

Autoreactive T cells bypass negative selection and respond to self-antigen stimulation during infection

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Central and peripheral tolerance prevent autoimmunity by deleting the most aggressive CD8⁺ T cells but they spare cells that react weakly to tissue-restricted antigen (TRA). To reveal the functional characteristics of these spared cells, we generated a transgenic mouse expressing the TCR of a TRA-specific T cell that had escaped negative selection. Interestingly, the isolated TCR matches the affinity/avidity threshold for negatively selecting T cells, and when developing transgenic cells are exposed to their TRA in the thymus, only a fraction of them are eliminated but significant numbers enter the periphery. In contrast to high avidity cells, low avidity T cells persist in the antigen-positive periphery with no signs of anergy, unresponsiveness, or prior activation. Upon activation during an infection they cause autoimmunity and form memory cells. Unexpectedly, peptide ligands that are weaker in stimulating the transgenic T cells than the thymic threshold ligand also induce profound activation in the periphery. Thus, the peripheral T cell activation threshold during an infection is below that of negative selection for TRA. These results demonstrate the existence of a level of self-reactivity to TRA to which the thymus confers no protection and illustrate that organ damage can occur without genetic predisposition to autoimmunity.

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Abbreviations used: APL, altered peptide ligand; LCMV, lymphocytic choriomeningitis virus; Lm, *Listeria monocytogenes*; mTec, medullary thymic epithelial cell; Rip, rat insulin promoter; TRA, tissue-restricted antigen; VSV, vesicular stomatitis virus.

The random rearrangement of TCR coding sequences creates an enormously diverse repertoire that includes many autoreactive specificities. Several mechanisms are involved in eliminating and silencing these T cells to safeguard against autoimmunity. The majority of autoreactive CD8⁺ T cells are removed in the thymus where T cells bearing receptors which react strongly to self-peptides presented by MHC undergo apoptotic cell death (negative selection; Kisielow et al., 1988; Palmer, 2003; Starr et al., 2003; Stritesky et al., 2012). A key mechanism, which enables the thymus to eliminate not only T cells specific for ubiquitously expressed antigen but also T cells responding to antigens that are otherwise only found in specialized peripheral tissues (tissue-restricted antigen [TRA]; Klein et al., 2009), is the ability of medullary thymic epithelial cells (mTecs) to ectopically express TRA in the thymus. The critical importance of providing

these antigens for negative selection is underscored by the fact that mice and humans develop severe autoimmunity when they lack the transcription factor AIRE, which is required for the ectopic expression of TRA by mTec (Anderson et al., 2002; Liston et al., 2004; Mathis and Benoist, 2009). In addition, autoreactive T cells can be eliminated by lymph node-resident immature dendritic cells that present tissue-derived antigen (Kurts et al., 1997; Morgan et al., 1999; Belz et al., 2002) as well as by lymph node stromal cells. The latter are also able to ectopically express TRA (Lee et al., 2007; Metzger and Anderson, 2011). Together, these thymic and peripheral mechanisms ensure that T cells are

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negatively selected against all self-antigens. However, the frequent occurrence of T cell-mediated autoimmune diseases such as type 1 diabetes and multiple sclerosis indicate that the establishment or maintenance of tolerance can fail. How this occurs and if autoimmunity inevitably requires defects in tolerance enforcing mechanisms or if even healthy individuals with a normally selected T cell repertoire and without any genetic predisposition can develop autoimmunity remains unknown.

The critical contribution of regulatory T cells in maintaining tolerance underlines the fact that T cells with autoreactive potential are part of the normal T cell repertoire in healthy individuals (Josefowicz et al., 2012). Moreover, there is conclusive evidence that the mechanisms which select the T cell repertoire remove only the most aggressive TRA-specific T cell clones but spare T cells with less avidity for TRA. These lower avidity T cells persist in the periphery but they have the potential to cause autoimmunity even in the presence of regulatory T cells (von Herrath et al., 1994; Zehn and Bevan, 2006). Despite many observations which imply that low avidity T cells are key mediators of autoimmunity (Bulek et al., 2012), we lacked strategies and tools to study these T cells in defined experimental systems. Hence, most prior investigations were limited to observing autoimmune responses induced by high avidity T cells and thus dealt with T cells that are normally effectively eliminated by central and peripheral tolerance. In contrast, the biological properties of T cells with physiologically relevant levels of self-reactivity and which are not eliminated in the thymus or the periphery remained unknown. Most importantly, there was a lack of knowledge regarding the phenotypic and functional characteristics of these T cells before their activation and expansion in ongoing autoimmune responses and we did not know if any tolerance mechanisms normally prevent these cells from causing autoimmunity in healthy individuals. Finally, it was not known what stimuli are needed to convert cells that escaped negative selection into self-reactive effector T cells in the periphery and to what degree they can respond to self-antigen.

To address these questions in a well defined experimental system, we developed a novel transgenic mouse expressing a TCR that barely escapes negative selection by a TRA, i.e., one that is at the threshold of thymic negative selection versus survival. We show that in this model system, escaped low avidity autoreactive T cells are quiescent under steady-state conditions in the presence of their target TRA, but during an infection they are able to respond to antigen stimulation as well as foreign antigen-reactive T cells. This includes their differentiation to fully functional effector T cells and the formation of memory upon stimulation by cross-reactive foreign ligands. This occurs even in response to altered peptide ligands (APLs) that provide much lower levels of stimulation than the native ligand that failed to negatively select a large fraction of the cells. Importantly, in this model system even during a bystander infection the low avidity autoreactive T cells have the ability to respond to endogenous levels of self-antigen. Thus, autoreactive T cells can respond during an infection to a self-ligand that failed to delete T cells in thymus. This difference and the lack

of imprinting of any tolerance mechanisms on cells that escape negative selection reveal a significant deficit in tolerance enforcement and the existence of a range of self-reactivity against TRA to which thymic and peripheral tolerance confer no protection. Together, our observations imply that autoimmunity can occur in the absence of any defects in negative selection and in the presence of functional regulatory T cells.

RESULTS

Detection of low avidity autoimmune T cells in the periphery of normal mice

Low avidity autoreactive T cells have been detected in several experimental setups and they can best be studied in transgenic systems expressing a model antigen as a neo self-antigen. Such a setup allowed us to directly compare the quality of antigen-specific T cells that can be found in the presence or the absence of the neo self-antigen. Rat insulin promoter (Rip)-mOva mice express a membrane-bound form of Ova in mTec in the thymus, the β cells in the islets of Langerhans in the pancreas, the proximal tubular epithelial cells in the kidneys, and, in the case of male mice, in the testis (Kurts et al., 1997). OT-1 TCR transgenic T cells, which respond with high avidity to an Ova epitope, are negatively selected in the thymus of Rip-mOva mice and are eliminated in the periphery when injected into Rip-mOva mice (Kurts et al., 1997; Gallegos and Bevan, 2004). We previously used Rip-mOva mice with an elevated precursor frequency of K^b /Ova-specific T cells (by crossing them with a mouse expressing a transgenic K^b /Ova-specific TCR- β chain) to show that high avidity Ova-reactive T cells were efficiently deleted but low avidity T cells could be found in the periphery of these mice (Zehn and Bevan, 2006). To ask if the same would happen in Rip-mOva mice with a nontransgenic endogenous T cell repertoire, we infected Rip-mOva mice and transgene negative littermate controls with a strain of *Listeria monocytogenes* (Lm) that expresses Ova (Lm-Ova). After the infection, only very low numbers of Ova/ K^b reactive $CD8^+$ T cells were observed in Rip-mOva mice (Fig. 1, A and B) and none of the infected mice developed signs of autoimmunity (not depicted). However, when Lm-Ova-primed Rip-mOva mice were challenged with a second Ova-expressing pathogen, vesicular stomatitis virus (VSV)-Ova, then the Ova-reactive T cells expanded to much higher numbers (Fig. 1, A and B). This higher frequency allowed us to assess the functional avidity of these Ova-specific T cells and we noted that ~ 50 – 100 -fold higher peptide concentration was needed to trigger a half-maximum (EC_{50}) IFN- γ response by K^b /Ova-specific T cells in Rip-mOva mice compared with control C57BL/6 mice (Fig. 1, C and D). These data show that T cells responding with high functional avidity to the K^b /Ova antigen are eliminated from the T cell repertoire in Rip-mOva mice, whereas lower avidity T cells remain.

