), $OVA_{257–264}$ (SIINFEKL), $OVA_{323–339}$ (ISQAVHAAHAEINEAGR) and SIY (SIYRYYGL), were synthesized by Dr. Meredith (The University of Chicago, Chicago, IL). p2Ca (LSPFPFDL) was synthesized by the Macromolecular Core Facility of the Section of Research Sources, Penn State College of Medicine.

Mice. C57BL/6J were purchased from Jackson Laboratories (Bar Harbor, ME), C3H/HeNCrl from Charles River (Wilmington, MA), and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ from Taconic (Hudson, NY). Colonies of 2C (provided by J Chen, Massachusetts Institute of Technology, Cambridge, MA), Bcl-2-transgenic (provided by H. Huang, The University of Chicago, Chicago, IL), Bcl-xL-transgenic (provided by A. Sperling, The University of Chicago, Chicago, IL), C3H *Rag2*$^{-/-}$ (obtained from D Hanahan, University of California, San Francisco, CA), $CD4^{-/-}$ (B6.129S2-*Cd4*^{tm1Mak}/J; Jackson Laboratories), $CD8^{-/-}$ (B6.129S2-*Cd8a*^{tm1Mak}/J; Jackson Laboratories), OT-I (provided by M Mescher, University of Minnesota, Twin Cities, MN), OT-I/Thy1.1 (provided by T Gajewski, The University of Chicago, Chicago, IL), OT-II (provided by CD Surh, The Scripps Research Institute, La Jolla, CA), OT-II/$CD8^{-/-}$ (obtained by crossing OT-II to $CD8^{-/-}$), *Prf*$^{-/-}$ (C57BL/6-*Prf*^{tm1Sdz}/J; Jackson Laboratories), *Rag1*$^{-/-}$ (B6.129S7-*Rag1*^{tm1Mom}/J; Jackson Laboratories), and *Rag1*$^{-/-}$$K^{b-/-}$$D^{b-/-}$ (obtained by crossing Rag$^{-/-}$ to $K^{b-/-}D^{b-/-}$, which were provided by A Chervonsky, The University of Chicago, Chicago, IL) were maintained at the University of Chicago facilities. The Institutional Animal Care and Use Committee at the University of Chicago approved all animal experiments.

T cell cultures and transductions. NH_4Cl-treated splenocytes were cultured at 4×10^6 cells/ml, 3 ml/well of a 6-well plate in RPMI, 10% FCS (Sigma-Aldrich, St Louis, MO), 2 mmol/l glutamine, 50 μmol/l β-mercaptoethanol, 1 mmol/l Hepes, 1 mmol/l sodium pyruvate, 1× nonessential amino acids, 100 U/ml/100 μg/ml penicillin/streptomycin, 50 μg/ml gentamicin (all Gibco/Invitrogen, Carlsbad, CA). T cells were activated for 24 hours with either 1 μmol/l SIINFEKL (for OT-1) or with 0.2 μg/ml anti-CD3 (2C11), 0.5 μg/ml anti-CD28 (37.51) (eBioscience, San Diego, CA), and 6 U/ml IL-2 (for $CD8^{-/-}$, C57BL/6, and C3H). Ecotropic TCR retroviruses were obtained after transient transfection of the packaging cell line Plat-E with retroviral vector plasmids using calcium phosphate precipitation. For CD8αβ cotransductions, TCR- and CD8-retroviral plasmids were mixed at a 1:1 ratio. 48 hours after transfection, viral supernatant was harvested, filtered (0.45 μm pore size), and either used directly or stored at −20 °C. Stimulated T cells and viral supernatant were added to RetroNectin-coated 6-well plates (12.5 μg/ml coating solution; Takara, Otsu, Japan), and supplemented with protamine sulfate (final concentration: 4 μg/ml; Sigma-Aldrich). Finally, cells were spinoculated for 90 minutes at 800*g* and 32 °C. Transduction of $CD4^+$ T cells for tumor treatment was performed as described above with the following changes: Wild-type C57BL/6 T cells were enriched for $CD4^+$ T cells by MACS (Miltenyi, Auburn, CA). OT-II or enriched wild-type or $CD4^+$ T cells were cultured in complete IMDM (10 % FCS, 2 mmol/l glutamine, 50 μmol/l β-mercaptoethanol, 100 μg/ml penicillin/streptomycin) and stimulated with 10 U/ml IL-2 and Dynabeads Mouse T-Activator CD3/CD28 (Invitrogen) or plate-bound anti-CD3 (2C11) and anti-CD28 antibodies (37.51; both 1 μg/ml, eBioscience) in 24-well plates; protamine sulfate was replaced with Lipofectamine (7.7 ml/ml of viral supernatant; Invitrogen). Transduced T cells were cultured for a total of 3–4 days before adoptive transfer and 4–5 days before assays *in vitro*.

