Targeting Antigen to Diverse APCs Inactivates Memory CD8+ T Cells without Eliciting Tissue-Destructive Effector Function

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Targeting Antigen to Diverse APCs Inactivates Memory CD8+ T Cells without Eliciting Tissue-Destructive Effector Function

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Memory T cells develop early during the preclinical stages of autoimmune diseases and have traditionally been considered resistant to tolerance induction. As such, they may represent a potent barrier to the successful immunotherapy of established autoimmune diseases. It was recently shown that memory CD8+ T cell responses are terminated when Ag is genetically targeted to steady-state dendritic cells. However, under these conditions, inactivation of memory CD8+ T cells is slow, allowing transiently expanded rapidly inactivated by MHC class II+ hematopoietic APCs through a mechanism that involves a rapid and sustained down-regulation of TCR, in which the effector response of CD8+ memory cells is rapidly truncated and Ag-expressing target tissue destruction is prevented. Our data provide the first demonstration that genetically targeting Ag to a broad range of MHC class II+ APC types is a highly efficient way to terminate memory CD8+ T cell responses to prevent tissue-destructive effector function and potentially established autoimmune diseases. The Journal of Immunology, 2010, 184: 000–000.

The immunotherapeutic goal for autoimmune disease is restoration of Ag-specific tolerance; in established autoimmune disease, this requires purging the repertoire of established autoaggressive effector and memory T cell populations. The use of genetically targeted Ag expression, achievable through transplantation of genetically engineered hematopoietic stem cells (10, 11), has been proposed as a powerful tool for immunotherapy of autoimmune disease. Although naive T cells exhibit a high degree of developmental plasticity and are readily inactivated upon abortive activation by steady-state dendritic cells (DCs) (12) or DCs engineered to express cognate Ag (13), effector and memory T cells are terminally differentiated and are specialized to exert direct or, in the case of memory cells, elicited effector function; thus, they are traditionally considered to be resistant to inactivation. Indeed, memory CD8+ T cells generated through heterologous responses to viruses were shown to represent a significant hurdle to the induction of transplantation tolerance (14–16). However, we recently demonstrated that Ag-expressing DCs inactivate central memory and mixed effector/memory populations of CD8+ T cells (17). However, as a consequence of their differentiated nature, memory T cells exhibit increased expansion and effector function relative to naive T cells upon encounter with Ag-expressing DCs (13, 17). This transient phase of effector function that memory T cells undergo prior to inactivation could be detrimental to cognate Ag-expressing target tissues. To determine the most suitable strategy for applying genetically targeted Ags to tolerance induction, we compared T cell inactivation by Ags targeted specifically to DCs using the CD11c promoter or targeted to more diverse MHC class II+ APC types using an MHC class II promoter.

Materials and Methods

Animals

Mice were bred and maintained at the Biological Research Facility, Princess Alexandra Hospital, or purchased from Animal Resources Centre (Perth, Australia). OT-I mice carry an MHC class I-restricted transgenic TCR for the OVA peptide OVA257–264 (18) and were crossed with CD45.1 congenic C57BL/6.SJL-Ptprc<sup>+</sup> mice to generate mice bearing CD45.1<sup>+</sup> OT-I cells. 11c.OVA mice express a membrane-bound OVA construct under the
control of the CD11c promoter, which targets OVA expression and presentation to CD11c<sup>+</sup> conventional DCs (13). MILO-VA mice expressing a membrane-bound OVA construct under control of an MHC class II promoter (pDOI-5) (19) were kindly provided by Dr. Francis Carbone (University of Melbourne). OT-I mice deficient for Bim were described previously (20). Nontransgenic controls for 11c.OVA and MILO-VA mice were C57BL/6. RIP.OVA<sup>+</sup> mice expressing OVA directed to pancreatic β-cells by a rat insulin promoter; they were described previously (21). RIP.OVA<sup>+</sup> x 11c.OVA (RIP x 11c), RIP.OVA<sup>+</sup> x MILO-VA (RIP x MIL), and RIP.OVA<sup>+</sup> x C57BL/6 (RIP x non-tg) mice were FL offspring of RIP.OVA<sup>+</sup> mice with 11c.OVA, MILO-VA, or C57BL/6 mice, respectively. Mice were matched for sex within the experiments. Animal studies were approved by the University of Queensland Animal Ethics Committee.