Autoimmune responses targeting pancreatic β cells in Rip-mOva mice are usually induced upon transferring high avidity Ova-specific $CD8^+$ T cells into the mice. To our knowledge, no disease induction has been reported in Rip-mOva mice relying entirely on endogenous T cells with physiological

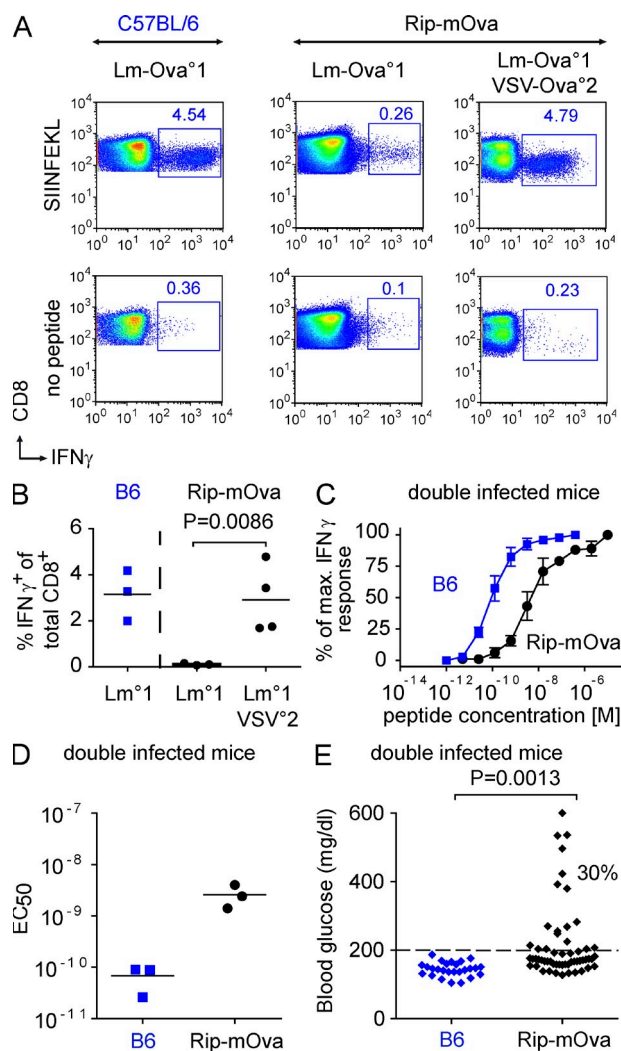


Figure 1. Low avidity K^b/Ova-specific T cells in the periphery of Rip-mOva mice. Rip-mOva or transgene negative littermate control C57BL/6 mice were infected with Lm-Ova. A fraction of the mice were rechallenged 4 wk later with VSV-Ova. 6 d after the last infection, splenocytes were isolated and either left untreated or were briefly *in vitro* restimulated with SIINFEKL peptide. In A, CD8⁺ gated flow cytometry plots of cells intracellularly stained for IFN- γ , and in B, the frequencies of Ova-specific T cells are depicted ($n = 3-4$ mice per group). Dose-response curves graphing peptide concentration against the fraction of maximum IFN- γ response in splenic CD8⁺ T cells are shown in C ($n = 4$ and 5 per group, error bars show SD). The data shown in A-C are representative of three independently performed experiments. (D) Calculated mean half-maximum effective concentrations (EC₅₀) obtained in three independent experiments (each including three to five mice per group) are presented. (E) Maximum blood glucose levels between days 7 and 13 after the secondary infection. The data are pooled from eight independent experiments (total number of mice: 56 Rip-mOva and 26 littermate control, transgene-negative C57BL/6 mice). The horizontal bars in B and D represent the mean value of the individual data points.

levels of self-reactivity and in the presence of an intact regulatory T cell compartment. Notably, we found T cell infiltrates in the pancreatic islets of 60–80% of doubly infected Rip-mOva

mice, whereas such infiltrates were absent in littermate controls (Fig. 2). In addition, 30% of the Rip-mOva mice primed with Lm-Ova and boosted with VSV-Ova had blood glucose levels exceeding 200 mg/dl, an elevated level which we did not observe in the parallel littermate control group (Fig. 1 E). Reversing the order of infections or challenge of low-dose Lm-Ova-primed mice with a high dose of Lm-Ova did not lead to higher disease incidence (unpublished data). From these observations, we conclude that low avidity self-reactive T cells can be found in all Rip-mOva mice and that when these cells are expanded to high numbers, they are able to cause tissue destruction and autoimmune diabetes.

OT-3: a novel transgenic mouse expressing a low avidity K^b/Ova-specific TCR

Although the aforementioned data, as well as other observations (von Herrath et al., 1994; Zehn and Bevan, 2006; Bulek et al., 2012), demonstrate the existence of low avidity autoreactive T cells in the periphery, these T cells have only been characterized after they had been expanded by immunization. However, their phenotypic and functional characteristics before expansion, and how low avidity autoreactive T cells respond to antigen stimulation in healthy individuals, remained unknown. To investigate this, we isolated a K^b/Ova-specific CD8⁺ T cell receptor from a diabetic Rip-mOva mouse, as

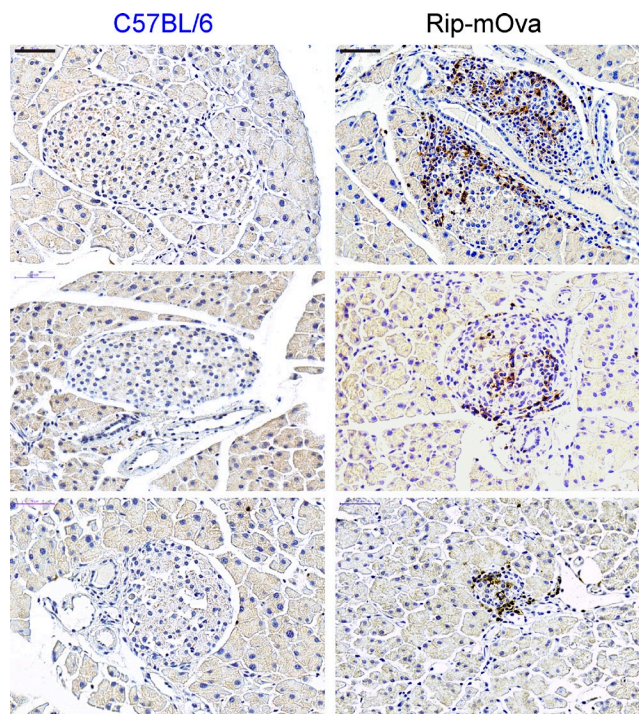


Figure 2. T cells infiltrate the islets of Rip-mOva mice after sequential Lm-Ova and VSV-Ova infection. C57BL/6 or Rip-mOva mice were infected with Lm-Ova and, 4 wk later, with VSV-Ova. Representative images of anti-CD8-stained pancreas sections obtained at day 8 after the secondary infection are presented. Bars, 50 μ m. Results from one of two representative experiments are shown with $n = 5$ mice per group.