Flow cytometry. Cells were stained using FITC-, PE-, or APC-labeled mAb directed against mouse Vβ8.1/8.2 (KJ16), CD90.1 (Thy1.1, OX-7), CD8α (53–6.7), CD4 (RM4-5), or mouse IgG1 (RMG1-1), and with Annexin V, Streptavidin-PE, mouse IgG1 isotype control (P3), and K^b-DimerX [$(K^b)_2$-IgG] (purchased from BD (Franklin Lakes, NJ) or eBioscience). The biotinylated anti-2C clonotypic antibody 1B2 was provided by Y-X Fu (The University of Chicago, Chicago, IL). Flow cytometry data was acquired on FACSCalibur or FACSCanto machines (BD) and data analyzed using FlowJo (Tree Star, Ashland, OR) software. Cell sorting was performed using FACSAria (BD) or MoFlo-HTS (Beckman Coulter, Brea, CA) at the Flow Cytometry Facility of the University of Chicago.

Cytokine release. 1×10^5 responders were cultured with 1×10^5 cancer or spleen cells or 5×10^4 peptide pulsed T2-K^b stimulators per well of a 96-well U-bottom plate for 24 hours. Plate-coated anti-CD3 (2C11) and anti-CD28 (37.51; both 1 μg/ml; eBioscience) served as positive stimulation control. For the analysis of T cells *ex vivo*, popliteal lymph nodes were isolated and single cell suspension generated 10 days after peptide/complete Freund's adjuvant immunization of the mouse. Isolated cells were cultured with T cell-depleted B6 wild-type feeder cells and 5 μmol/l of peptide for 24 hours at 5, 2.5, 1.25, and 0.625:1 responder to stimulator ratios; 1×10^5 stimulators were seeded per well of a 96-well U-bottom plate. T cells were depleted from single splenocyte suspensions using anti-CD90.2 beads and LS-columns (both Miltenyi). All supernatants were removed and tested for IFN-γ using an ELISA kit ("Femto-HS" High Sensitivity; eBioscience) according to the manufacturer's protocol or frozen at −80 °C and tested at a later time point.

Cytotoxicity assay. Target cells were labeled for 1 hour with 100 μCi sodium chromate-51 (Perkin Elmer, Waltham, MA) and incubated with T cells using E:T ratios from 50:1 to 1.3:1 by employing 5×10^3 target cells. The ^{51}Cr released was measured using a gamma counter (Titertek, Huntsville, AL). The percentage of specific lysis was calculated as: % specific lysis = [(experimental release-spontaneous release)/(maximum release − spontaneous release)] × 100.

T cell transfers. T cell single cell suspensions were injected into the retro-orbital plexus in 200 μl phosphate-buffered saline. Blood samples of 75 μl volume were taken from the retro-orbital plexus for flow cytometry analysis at times indicated.

***Analysis of $CD4^+$ T cell function* ex vivo *and* in vivo.** For restimulation of $CD4^+$ T cells, mice were immunized with peptide at least 1 month after adoptive T cell transfer of transduced $CD4^+$ T cells. 20 nmol $OVA_{323–339}$ or nucleoprotein peptides 4445 and 5253/50 μl of complete Freund's adjuvant and saline [1:1 (vol/vol)] were injected in each posterior foot pad. Popliteal lymph nodes were isolated 10 days after immunization.

For tumor treatment, Rag$^{-/-}$ mice were injected with 2×10^6 MC57-SIY cells subcutaneously. 5×10^6 m33 or P14 TCR-transduced OT-II or $CD4^+$ C57BL/6 T cells were transferred on day 6 after cancer cell inoculation. Tumor volumes were measured along three orthogonal axes (a, b, and c) every 3–4 days and calculated as *abc*/2. Results of treatment of small groups of mice were analyzed using the two-tailed probability calculated by the Fisher's exact probability test ($P \leq 0.01$ is considered highly significant).

SUPPLEMENTARY MATERIAL

Figure S1. m33 TCR-expressing $CD8^+$ T cells have a higher peptide sensitivity for dEV-8, but a similar sensitivity for p2Ca when compared to 2C-transduced cells.

Figure S2. m33 TCR-expressing $CD8^+$ T cells do not remain in circulation by down-modulating the m33 TCR.
Figure S3. m33, but not 2C-expressing OT-II $CD4^+$ T cells bind SIY presented on the MHC class I molecule K^b.
Figure S4. Wild-type $CD4^+$ T cells transduced with a high-affinity TCR persist while $CD8^+$ T cells transduced with the same TCR disappear.
Figure S5. Nanomolar-affinity TCR-expressing $CD4^+$ T cells obtained from $CD8^{-/-}$ mice retain their specificity for peptide-MHC class I.
Figure S6. The disappearance of m33 TCR-expressing $CD8^+$ T cells is not a result of cell elimination by the cytotoxic cells of the host.
Materials and Methods.

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