**Cell preparation and adoptive transfers**

Memory OT-I T cells were generated as previously described (17). Briefly, lymph node cells from CD45.1<sup>+</sup> OT-I mice were cultured in six-well plates (2 x 10<sup>6</sup>/well) in 3 ml complete RPMI (RPMI 1640 supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids [all from Life Technologies, Rockville, MD], and 50 μM 2-mercaptoethanol [Sigma-Louis, St. Louis, MO]) with 1% normal mouse serum, 0.1 mM g/ml OVA<sub>257–264</sub> (Mimotopes, Melbourne, Australia) and 10 g/ml IL-2 [PeproTech, Rocky Hill, NJ]. After 3 d, cells were harvested and washed three times with RPMI 1640 and recultured in six-well plates at 2 x 10<sup>6</sup>/well in the absence of Ag but with 10 g/ml IL-2 (PeproTech) for an additional 2 d. Cells were harvested and washed in PBS prior to transfer to experimental mice. For some experiments, memory CD8<sup>+</sup> T cells were generated in vivo as described (17). Unless stated otherwise, 2 x 10<sup>5</sup> OT-I T cells were transferred i.v. in all experiments. Where indicated, Flt3 ligand (FL) (Amgen, Thousand Oaks, CA) or PBS was injected i.p. (10 g/ml) for an additional 2 d. Cells were harvested and stained with hematoxylin and eosin, and at least five sections separated by 4 μm were examined from each pancreas.

**Statistical analysis**

Comparison of means was performed using the Student’s t test, and multiple groups were compared using one-way ANOVA followed by the Newman–Keuls posttest (GraphPad Prism, GraphPad, San Diego, CA).

**Results**

OVA expressed in diverse APC types induces limited expansion of OT-I CD8<sup>+</sup> effector/memory T cells

Previously, we showed that central memory or mixed populations of effector and memory CD8<sup>+</sup> T cells are inactivated upon transfer to mice in which steady-state DCs express cognate Ag (17). To determine the response of memory and effector CD8<sup>+</sup> T cells when Ag expression was more widespread, we compared the CD8<sup>+</sup> effector/memory T cell response in mice in which cognate Ag expression was restricted to DCs using the CD11c promoter (11c.OVA) or more widely expressed under control of an MHC class II promoter (MILO-VA). For transfer, a mixed population of effector/memory cells was generated by culturing OT-I lymph node cells with IL-2 and OVA<sub>257–264</sub> for 3 d, followed by 2 d with IL-15 in the absence of peptide. These conditions yield a population of T cells comprising approximately equal proportions of CD44<sup>hi</sup>/CD62L<sup>lo</sup> effector/memory phenotype or CD44<sup>hi</sup>/CD62L<sup>hi</sup> central memory phenotype cells that, in vivo, exhibit strong CTL activity and establish long-lived memory (17). When transferred to 11c.OVA mice, effector/memory OT-I T cells proliferated extensively, with the number of OT-I cells in spleen reaching a maximum 7 d after transfer (Fig. 1A). After expanding, the OT-I population in 11c.OVA recipients contracted substantially within the next 7 d and then showed a slower, but sustained, contraction, as described previously (17). In contrast, in MILO-VA recipients, the expansion of OT-I effector/memory cells in spleen was substantially truncated, such that the bulk of expansion and contraction had occurred within 7 d of transfer (Fig. 1A). The number of OT-I T cells in lymph node, liver, and BM 7 d after transfer relative to that in spleen was similar across recipient strains (data not shown), indicating that the rapid contraction of the OT-I population in MILO-VA lymphoid tissues was not due to retrafficking of OT-I cells to nonlymphoid tissue sites where memory T cells normally accumulate.