described in Materials and methods, and then created a TCR transgenic mouse (designated as OT-3). To exclude any contribution by secondary, endogenously rearranged TCR- α chains, we crossed the OT-3 mice onto a Rag2^{-/-} or TCR- α ^{-/-} background and only such OT-3 T cells were used in the entire study. We observed that TCR transgenic OT-3 T cells required $\sim 50\times$ higher Ova peptide concentration for half-maximum IFN- γ production than OT-1 T cells (Fig. 3, A and C). Comparing the OT-3 T cells with the cells seen in doubly infected Rip-mOva mice, we noticed that the OT-3 T cells were slightly more sensitive to K^b/Ova and showed a steeper titration slope than the endogenous population in Rip-mOva mice (Fig. 3 A). This result is not unexpected as OT-3 T cells are a monoclonal population in which all cells have the same functional avidity for K^b/Ova. In contrast, the slower increase in the titration curve of endogenous T cells indicates that this is a mixed population of cells, some of which have similar and others lower functional avidity for K^b/Ova compared with OT-3 T cells. To characterize the OT-3 T cells further, we examined their ability to induce diabetes in Rip-mOva mice and found that 30,000 transferred OT-3 T cells caused severe diabetes within 6 d after an Lm-Ova infection, and as few as 3,000 OT-3 T cells increased blood glucose levels (Fig. 3 B). We observed that the avidity of the OT-3 T cells was too low to reliably stain them with conventional tetramers (unpublished data).

OT-3 T cells match the TCR stimulation threshold for negative selection against the Ova antigen

Several APLs have been identified that provide lower levels of stimulation to OT-1 T cells than the native Ova-derived

SIINFEKL (N4) ligand (Alam et al., 1996; Daniels et al., 2006). Interestingly, OT-1 T cells respond to the SIITFEKL (T4) APL with a similar functional avidity as OT-3 T cells respond to K^b/Ova (Fig. 3 C), and the ligands induce comparable numbers of OT-1 and OT-3 effector and memory T cells after Lm-T4Ova or Lm-Ova infection, respectively (Fig. 3 D). Note that T4 is the weakest ligand described that still induces negative selection of OT-1 T cells in fetal thymic organ cultures (Daniels et al., 2006). Because of this, the T4 ligand is thought to mark the avidity/affinity threshold between positive and negative selection of OT-1 cells. The similar functional avidity of OT-3 T cells to K^b/Ova and OT-1 T cells to K^b/T4 suggests that K^b/Ova is the threshold ligand for OT-3 T cells.

This conclusion was further supported by grafting lethally irradiated Rip-mOva or C57BL/6 recipient mice with a mixture of C57BL/6 and OT-3 bone marrow cells. After reconstitution, we observed that Rip-mOva recipient mice contained, compared with the numbers in control C57BL/6 recipients, only half the number of OT-3 T cells in the peripheral blood, in pancreatic lymph nodes, and among CD8⁺ single-positive CD24^{low} thymocytes (Fig. 4). This difference was also seen at 20 wk after bone marrow reconstitution (Fig. 4 A). The stability in the ratio of peripheral OT-3 T cells between C57BL/6 and Rip-mOva mice indicates that OT-3 T cells were largely eliminated in the thymus but not in the periphery of Rip-mOva mice. Overall, these results indicate that $\sim 50\%$ of the OT-3 T cells bypass negative selection in Rip-mOva in a stochastic manner and that K^b/Ova appears to be the threshold ligand for OT-3 T cells in these mice.

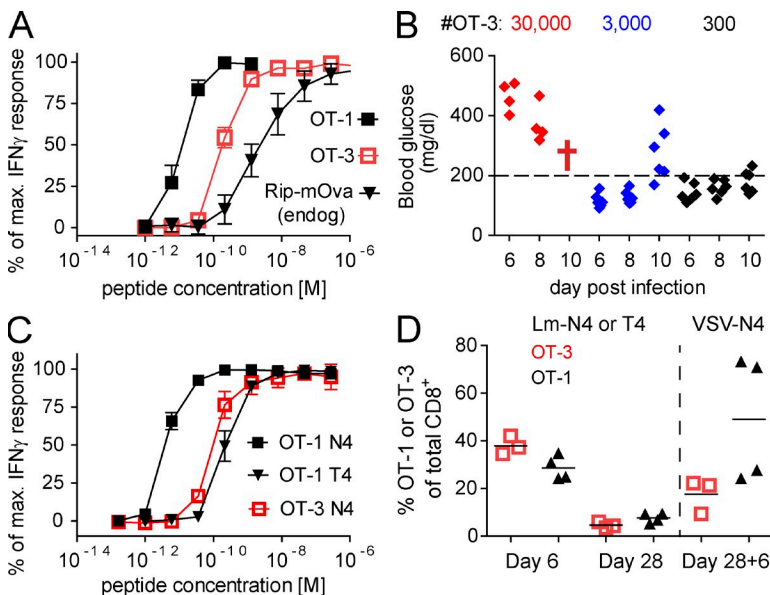


Figure 3. OT-3 T cells respond with similar functional avidity to K^b/Ova as OT-1 T cells respond to SIINFEKL.

(A) 800 OT-3 T cells were transferred into Rip-mOva mice which were infected with Lm-Ova and, 4 wk later, with VSV-Ova. 10⁴ OT-1 T cell-engrafted control C57BL/6 mice were infected with VSV-Ova. Splenocytes from both groups were restimulated with SIINFEKL peptide and stained for IFN- γ . The corresponding peptide dose-response curves for OT-3, OT-1, and endogenous T cells in Rip-mOva mice are shown ($n = 3$ or 4, error bars show SD). The data are representative of two independent experiments. (B) Rip-mOva mice, supplemented with titrated numbers of OT-3 T cells, were infected with Lm-Ova, and blood glucose levels were determined 6, 8, and 10 d after infection. Data are representative of three independent experiments ($n = 4-6$ mice). Because of the diabetes, the mice engrafted with the highest number of OT-3 T cells had to be sacrificed after day 8 (marked with a cross). (C) 2×10^4 naive OT-1 and 10^5 OT-3 T cells were transferred into CD45.1 congenic mice which were infected with Lm-Ova. Splenocytes were stimulated with titrated doses of SIINFEKL (N4) or SIITFEKL (T4) peptide (OT-1 only) at 7 d after infection and intracellularly stained

for IFN- γ . The resulting dose-response curves are shown ($n = 4$, data are representative of three experiments, SD error bars are shown). (D) 10^5 OT-1 and OT-3 T cells were transferred into CD45.1 congenic hosts. OT-1 mice were infected with Lm-T4Ova and OT-3 mice with Lm-Ova. OT-1 and OT-3 T cell frequencies at days 6 and 28 after infection and 6 d after a secondary VSV-Ova infection are presented. The horizontal bars represent the mean value of the individual data points. The data are representative of two independent experiments with $n = 3-4$ mice per group.

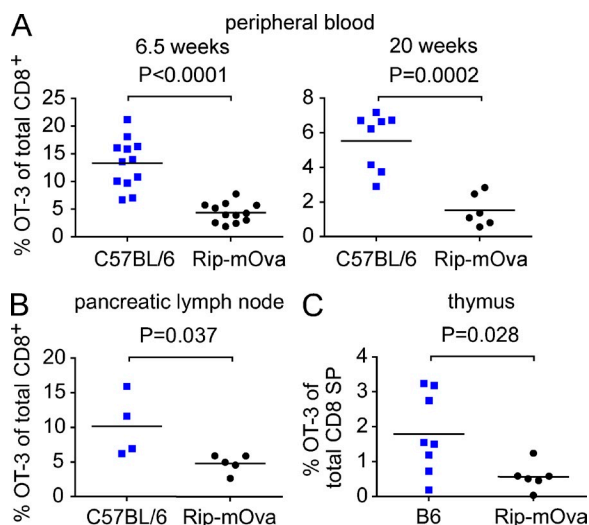


Figure 4. Survival of a large fraction of OT-3 T cells in the thymus of Rip-mOva mice. (A and B) Lethally irradiated Rip-mOva or C57BL/6 mice (both coexpress CD45.1 and CD45.2) were injected with a 4:1 mix of C57BL/6 (CD45.1) and OT-3 (CD45.2) bone marrow. (A–C) Frequency of OT-3 T cells among total CD8⁺ T cells in these mice was measured at 6.5 wk ($n = 12$ per group) or 20 wk ($n = 8$ and 6 per group) after reconstitution in the peripheral blood (A), at 9 wk in the pancreatic lymph nodes ($n = 4$ and 5 mice per group; B), and at 5 wk among CD8⁺ CD24 low single-positive thymocytes ($n = 8$ and 6 per group; C). Six independent experiments were performed to assess the frequency of OT-3 at 5.5–7 wk in the blood and three experiments at the later time point. The measurements in the pancreatic lymph nodes are representative of two independent experiments. The horizontal bars in A, B, and C represent the mean value of the individual data points.

Endogenous antigen level provides sufficient stimulation to activate OT-3 cells in a bystander infection

The escape of low avidity self-reactive T cells from negative selection would only be a problem for self-tolerance if those cells could efficiently be activated in the periphery. The data shown in Fig. 3 B already demonstrate that effector OT-3 T cells bear sufficient functional avidity to destroy pancreatic islet cells in Rip-mOva mice after Lm-Ova immunization. In addition, we wanted to know if OT-3 T cells could be activated by endogenous K^b/Ova presentation. It is well documented that K^b/Ova is presented in Rip-mOva mice by immature DC-draining Ova-expressing organs such as the pancreas or kidneys (Kurts et al., 1997; Belz et al., 2002).

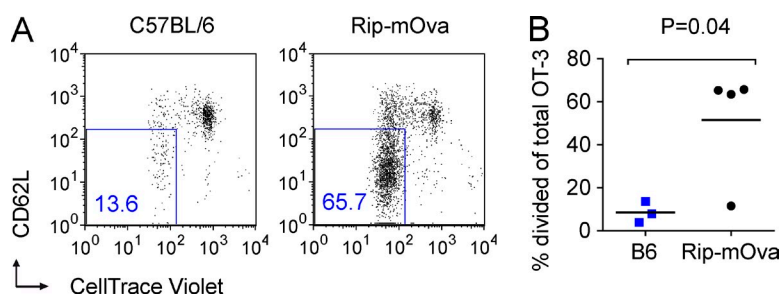


Figure 5. Endogenous K^b/Ova complexes activate OT-3 T cells during an infection. CD45.1 Rip-mOva or C57BL/6 mice were injected with 5×10^5 CellTrace Violet-labeled OT-3 T cells and infected with LCMV Armstrong. 4 d after infection, splenocytes were analyzed for dye dilution and CD62L expression levels. (A) Representative OT-3 gated flow cytometry plots. (B) Data for all mice ($n = 3$ or 4 per group). The data are representative of three independent experiments. The horizontal bars represent the mean value of the individual data points.

Moreover, lymphocytic choriomeningitis virus (LCMV) infection matures these DCs and it has been shown that LCMV-infected Rip-mOva mice facilitate OT-1 T cell expansion, effector differentiation, and autoimmunity triggered by endogenous K^b/Ova (Zehn and Bevan, 2006). We used the same system to test if OT-3 T cells respond to endogenous levels of K^b/Ova. Interestingly, we observed that even low avidity OT-3 T cells proliferated and down-regulated CD62L in LCMV-infected Rip-mOva mice (Fig. 5). This indicates that recognition of endogenous K^b/Ova is able to trigger the activation of naive OT-3 T cells after LCMV infection. The activation of OT-3 T cells by peripheral K^b/Ova contrasts with the inefficient negative selection against K^b/Ova. The result indicates that autoreactive T cells which escape negative selection can nevertheless respond to peripheral self-antigen during a systemic viral infection.

Even weaker ligands than K^b/Ova trigger the activation of OT-3 T cells during an infection

To directly address the sensitivity of OT-3 T cells to antigen stimulation in the periphery, we screened a large collection of SIINFELK-derived APL to identify ligands that are weaker in stimulating OT-3 T cells than K^b/Ova. We identified SIFNFEKL (F3) and SIVNFEKL (V3) to be such ligands (Fig. 6 A). To our surprise, we observed that Lm expressing these ligands induced robust OT-3 T cell expansion (Fig. 6 B). This occurred even though they have a 35- or 200-fold lower EC₅₀ value than the negative selection threshold ligand SIINFELK (Fig. 6 A). V3- and F3-primed effector T cells exhibited a normal pattern of CD44 and CD62L expression and secreted IFN- γ and TNF. Most of the OT-3 T cells from both groups produced granzyme B (Fig. 6, C and D). When Lm-V3-primed OT-3 T cells were challenged 4 wk later with VSV-Ova, we observed much higher numbers of OT-3 T cells (Fig. 6 E, left) compared with recipients that received OT-3 T cells but were primed with WT Lm (Lm-wt) that do not express Ova (Fig. 6 E, right). The few cells seen in the Lm-wt group on day 4 after the VSV-Ova infection are OT-3 T cells that persisted throughout the Lm infection and that mounted a primary response after the VSV-Ova infection. This indicates that even a sub-threshold level of stimulation is sufficient to generate functional memory T cells with the potential to mount a secondary effector T cell response. Moreover, Lm-V3-primed OT-3 T cells lysed antigen-pulsed target cells in vivo (Fig. 6 F). To test the ability of these subthreshold ligands to induce functional

effector T cells capable of causing autoimmunity, we infected Rip-mOva mice containing OT-3 T cells with Lm-F3. The mice became diabetic within 8 d after infection (Fig. 6 G). The overall picture that emerges from these observations is that although K^b/Ova fails to effectively induce negative selection of OT-3 T cells in Rip-mOva mice, ligands that are much less potent activate OT-3 in the periphery and induce normal T cell differentiation. This leads to the conclusion that there is a substantial difference in the threshold for negative selection to this TRA and peripheral T cell activation after an infection.

Low avidity T cells that escape negative selection are not tolerant and differentiate into functional effector and memory T cells

Our data show that negative selection in Rip-mOva mice fails to eliminate a large fraction of the OT-3 T cells. We asked if

OT-3 T cells that matured in C57BL/6 mice are phenotypically or functionally different from OT-3 T cells that developed in Rip-mOva mice. Because Ova is a self-antigen for OT-3 T cells from Rip-mOva mice, we refer to these as self-OT-3 T cells in contrast to OT-3 T cells from C57BL/6 mice for which Ova is a foreign antigen (foreign-OT-3). Using chimeric Rip-mOva or C57BL/6 mice reconstituted with a mixture of C57BL/6 and OT-3 bone marrow stem cells, we asked if there were any phenotypic differences between self- or foreign-OT-3 T cells. CD5, CD44, CD25, and PD-1 did not show different levels of expression. The similar expression of CD44 indicates that in both hosts the OT-3 T cells are naive (Fig. 7 A). Consistent with this, self- and foreign-OT-3 T cells responded very similarly during an Lm-Ova infection and gave rise to effector T cells with comparable IFN- γ and TNF secretion patterns, granzyme B expression levels, and CD127 and KLRG1 expression (Fig. 7 B). Consistent with these results, self- and foreign-OT-3 T cells transferred into Rip-mOva