**In vitro and in vivo assays**

Intracellular cytokine staining was performed as described (13) in cells stimulated with 0.5 μg/ml SIINFEKL (AUSp, Parkville, Victoria, Australia) or 25 μg/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Calbiochem, San Diego, CA) for 3 h. To assess proliferation in vivo, OT-I effector/memory T cells were labeled with 2.5 μM CFSE as described (24) and injected i.v. Three days later, recipient spleens were harvested, and CFSE dilution in CD45.1<sup>+</sup> cells was assessed by FACS. To determine the responsiveness to Ag, mice were immunized s.c. at the tailbase with OVA/QuilA (100 μg OVA [Grade V, Sigma-Aldrich], 20 μg QuilA [Soperfos Biosector, Vedbaek, Denmark]). In vivo CTL activity was determined as described previously (17). To test the effects of Fas-L signaling, anti-Fas-L was injected i.p. (200 g/ml) prior to OT-I cell transfer and every 3 d thereafter for the next 7 d after transfer. For diabetes incidence, studies, blood glucose levels were determined with a portable glucometer (Accu-Check II, Roche Diagnostics, Castle Hill, Australia) prior to and at defined times after OT-I effector/memory cell transfer.

**Generation of bone marrow chimeras**

Hind limb bones were collected from CO<sub>2</sub> euthanized donor mice, and bone marrow (BM) cells were harvested by flushing with cold PBS/2.5% FCS. Erythrocytes were lysed (NH<sub>4</sub>Cl/Tris buffer), and for H-2<sup>d</sup>/H<sup>M2</sup> chimeras, T and B cells were depleted using immunomagnetic depletion (Miltenyi MACS, Miltenyi Biotec, Auburn, CA), according to the manufacturer’s instructions. Where indicated, different proportions of BM were mixed and transferred (5 x 10<sup>6</sup>) i.v. to irradiated (2 x 550 cGy 3 h apart, 13<sup>7</sup>Cs) recipients. Recipients were provided with neomycin-supplemented (1 μg/ml) drinking water for 3 wk and used for experiments 6–7 wk after transfer. The degree of donor marrow engraftment was determined on completion of the experiments by flow cytometry using alleloype-specific anti–H-2-K Abs for H-2<sup>d</sup>/H<sup>M2</sup> or CD45 alleloype-specific Abs for mixed MILO-VA chimeras.

**Iset infiltration analysis**

Pancreata were collected from CO<sub>2</sub> euthanized animals at the termination of experiments and embedded for paraffin sectioning. Sections were stained with hematoxylin and eosin, and at least five sections separated by ≥100 μm were examined from each pancreas. Iset infiltration was scored in a masked fashion according to an established method, where 0 = no infiltration, 1 = <10 peri-islet lymphoid cells, 2 = 10–20 peri-islet and intraislet lymphoid cells, 3 = ≥20 intraislet lymphoid cells, and 4 = ≥50% islet cells destroyed, and 4 = ≥50% islet cells destroyed (25).
accumulation in spleen (Fig. 1B) was almost identical to that of memory cells generated in vitro (Fig. 1A). These data are consistent with our previous demonstrations that effector/memory T cells generated using the IL-2/IL-15 conditions in this study behave similarly to long-lived memory T cells arising after immunization in vivo.

To determine whether accelerated cell death contributed to rapid contraction of OT-I cells in MILOVA recipients, we tested the contraction of OT-I effector/memory T cells lacking Bim, which is required for T cell deletion during the contraction phase of DC-induced tolerance in memory CD8+ T cells (17). Bim deficiency weakly reduced the extent of effector/memory OT-I contraction between days 3 and 7 posttransfer in MILOVA recipients but not in 11c.OVA or nontransgenic recipients (Fig. 1C); however, it did not reverse the rapid onset of OT-I contraction in MILOVA recipients. Similarly, blockade of Fas–Fas-L interactions did not prevent early OT-I contraction in MILOVA recipients (Fig. 1D).

To independently verify that the two major cell death pathways tested, the mitochondrial and receptor-mediated pathways, made little contribution to OT-I contraction in MILOVA recipients, we compared apoptosis of OT-I cells in liver, a key site at which apoptotic CD8+ T cells are cleared, in 11c.OVA and MILOVA recipients. Staining with Annexin V, a marker of apoptotic cell death, indicated no significant difference in the rate of apoptosis in OT-I cells isolated from liver between 11c.OVA and MILOVA mice (2.2% ± 0.7% versus 2.5% ± 1.8%, respectively) 3 d after effector/memory OT-I transfer, at the onset of the contraction phase in MILOVA recipients (Fig. 1E). Together, these data indicated that Bim-dependent cell death contributed in a minor way to OT-I contraction in MILOVA recipients, but this was not solely responsible for the difference in population expansion and contraction between 11c.OVA and MILOVA recipients.

We next tested an alternate possibility, that early OT-I contraction in MILOVA recipients resulted from a lack of sustained OT-I proliferation. Analysis of CFSE dilution showed that, 3 d after transfer, OT-I effector/memory cells had undergone a similar number of divisions in 11c.OVA and MILOVA recipients (Fig. 1F), which was consistent with the similar accumulation of OT-I cells.

**FIGURE 1.** Ag targeted to diverse APCs induces limited expansion of CD8+ effector/memory T cells. A, The total number of OT-I (CD45.1+/CD8+/Vα2+) cells in spleens was determined after transfer of CD45.1+ OT-I effector/memory cells to 11c.OVA, MILOVA, or nontransgenic recipients. Data (mean ± SD, n = 4) are pooled from two independent experiments at each time point. B, FACS-sorted CD45.1+ OT-I memory T cells (CD8+/CD44hi) were transferred, and the total number of OT-I T cells in spleens of MILOVA, 11c.OVA, or non-tg recipients was determined 7 d later. Data are pooled from two independent experiments of two mice per group. C, Wild-type (WT) or Bim−/− OT-I effector/memory cells were transferred, and the total number of OT-I cells in recipient spleens was determined 3 and 7 d later. Data (mean ± SD, n = 4) are pooled from two independent experiments at each time point. *Number of OT-I bim−/− cells was significantly greater than OT-I wild-type cells in MILOVA recipients 7 d posttransfer. D, CD45.1+ OT-I effector/memory cells were transferred to anti–Fas-L- or PBS-treated recipients, and total OT-I cells in spleen were determined 7 d later. Data are pooled from two independent experiments. E, CD45.1+ OT-I effector/memory cells were transferred, and OT-I cells in liver were analyzed for Annexin V binding 3 d later. Data are representative of two experiments with two mice per group. F, OT-I effector/memory cells were labeled with CFSE, transferred, and CFSE dilution was analyzed in spleen cells 3 and 5 d later. Data are representative of two mice per group from each of two independent experiments.
in both recipient sets at this time point (Fig. 1A). In contrast, 5 d after transfer, OT-I T cells in MII.OVA recipients had undergone little additional division (Fig. 1F), but considerable further dilution of CFSE indicated sustained proliferation of transferred effector/memory OT-I T cells in 11c.OVA recipients (Fig. 1F). Therefore, we concluded that in MII.OVA recipients, limited expansion of the OT-I population resulted primarily from a rapid termination of the proliferative response rather than accelerated onset of cell death.

**Effector/memory OT-I T cells rapidly lose TCR responsiveness in MII.OVA recipients**

To determine the possible mechanisms that led to truncated effector/memory OT-I proliferative responses in MII.OVA recipients, we first performed phenotypic analysis of transferred OT-I cells. Substantial downregulation of surface TCRα- and β-chains was observed on transferred effector/memory OT-I cells in MII.OVA recipients as early as 3 d after transfer (Fig. 2A); however, little or no downregulation of TCRα or β was seen on transferred effector/memory OT-I cells in 11c.OVA or nontransgenic recipients (Fig. 2A) or host CD8⁺ T cells (data not shown), all of which carried similar levels of TCRα and β. Similarly, CD3 expression was reduced, but to a lesser extent, only on OT-I T cells in MII.OVA recipients, whereas no change was seen in CD8 expression (Fig. 2A). Downregulation of TCR was also observed in MII.OVA, but not 11c.OVA, recipients when long-lived OT-I cells generated by immunization in vivo were transferred (data not shown). Downregulation of TCR was observed on effector/memory OT-I cells recovered from lymph node, liver, and lung (Fig. 2B) as soon as 3 d after transfer to MII.OVA mice demonstrating rapid systemic inactivation of memory cells. Because T cell activation can lead to loss of surface TCR through internalization, we compared surface TCR expression with total (surface and internalized) TCR revealed by permeabilization of cells prior to staining. Although surface TCR, indicated by Vα2 staining, was markedly downregulated only on OT-I T cells in MILOVA recipients, total TCR staining differed little among MILOVA, 11c.OVA, and nontransgenic recipients (Fig. 2C). This indicated that surface TCR from OT-I T cells in MILOVA recipients was lost primarily through internalization. TCRα- and β-chains remained downregulated on OT-I T cells in MILOVA recipients for ≥42 d after transfer (data not shown), indicating TCR downregulation developed as a stable phenotype of OT-I T cells in MILOVA recipients.