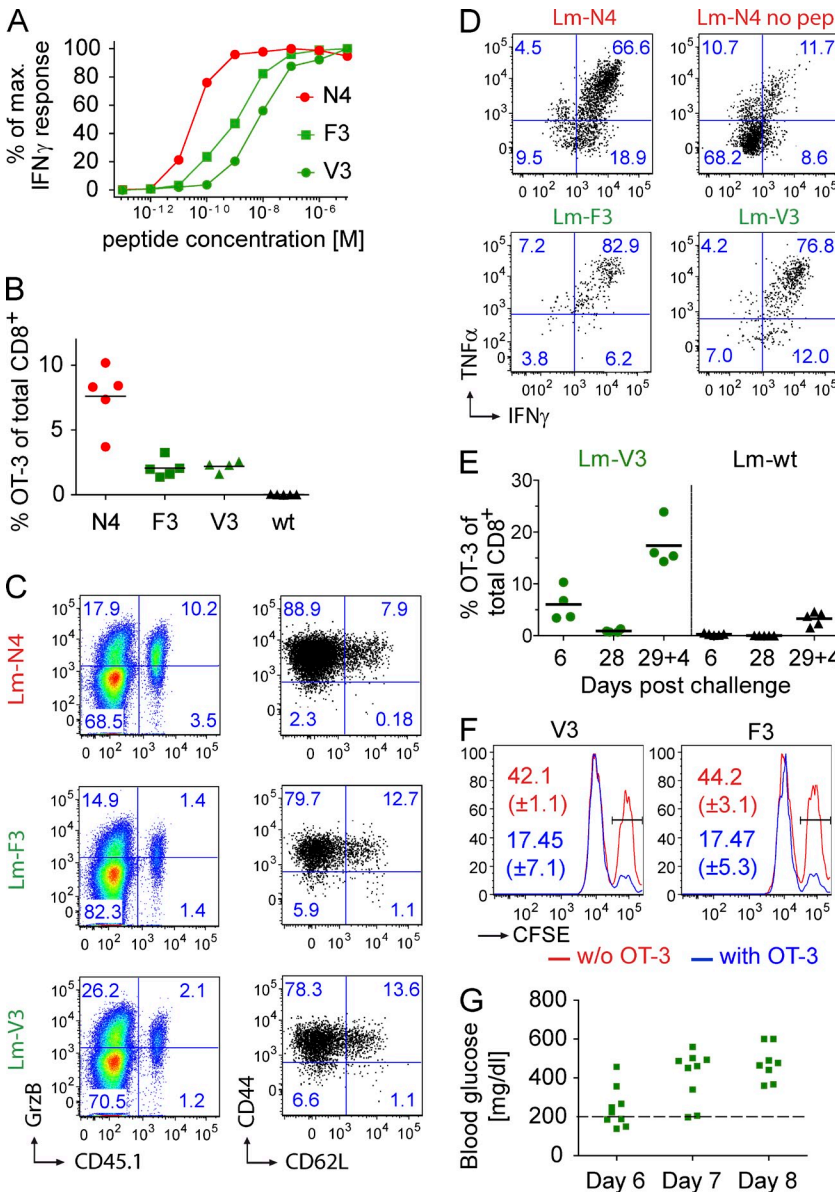


Figure 6. pMHC ligands with avidities far below the negative selection threshold induce functional effector and memory T cells. (A) An OT-3 T cell line was briefly stimulated with SIINFEKL (N4) or the APLs SIFNFEKL (F3) or SIVNFEKL (V3). Shown are dose-response curves graphing the peptide concentration against the fraction of IFN- γ -producing T cells. (B–E) 10⁵ CD45.1 congenic OT-3 T cells were transferred into C57BL/6 mice which were then infected with Lm-Ova, Lm-F3, or Lm-V3. Another group of mice was infected with nonrecombinant wild-type Lm (wt). (B) The frequency of OT-3 T cells on day 7 after infection ($n = 4$ or 5 mice per group). (C) Representative flow cytometry plots of total CD8⁺ T cells (left) or OT-3 T cells (right) analyzed on day 5.5 after infection for granzyme B, CD44, and CD62L expression. (D) The same cells briefly restimulated with SIINFEKL peptide and stained for TNF and IFN- γ are shown ($n = 3$ or 4 mice per group in C and D). (E) The frequency of OT-3 T cells in mice infected with Lm-V3 or control Lm-wt at 6 and 28 d and at 4 d after a secondary VSV-Ova infection are displayed ($n = 4$ or 5 mice per group). (F) Rip-mOva mice received nothing or 2×10^4 OT-3 T cells and infected with Lm-V3. At 5.5 d after infection, peptide-pulsed CFSE^{bright} and control CFSE^{low} splenocytes were injected and analyzed 24 h later for the elimination of the CFSE^{bright} population. The numbers indicate the percentage and SD of CFSE^{bright} cells in Rip-mOva mice supplemented with or without OT-3 T cells ($n = 3$ per group). (G) Blood glucose levels at 6–8 d after Lm-F3 infection and 10⁵ OT-3 transfer ($n = 8$ mice). The horizontal bars in B and E represent the mean value of the individual data points. All the experiments shown were performed two times and we observed similar outcomes.

mice exhibited a similar capacity to expand and induce diabetes (Fig. 7 C).

Next, we tested if the constitutive presentation of K^b/Ova in lymph nodes of Rip-mOva mice affects the formation of OT-3 memory cells. To address this issue, we had to use a special experimental setup to ensure that the expanded effector OT-3 T cells did not cause diabetes. This was achieved by using Rip-mOva mice expressing the variant H-2K^{bm1} MHC-I molecule which is unable to present the SIINFEKL epitope. According to a published strategy, we lethally irradiated H-2K^{bm1}/Rip-mOva mice and reconstituted them with C57BL/6 bone marrow. This creates a situation in which all nonhematopoietic cells express H-2K^{bm1} and fail to present SIINFEKL. As a consequence, OT-3 T cells cannot destroy the pancreatic islets. In the same animals, however, all hematopoietic cells express H-2K^b and DC cross-present tissue-derived Ova antigen (Kurts et al., 1997). Upon transferring OT-3 T cells into C57BL/6 >H-2K^{bm1}/Rip-mOva chimeric mice and infecting the hosts with Lm-Ova, we observed that the OT-3 cells formed memory T cells. Moreover, when the mice were challenged with VSV-Ova 4 wk later, the memory OT-3 T cells mounted a robust secondary response (Fig. 7 D). The latter findings are particularly important as they indicate that even self-reactive T cells with autoimmune potential can form functional memory T cells that can expand to larger numbers of autoreactive T cells upon reencountering antigen.

In conclusion, our data show that cells with an avidity/affinity for self-antigen at or just below the threshold for negative selection are not impaired in their ability to respond to self-antigen and do not show any signs of cell intrinsic anergy or tolerance.