To test the functional capacity of transferred effector/memory OT-I cells, IFN-γ production in response to in vitro OVA257–264 stimulation was compared across recipient strains. Approximately 50% of OT-I cells recovered from 11c.OVA and nontransgenic recipients 3 d after transfer produced IFN-γ detectable by intracellular cytokine staining in response to peptide stimulation (Fig. 2D), whereas few OT-I T cells recovered from MILOVA recipients did (Fig. 2D, upper panel). Because the loss of OVA257–264-induced IFN-γ production in MILOVA recipients was possibly due to the loss of surface TCR expression, we tested whether bypassing TCR-dependent activation would restore IFN-γ production. In response to TCR-independent activation with PMA and ionomycin, OT-I T cells recovered from MILOVA, 11c.OVA, and nontransgenic recipients all produced IFN-γ to a similar degree (Fig. 2D, lower panel, 2E). TCR-dependent activation by OVA257–264 or anti-CD3 mAb induced IFN-γ production only in OT-I cells recovered from 11c.OVA or nontransgenic recipients (Fig. 2E).

Because CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) are positively selected by MHC class II-expressing thymic epithelial cells on the basis of self-reactivity (26), it is conceivable that differences in the specificity or function of Tregs could exist between 11c.OVA and MILOVA mice and contribute to OT-I memory T cell inactivation. To explore this, MILOVA and 11c.OVA recipients were depleted of Tregs by the administration of anti-CD25 mAb 3 d prior to transfer of effector/memory OT-I cells. Rapid TCR downregulation was unaltered in MILOVA mice.

**FIGURE 2.** OVA-specific T cells rapidly lose TCR responsiveness in MILOVA mice. CD45.1⁺ OT-I effector/memory cells were transferred to 11c.OVA, MILOVA, or nontransgenic recipients. Three days later, OT-I cells (CD45.1⁺/CD8⁺) were analyzed by flow cytometry without (A and B) or with and without (C) permeabilization. Data are representative of more than two experiments with two mice per group. D and E, CD45.1⁺ OT-I effector/memory cells were transferred to nontransgenic, 11c.OVA, or MILOVA recipients, and IFN-γ production was determined by intracellular cytokine staining 3 d later. Data shown are gated on OT-I (CD45.1⁺/CD8⁺) cells (C) and are representative of (D) or pooled from (E) two experiments with two mice per group.
by depletion of Tregs (data not shown). Additionally, no differences were observed in total CD4^+CD25^+FoxP3^+ Treg numbers or the in vitro suppressive activity of Tregs among 11c.OVA, MIL.OVA, and nontransgenic control mice (data not shown). Together, these data indicate that effector/memory OT-I T cells transferred to MIL.OVA mice rapidly lost responsiveness to TCR-dependent activation through rapid and sustained loss of surface TCR.

**Ag targeted to diverse APC types elicits little effector function from effector/memory CD8^+ T cells during inactivation**

We showed previously that effector/memory T cells exhibit a transient phase of effector function while undergoing inactivation in response to steady-state Ag-expressing DCs (17). Because expansion was limited and TCR-mediated signaling was rapidly lost from OT-I effector/memory cells in MIL.OVA mice, we compared effector function in MIL.OVA and 11c.OVA recipients. We first tracked IFN-γ production as a surrogate of effector function in effector/memory T cells undergoing inactivation. As described earlier, the proportion of OT-I cells producing IFN-γ in response to OVA257–264 was considerably reduced in MIL.OVA recipients relative to nontransgenic and 11c.OVA recipients within 3 d of transfer (Fig. 3A). In contrast to the rapid decrease in IFN-γ production in MIL.OVA recipients, the proportion of OT-I cells producing IFN-γ in nontransgenic recipients remained relatively consistent, with only a moderate decrease in the proportion between days 3 and 42 posttransfer (Fig. 3A). In 11c.OVA mice, the proportion of OT-I cells producing IFN-γ was initially similar to that in nontransgenic recipients, but it decreased to low levels between days 7 and 28 after transfer (Fig. 3A), indicating that inactivation of OT-I cells was slower than in MIL.OVA recipients. Because of the limited expansion and rapid loss of IFN-γ production, the total number of IFN-γ-producing OT-I T cells in spleens (Fig. 3B) of MIL.OVA recipients was reduced relative to nontransgenic and, in particular, to 11c.OVA recipients in which relatively large numbers of IFN-γ-producing OT-I cells accumulated. Comparison of systemic cytolytic capacity showed, consistent with the number of IFN-γ-producing OT-I T cells, that killing of OVA257–264-pulsed targets was reduced by >80% in MIL.OVA recipients relative to 11c.OVA or nontransgenic recipients at 7 and 28 d after transfer (Fig. 3C). Cytometric analysis showed that expression of granzyme B was slightly reduced in only a small proportion of OT-I cells in MIL.OVA recipients 7 d after transfer, indicating that impaired CTL activity was not due to a lack of effector molecule expression (Fig. 3D) but was most likely due to the loss of surface TCR and subsequent TCR signaling. Collectively, these data indicate that although CD8^+ memory T cells were ultimately inactivated when encountering DCs expressing cognate Ag, substantial effector function was elicited. In contrast, little effector function was detected when Ag was expressed in diverse MHC class II^+ APC types.