DISCUSSION

Most autoreactive T cells are exposed to self-antigen either in the thymus or the periphery, and high avidity T cells are eliminated in both compartments. In contrast, lower avidity T cells are often found in ongoing autoimmune diseases (Bulek et al., 2012) and in anti-tumor responses (McMahan and Slansky, 2007). Thus, expression of a low avidity TCR, allowing autoreactive T cells to bypass elimination, is a major mechanism by which autoreactive T cells escape tolerance (von Herrath et al., 1994; Nugent et al., 2000; Zehn and Bevan, 2006). However, up to this point, we had limited insight into the phenotypic and functional characteristics of these low avidity self-reactive T cells. Thus, it was unclear how self-antigen recognition in the thymus or periphery impacts the function of these T cells and whether such exposure imprinted mechanisms that restrict their activation. The failure to negatively select low avidity self-reactive T cells stimulated us to study how well these T cells respond to self-antigen in the periphery. To address these questions, we needed to establish a well defined experimental system in which we could study T cells

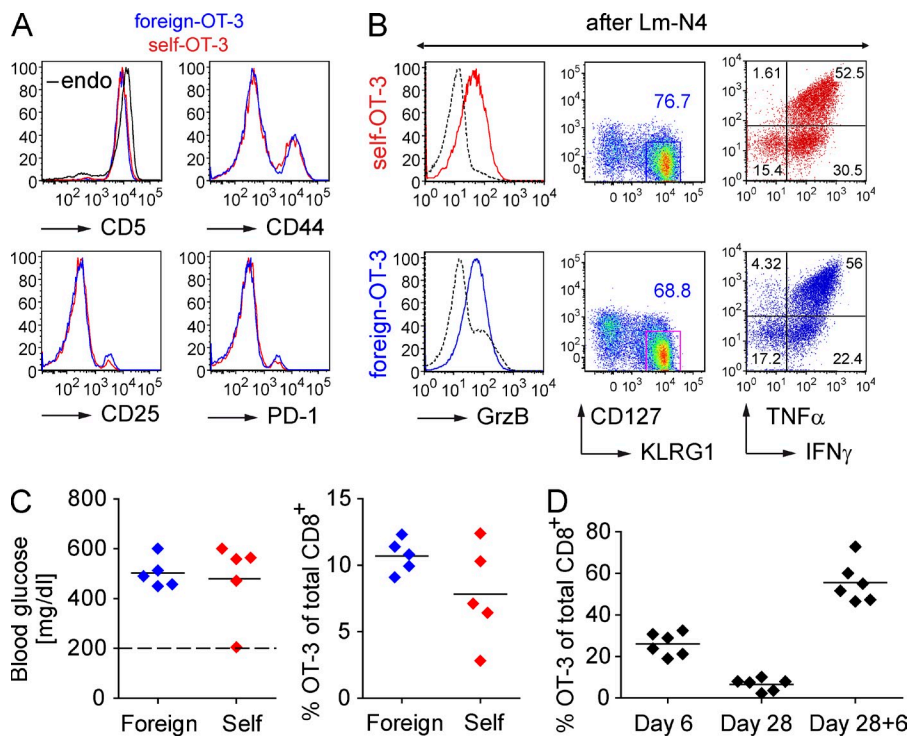


Figure 7. Autoreactive OT-3 T cells differentiate into normal effector and memory T cells. (A) Lethally irradiated Rip-mOva or C57BL/6 mice were reconstituted as described in Fig. 4. 6 wk later, expression levels of CD5, CD44, CD25, and PD-1 on OT-3 T cells in C57BL/6 mice (termed foreign-OT-3) and in Rip-mOva mice (self-OT-3 T cells) were determined (representative data for $n = 5$ mice per group are shown). The black line represents the level of CD5 expression by endogenous T cells. The data are representative of two experiments. (B) Similar chimeric mice were infected at 11 wk after reconstitution with Lm-Ova and, 7 d later, splenocytes were stained with GrzB, anti-CD127, and anti-KLRG1. They were also restimulated with SIINFEKL peptide and then stained intracellularly for IFN- γ and TNF. Shown are OT-3 gated flow cytometry plots representative of $n = 3$ mice per group. The dashed lines on the left are endogenous CD8⁺ T cells. (C) Rip-mOva mice received 5×10^4 foreign- or self-OT-3 T cells (described in A), and blood glucose levels and T cell expansion were determined after an Lm-Ova infection ($n = 5$ mice per group). Values >200 mg/dl were

considered diabetic. The experiments shown in B and C were performed two times and showed a similar outcome. (D) H-2K^{bm1}/Rip-mOva mice were lethally irradiated and grafted with C57BL/6 bone marrow. 8 wk after the reconstitution, 5×10^4 OT-3 Rag2^{-/-} T cells were injected and the mice were infected with Lm-Ova. The frequency of OT-3 T cells was determined 6 and 28 d after the Lm-Ova infection and at day 6 after a VSV-Ova rechallenge. The data are pooled from two independently performed experiments with $n = 3$ mice per group and experiment. The horizontal bars in C and D represent the mean value of the individual data points.

expressing a TCR with physiologically relevant functional avidity for a defined self-antigen. We built such a model based on Rip-mOva mice. After identifying what types of K^b/Ova-reactive T cells can be found in Rip-mOva mice, we generated the novel OT-3 TCR transgenic line to: determine the activation threshold of low avidity autoreactive T cells in the periphery; track the phenotype, cell fate, and function of these low avidity autoreactive T cells; and assess the effect of encountering a ligand at the threshold of negative selection in the thymus for T cell function in the periphery.

We observed that during an infection, T cells respond to a self-antigen that inefficiently induces negative selection when promiscuously expressed by mTec in the thymus. Moreover, even variants of the self-antigen that are weaker in stimulating the OT-3 T cells than K^b/Ova efficiently activate the T cells, induce normal effector and memory T cells, and cause diabetes. Why T cells respond differently to the same antigen in the thymus and the periphery remains unknown. Possibly, the number of peptide-MHC complexes resulting from the ectopic expression of antigen in the thymus is lower than the number of complexes found in an Lm-Ova infection or during presentation of endogenous Ova in the periphery. Thus, a weak ligand that fails to eliminate T cells in the thymus might sufficiently activate T cells in the periphery as the result of a higher epitope abundance. Along with higher ligand density, inflammation, higher levels of co-stimulation, and presentation by activated DC may create an environment that supports the activation of T cells even though they receive only very weak TCR stimulation.

The fact that inflammation and high levels of antigen presentation can activate T cells that survive negative selection appears less significant to us than the observation that the spared T cells induce autoimmunity and effectively destroy peripheral tissue cells. This indicates that there is a discrepancy between the safety margin imprinted by the thymus and what is needed to completely protect peripheral tissues from autoimmunity. Again, the question is why there is such a mismatch. Also in this situation, simply more peptide-MHC complexes could be presented on peripheral cells than during the ectopic expression and presentation in the thymus. Thus, instead of a T cell affinity threshold difference there could be an avidity (epitope abundance) mismatch between the thymus and the periphery. Whether or not such differences apply to all antigens or just a fraction of ectopically expressed TRA remains to be determined. An additional and non-mutually exclusive explanation for why there is a mismatch between thymic tolerance and peripheral T cell activation is that effector T cells might be more sensitive to antigen recognition than thymocytes when they are exposed to TRA in the thymic medulla. Consistent with this, it has been reported that effector T cells are very sensitive to antigen recognition and that sensitivity may increase as cells differentiate into effector T cells (Slifka and Whitton, 2001). Overall, we interpret our findings to mean that cells which are at the threshold of negative selection pose sufficient reactivity to peripheral tissue antigen to cause disease.

Our observations also raise the question of what is needed to activate low avidity T cells that bypass negative selection. Our experiments shown in Fig. 1 indicate that it is rather difficult to expand the endogenous K^b/Ova-specific T cells in Rip-mOva mice. We considered the possibility that low avidity self-reactive T cells are exposed to antigen either in the thymus or in the periphery and that this exposure might render the cells less responsive to subsequent stimulation. We therefore asked whether K^b/Ova-exposed OT-3 T cells showed any phenotypic or functional differences compared with OT-3 T cells obtained from control C57BL/6 mice but we found no evidence for such differences. Instead, OT-3 T cells from either source expanded and caused diabetes to a similar extent. This indicates that the impaired expansion of the endogenous population in Rip-mOva mice is not related to tolerance mechanisms but rather to the fact that the cells receive a very low TCR stimulation. In fact, we recently reported that the duration of T cell expansion (i.e., the number of cell divisions) directly correlates with TCR stimulation strength and that weakly stimulated T cells expand much less vigorously than strongly stimulated T cells (Zehn et al., 2009). Thus, the critical factors determining whether or not autoimmunity develops is not only the ability to activate these T cells but, equally importantly, to expand them to numbers high enough that they cause noticeable tissue destruction.

Applying the latter consideration to the development of autoimmunity, we suggest that pathogens which provide cross-reactive ligands that activate the T cells more strongly than the self-antigen would effectively trigger autoimmunity. This conclusion reinforces the importance of activation of autoreactive T cells via molecular mimicry. Molecular mimicry proposes that T cells responding to a pathogen may cross-react with a self-antigen and thereby cause autoimmunity (Oldstone, 2005). We mimicked the mechanisms that would occur during molecular mimicry by using pathogens that provide an antigen that is identical to a self-antigen. In a real molecular mimicry situation, a pathogen could introduce a cross-reactive antigen that stimulates autoreactive T cells with higher avidity than in our setup. This would lead to more vigorous expansion of autoreactive T cells and likely lead to higher disease incidence.

The finding that ligands which are weaker than the thymic selection threshold activate T cells in the periphery contrasts with earlier observations showing that T cells are more sensitive to antigen during negative selection in the thymus than they are in the periphery (Pircher et al., 1991; Sant'Angelo and Janeway, 2002). The earlier studies investigated selection against ubiquitously expressed antigens, whereas we studied TRA. This has consequences in so far as negative selection to ubiquitously expressed antigen can occur already in the thymus cortex (Baldwin et al., 2005). Notably, miR-181a, a microRNA which enhances antigen sensitivity, is expressed at higher levels by double-positive thymocytes than by cells in the medulla or in peripheral T cells (Li et al., 2007). miR-181a-mediated higher antigen sensitivity could enable negative selection against antigen in the thymus cortex, whereas the same antigen might

fail to eliminate T cells in the medulla. Thus, exposure to ubiquitous self-antigen in the cortex could indeed impose a safety window, but we propose that this is not the case when T cells are negatively selected against TRA in the medulla. A less stringent selection against TRA than against ubiquitously expressed self-antigen is consistent with the fact that T cell-mediated autoimmune responses often target TRA.

Although high avidity T cells are normally deleted in the thymus (Kisielow et al., 1988) or the periphery (Kurts et al., 1997), it has also been shown that these T cells sometimes persist in the periphery (Schietinger et al., 2012). Interestingly, these high avidity T cells show signs of prior activation and proliferation, they express high levels of CD44, and they are tolerant or anergic. In contrast, low avidity OT-3 T cells in Rip-mOva mice resembled naive T cells both phenotypically and functionally. They were CD44 low and showed no signs of previous antigen exposure. The observed tolerance of high but not low avidity T cells further supports the relevance of low avidity T cells in autoimmune pathology.

Although the induction of islet damage and autoimmune diabetes required two consecutive infections to initiate autoimmune diabetes in Rip-mOva mice, our data clearly demonstrate that severe autoimmunity can occur in normal mice with an unmanipulated TCR repertoire and in the presence of regulatory T cells (Fig. 1). This also indicates that low avidity autoreactive T cells form memory cells and mount a robust secondary response upon antigen reexposure. This is another significant difference from high avidity autoreactive memory T cells which are removed from the T cell repertoire over time (Kreuwel et al., 2002). Moreover, the low numbers of autoreactive T cells seen after the primary infection implies that formation of memory T cells and repetitive stimulation are likely required to generate sufficient numbers of autoreactive low avidity T cells to cause tissue damage. This is consistent with the proposed relapsing-remitting disease progression for type 1 diabetes (von Herrath et al., 2007), which proposes that multiple stimulations and increasing tissue damage cause autoimmune diabetes.

In conclusion, we demonstrate that T cells specific for TRA escape negative selection and can cause autoimmunity because of a mismatch between the threshold for negative selection to TRA and the threshold for T cell activation during an infection and the execution of effector function against peripheral tissues. This mismatch allows low avidity T cells to enter the periphery. Moreover, we showed that low avidity T cells, in contrast to high avidity T cells, persist without losing their self-destructive potential. We consider that these dormant autoreactive T cells constitute a significant source for cells which, even in the absence of genetic predisposition to develop autoimmunity, are able to cause organ-specific autoimmune damage.

MATERIALS AND METHODS

Mice. Rip-mOva mice expressing a membrane-bound form of Ova under the control of Rip (Kurts et al., 1997), OT-1 TCR transgenic mice recognizing $K^b/Ova_{257-264}$, CD45.1 congenic C57BL/6 mice, and H-2K^{bmi} C57BL/6 mice were all obtained from The Jackson Laboratory, and C57BL/6 mice

were from Charles River. H-2K^{bmi} mice were crossed with Rip-mOva mice and subsequently intercrossed to obtain Rip-mOva H-2K^{bmi} homozygous mice. The mAb clone 5F1 (provided by L. Sherman, Scripps Research Institute, La Jolla, CA) binds H-2K^b but not H-2K^{bmi} and was used to screen the F2 generation. V β 5 TCR- β chain-only transgenic mice (Dillon et al., 1994) were provided by P.J. Fink (University of Washington, Seattle, WA). OT-3 TCR transgenic mice responding with low avidity to $K^b/Ova_{257-264}$ (described in the next section) were crossed to Rag2^{-/-} mice or TCR- α ^{-/-} mice (The Jackson Laboratory). Mice were bred and maintained in SPF facilities and infected mice in conventional animal facilities. Experiments were performed in at least 6-wk-old mice in compliance with the University of Lausanne Institutional regulations and were approved by the veterinarian authorities of the Swiss Canton Vaud. Isolation of low avidity K^b/Ova -reactive T cells from V β 5xRip-mOva double transgenic mice and pronuclear injections were performed at the University of Washington (UW) in compliance with the UW Institutional Animal Care and Use Committee regulations.

Generation of the OT-3 TCR transgenic mice. For practical reasons, we decided to isolate a low avidity TCR that escapes negative selection from the previously described V β 5xRip-mOva mice (Zehn and Bevan, 2006). These mice express a fixed TCR- β chain (Dillon et al., 1994) that is identical to the β chain found in OT-1 mice, but V β 5xRip-mOva mice have endogenously rearranged TCR- α chains. The advantage of using the V β 5xRip-mOva was that, given the fixed TCR- β chain, we only needed to isolate an appropriate TCR- α chain to identify a low avidity K^b/Ova -reactive TCR- α/β combination. 6–8-wk-old V β 5xRip-mOva mice (Zehn and Bevan, 2006) were infected with Lm-Ova. 7 d later, CD8⁺ splenocytes were isolated and co-cultured with irradiated C57BL/6 splenocytes and titrated concentrations of SIINFEKL peptide. The cultures with the lowest peptide concentration that still induced T cell proliferation were restimulated after 7 d. After another week, RNA extracted from the cultured cells was reverse transcribed using a primer (5'-GATGGAGCTTGGGAGTCA-3') complementary to the 3' untranslated region of the TCR- α chain. A 5' RACE cDNA-based strategy (Invitrogen) was used to clone the TCR- α chains into a retroviral vector (MigR1; Pear et al., 1998). We sequenced 32 clones and analyzed them with algorithms provided by the International Immunogenetics Information System (www.imgt.org). By doing that, we identified four unique and in-frame rearranged TCR sequences. To test whether or not these TCR- α chains respond to $K^b/Ova_{257-264}$, we retrovirally transduced them into activated CD8⁺ T cells obtained from V β 5 transgenic mice. Without any manipulation, 1–5% of polyclonal CD8⁺ T cells in V β 5 mice respond to $K^b/Ova_{257-264}$ (Zehn and Bevan, 2006). In contrast, three of the four isolated TCR- α chains strongly elevated the frequency and this confirmed their reactivity to $K^b/Ova_{257-264}$. One TCR- α sequence (clone 7) was selected to generate the transgenic mouse. According to the sequence IMGT/V-QUEST analysis, clone 7 expresses TRAV19 paired with TRAJ26. Clone 7 and the IRES GFP sequences from MigR1 were introduced into the hCD2 construct using the described restriction sites (Zhumabekov et al., 1995). Clone7 hCD2 and the vector originally used to generate the V β 5 TCR- β -only transgenic mice (provided by F. Carbone, University of Melbourne, Melbourne, Australia) were digested as previously described (Kelly et al., 1993) and co-injected into the pronucleus of fertilized C57BL/6 zygotes. One of the resulting founder lines was selected and crossed with Rag2^{-/-} and TCR- α ^{-/-} C57BL/6 mice. All the experiments shown in our paper were performed with either OT-3xRag2^{-/-} or OT-3xTCR- α ^{-/-} mice and gave similar results.