**Inactivation of effector/memory T cells is more rapid when Ag is expressed in diverse APC types rather than DCs alone**

Because the effector function of transferred effector/memory cells was rapidly damped in MIL.OVA relative to 11c.OVA recipients, we next tested whether the ability of OT-I effector/memory cells to respond to immunogenic Ag challenge in vivo was also abrogated. Challenge with OVA, along with the highly immunogenic adjuvant QuilA, demonstrated that population expansion and induction of IFN-γ production by effector/memory OT-I cells was blocked in MIL.OVA mice within 7 d of transfer (Fig. 4A). In 11c.OVA recipients, a substantial expansion and increase in the total number of IFN-γ-producing OT-I cells was induced by immunogenic OVA challenge at the same time point (Fig. 4A, 4B). Additionally, as previously reported (17), the capacity of OT-I T cells to expand in response to OVA challenge was retained for ≥21 d after transfer to 11c.OVA recipients (Fig. 4C), although the induction of IFN-γ production by immunogenic OVA challenge was damped (Fig. 4D). Together, these data indicate that although Ag-expressing DCs are capable of inactivating the in vivo responsiveness of effector/memory CD8^+ T cells, this process is considerably slower than the rapid inactivation seen when Ag is targeted to diverse APCs.

**Ag targeted to diverse APC types prevents development of tissue-destructive CD8^+ T cell effector function**

Ag-expressing APCs have been proposed as an immunotherapeutic tool for the inactivation of Ag-specific T cells (10). Ideally, such immunotherapeutics should be suitable for application to the inactivation of pre-existing memory and/or effector cell populations, without eliciting detrimental T cell functions. However, although the inactivation of memory T cells is achievable with Ags targeted to DCs (17), effector function elicited from memory or effector T cells during inactivation could be detrimental and result in accelerated destruction of the target tissues intended for protection. To determine whether activation by DCs or diverse MHC class II^+ APC types expressing cognate Ag differentially elicited detrimental effector function from effector/memory T cells undergoing inactivation, we tested the effect of transferring OT-I effector/memory T cells to mice expressing OVA in pancreatic β cells alone (RIP × non-tg), in β cells and DCs (RIP × 11c), or in β cells and diverse APC types (RIP × MII). Titrated doses of effector/memory OT-I T cells induced a dose-dependent loss of glycemic control (autoimmune diabetes) in RIP × non-tg controls (Fig. 5A) that was associated with islet infiltration and β-cell destruction (Fig. 5B, 5C). In RIP × 11c mice, the onset of elevated blood glucose was accelerated, particularly at a suboptimal dose (2 × 10^5) of diabetogenic OT-I effector/memory cells, which
led to slow diabetes onset in RIP × nontransgenic mice, as well as at an optimally diabetogenic dose (Fig. 5A). In contrast, in RIP × MII recipients, no increase in blood glucose was observed, even after transfer of a dose of effector/memory OT-I T cells that was optimally diabetogenic in nontransgenic controls (Fig. 5A), and substantially less islet infiltration and β cell destruction was present (mean islet score: 1.00, 1.23, and 0.23, respectively, for RIP × non-tg, RIP × 11c, and RIP × MII; p < 0.05 for RIP × non-tg versus RIP × MII and for RIP × 11c versus RIP × MII (Fig. 5B, 5C). Systemic CTL activity did not seem to be altered by the additional presence of OV A expressed in β cells; in vivo CTL assays performed at the termination of the experiments showed, as expected, little CTL activity in OVA-expressing cells in vivo, and others has shown that additional expression of MHC class II in DCs alone promotes Ag-specific tissue destruction.