Generation of bone marrow chimera mice. Recipient mice were lethally irradiated with 900 rad and, 1 d later, engrafted with bone marrow cells isolated from femur and tibia. Before injecting, the bone marrow was stained with biotinylated anti-CD3 antibody (eBioscience), and anti-biotin microbeads and T cells were depleted by separation on LS columns (all Miltenyi Biotec). The host mice received 300 μ g anti-Thy1.2 monoclonal antibody 2–5 d after bone marrow transfer (Bio-XCell) to deplete residual T cells. Mice were treated with antibiotics (Bactrim; Roche) until 3 wk after irradiation.

Infections. Recombinant Lm stably expressing Ova containing the SIINFELK (N4) epitope or the APL SIITFEKL (T4) were previously described (Zehn et al., 2010). Strains expressing SIFNFEKL (F3) or SIVNFEKL (V3) were generated according to established protocols (Zehn et al., 2009), using previously generated expression constructs (Pope et al., 2001; Lauer et al., 2002) which were manipulated by site-directed mutation to modify the plasmids to encode the APLs. Frozen stocks of these strains were grown in brain-heart infusion broth (Thermo Fisher Scientific) to mid log phase. Bacterial numbers were determined by measuring the OD at 600 nm. 1,000–3,000 cfu were injected in PBS intravenously. VSV expressing Ova (VSV-Ova; provided by L. Lefrançois, University of Connecticut, Farmington, CT; Kim et al., 1998) and LCMV (strain 53b; Armstrong) were titered on BHK or on vero cells, respectively. Frozen stocks were diluted in PBS, and 2×10^6 pfu VSV-Ova or 2×10^5 pfu LCMV were injected intravenously or intraperitoneally.

Blood glucose measurement. Blood samples from the tail vein were read on a Contour blood glucose reader (Bayer). Mice with blood glucose levels >200 mg/dl were considered to have altered glucose homeostasis.

Immunohistology. Pancreatic tissue was prepared in Hepes-glutamic acid buffer-mediated organic solvent protection effect (HOPE) fixative (DCS Innovative) (Olert et al., 2001) and embedded in paraffin as described previously (Berghaler et al., 2007). Upon inactivation of endogenous peroxidases (phosphate buffered saline/0.3% hydrogen peroxide, 30 min) and blocking (phosphate buffered saline/10% fetal calf serum), sections were stained with primary rat anti-mouse CD8 (clone: YTS169). Bound primary antibodies were visualized using an avidin-biotin technique with 3,3'-diaminobenzidine as a chromogen (hemalaun counterstaining of nuclei). Stained sections were subsequently scanned using the Mirax slide scanner (Carl Zeiss) for histopathological evaluation.

Purification of T cells, adoptive cell transfer, and cell labeling. Spleen, thymus, and lymph node suspensions were obtained by mashing total organs through a 100- μ m nylon cell strainer (BD). Red blood cells were lysed with a hypotonic ACK lysis buffer. PBMCs were separated from heparinized blood samples using Lympholyte M (Cedarlane). The mouse CD8⁺ T cell enrichment kit LS columns (Miltenyi Biotec) were used for untouched isolation of OT-1 or OT-3 T cells. The indicated numbers of CD45-congenic OT-1 or OT-3 TCR transgenic T cells were injected into the tail vein of mice, and CD45.1 or CD45.2 staining were used to identify the T cells after the transfer. T cells were labeled with the CellTrace Violet cell proliferation kit (Invitrogen) according to the protocols provided by the manufacturer.

T cell activation and expansion. 10^6 OT-3 Rag2^{-/-} and OT-1 CD8⁺ T cells were seeded into 24-well plates, stimulated with anti CD3/CD28 Dynabeads (Invitrogen), and cultured in RPMI supplemented with 10% FCS, penicillin/streptomycin, 5 μ M 2-ME, and 5 mM Hepes (all Invitrogen). Culture volumes were increased and additional media was added as needed. 50 U/ml human IL-2 (Chiron) was added on day 0 and every other day. Cells were analyzed 6–7 d after the stimulation. A similar procedure was to expand K^b/Ova-specific T cells from V β 5xRip-mOva but those were stimulated with SIINFELK peptide.

Short-term stimulation to detect antigen-specific CD8⁺ T cells. Up to 4×10^6 total splenocytes were seeded into 96-well plates. In vitro expanded CD8⁺ T cells were mixed 1:4 with CD45 congenic splenocytes. Unless indicated otherwise, the cells were stimulated with 10 μ M SIINFELK, SIITFEKL, SIVNFEKL, or SIFNFEKL peptide (EMC Microcollections) in vitro for 30 min at 37°C, and then 7 μ M Brefeldin A (Sigma-Aldrich) was added and the cells were returned to 37°C for another 4.5 h. Afterward, the cells were washed and surface and intracellularly stained. Antigen-specific T cells were detected based on their ability to produce IFN- γ .

Surface and intracellular antibody staining and flow cytometry cell sorting. Surface staining was performed for 30 min at 4°C in PBS supplemented with 2% FCS and 0.01% azide (FACS buffer) with the following antibodies:

anti-CD8 PerCPCy5.5, anti-CD62L PE-Cy7, anti-CD44 PE, anti-CD5 PE, anti-PD-1 PE, anti-CD127 PE, anti-KLRG1 biotin, Streptavidin PE-Cy7 (all from eBioscience), and anti-V β 5 (BD). Anti-CD4, anti-CD45.1, anti-CD45.2, and the H-2K^b clone 5F1 were purified from hybridoma supernatants and coupled to Pacific blue, Alexa Fluor 647, or FITC using labeling reagents from Invitrogen. Cells were washed twice and fixed in PBS supplemented with 1% formaldehyde, 2% glucose, and 0.03% azide (Fix buffer). After 30 min, the Fix buffer was replaced with FACS buffer. For intracellular detection, cells were fixed and permeabilized using the Cytotfix/Cytoperm kit (BD) and stained with anti-IFN- γ (obtained from Bio-XCell, coupled to Alexa Fluor 647 or FITC using labeling reagents from Invitrogen), anti-IFN- γ PE (eBioscience), TNF (eBioscience), or Granzyme B (Invitrogen). For sorting living cells, cells were stained in RPMI media containing 10% FCS and sorted on a FACSAria instrument (BD).

Data analyses. Flow cytometry measurements were performed on an LSR-II (BD) machine and the data were analyzed with FlowJo software (Tree Star). Graphs were prepared and EC₅₀ concentrations were determined with Prism (GraphPad Software). The error bars or numbers show SD.

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S. Enouz performed all experiments with the assistance of L. Carrié in the laboratory of D. Zehn, D. Merkler performed the histological analysis, the OT-3 TCR was isolated by D. Zehn in the laboratory of M.J. Bevan, experimental conceptions and plans were made by D. Zehn, data analysis was performed by S. Enouz and D. Zehn, the manuscript was written by S. Enouz and D. Zehn, and critical advice and suggestions to the manuscript were provided by M.J. Bevan and D. Merkler.

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