**Rapid termination of CD8+ memory T cells responses is mediated by hematopoietic cells**

MHC class II expression can be induced in many cell types, particularly by IFN-γ, a key cytokine produced by effector/memory CD8+ T cells (Figs. 2 and 3). If MHC class II was induced in nonhematopoietic APCs in MII.OVA mice and led to OVA expression, it is possible these cells could contribute to the rapid inactivation of transferred OVA-specific effector/memory CD8+ T cells. Therefore, we sought to determine whether hematopoietic or nonhematopoietic APCs were responsible for the rapid inactivation of CD8+ T cells in MILOVA mice. BM chimeras of MILOVA and H-2K bm1 (bm1) mice, which have an H-2K unable to present OV A257–264, were generated so that the effect of restricting the expression and presentation of OV A257–264 to hematopoietic or nonhematopoietic cells could be tested. Following irradiation and reconstitution, analysis of spleen cell suspensions indicated >99% conversion of BM-derived cells to donor-type cells. When non-BM–derived cells could express and present OVA, but
BM-derived cells could not (bm1→MII.OVA), no reduction in OT-I effector/memory IFN-γ production was observed (Fig. 6B, 6C). Only when the hematopoietic compartment expressed OVA in conjunction with H-2Kb (MII.OVA→bm1, MII.OVA→MII.OVA) were IFN-γ production and the number of IFN-γ-producing OT-I T cells, damped as seen in MII.OVA controls (Fig. 6B, 6C). Therefore, OVA expression and presentation were required in hematopoietically derived APCs for rapid inactivation of transferred OT-I effector/memory T cells to occur, indicating that nonhematopoietic cells did not contribute to rapid effector/memory OT-I inactivation as a result of the transfer of IFN-γ–producing effector/memory OT-I cells. This indicates that rapid inactivation of memory CD8+ T cells could be achieved by gene therapeutic approaches targeting hematopoietic APCs alone.

Rapid termination of CD8+ memory T cells responses is promoted by increasing the density of OVA-expressing APCs

To determine whether the rapid inactivation of CD8+ effector/memory T cells resulted from increasing the frequency of OVA-expressing APCs, we generated mixed chimeras that carried titrated proportions of OVA-expressing APCs and effector/memory OT-I inactivation compared with MII.OVA and nontransgenic control mice. When the relative frequency of OVA-expressing APCs was high, damping of IFN-γ production was similar to that in MII.OVA controls (Fig. 6D). However, as the proportion of OVA-expressing APCs decreased to ≤25% of all APCs, the effectiveness of IFN-γ damping decreased dramatically as the proportion of OVA-expressing APCs declined (Fig. 6D). To further test whether this effect was dependent on the APC type expressing OVA or the relative frequency of Ag-expressing APCs, 11c.OVA mice were treated with FL to mobilize DCs (27). OT-I effector/memory cells were transferred on the ninth day of treatment, to coincide with the peak of DC mobilization. Treatment with FL resulted in an ~10-fold increase in the number of CD11c+ DCs in the spleens of treated mice but no increase in B cells relative to PBS-treated controls (Fig. 6E). When OT-I effector/memory T cells were transferred to FL-treated 11c.OVA mice, rapid downregulation of TCR was observed within 3 d of transfer (Fig. 6F), similar to that seen in MII.OVA mice (Fig. 2A). This indicated that rapid effector/memory CD8+ T cell inactivation is promoted by increasing the density of OVA-expressing APCs.

Discussion

The goal for immunotherapy of autoimmune diseases is restoration of Ag-specific tolerance. Because it is known that developing thymocytes and naive T cells are subject to tolerance enforced by interactions with APCs intrathymically or peripherally, harnessing these mechanisms is a conceivable approach for the prophylaxis of autoimmune disease. In the case of established autoimmune disease, treatment would require autoreactive memory and effector T cell populations to be purged from the peripheral immune repertoire. However, little is known about the capacity for established memory and effector T cell populations to be inactivated and how this could be achieved. Because some studies indicated that memory T cells could be resistant to tolerance induction, they have been considered a major hurdle for the therapy of autoimmune diseases. In this study, we compared different Ag-targeting strategies and showed that using an MHC class II promoter to drive Ag expression in a diverse range of APCs rapidly terminated the CD8+ memory response, without eliciting tissue-destructive effector function.

The use of genetically targeted Ag expression, achievable through transplantation of genetically engineered hematopoietic stem or progenitor cells, has been proposed as a powerful tool for immunotherapy of autoimmune disease (10, 11) and, more recently, allergies (28). We demonstrated previously that targeting DCs (29) or diverse APC types
is able to inhibit the development of autoimmune diabetes in the NOD mouse model. However, to exploit such an approach most effectively, detailed knowledge is required about which APC populations to genetically target. We have sought to determine strategies through which memory CD8+ T cell responses can be terminated; our previous studies showed, that in a manner similar to that demonstrated for naive T cells (13), steady-state DCs expressing cognate Ags terminate central memory and mixed effector/memory CD8+ T cell responses through a mechanism that requires persistent Ag exposure (17). However, targeting Ags to DCs alone results in a relatively slow process of inactivation accompanied by substantial transient expansion of the effector/memory CD8+ T cells during which tissue-destructive effector function is exerted. By diversifying the hematopoietic APC types capable of expressing and presenting cognate Ags through the use of an MHC class II promoter, we showed in this study that the CD8+ memory/effector T cell response can be rapidly terminated. These findings indicate that genetic targeting of Ags can be used for the induction of tolerance in memory CD8+ T cells and that the specific targeting strategy profoundly alters the outcome of tolerogenic Ag presentation to CD8+ memory T cells.

Targeting Ag to diverse APC types led to rapid and stable down-regulation of surface TCR expression, which limited the responsiveness of cognate Ag-specific CD8+ memory T cells. This differed substantially from the response elicited by Ags targeted solely to DCs, whereby extensive expansion was followed by deletion and induction of unresponsiveness, without the loss of surface TCR expression. These characteristics indicate that the biochemical pathways leading to memory T cell inactivation are likely to differ substantially between these two settings. It is plausible that the adaptive pathways that regulate the intracellular signaling cascades from the TCR are likely to play a critical role in the unresponsive state of residual memory OT-I T cells in 11c.OVA mice. Likely mediators of these effects include the classic regulators of TCR signaling, such as the anergy-associated E3 ubiquitin ligases gene related to anergy in lymphocytes, ictch, c-cbl, and cbl-b family members (30). The biochemical pathways that mediate rapid and sustained down-regulation of surface TCR expression upon activation, or indeed during tolerance, are poorly defined, although c-cbl and cbl-b are implicated (31), and these could be key controllers of rapid Ag-targeting OT-I T cell inactivation in MII.OVA recipients. Characterization of the relative roles of these pathways in the contrasting tolerance settings described in this study will provide important insights into opportunities for the regulation of memory T cell function.

DCs are highly specialized APCs capable of differentially controlling naive T cell differentiation. When activated, they perform a fundamental role in initiating primary immune responses; however, in the steady-state, they can also promote T cell inactivation or conversion of naive T cells to regulatory cells capable of suppressing immunity (32). The latter features can be exploited, using Ags targeted by genetic or other means (12), for the induction of peripheral tolerance in naive T cells. In contrast, the role of DCs in regulating memory T cell responses is less well understood; recent findings suggest that the nature of memory T cell activation by DCs may differ from that of naive T cells (33). Based on our previous findings, it is clear that persistent presentation of cognate Ags by steady-state DCs leads to the inactivation of fully differentiated CD8+ memory T cells (17). The mechanisms by which targeting cognate Ags to more diverse APCs alters memory T cell–APC interactions to induce rapid inactivation remain to be determined, although several possibilities exist. TaqMan analyses indicated a high level of OVA expression in DCs and B cells of MII.OVA mice but a much lower level of expression restricted to DCs in 11c.OVA mice (data not shown). Thus, increases in the absolute number of Ag-expressing APCs and the level of Ag expression by individual APCs could be important contributors. Consistent with this suggestion, previous studies indicated that high Ag “doses” can favor the induction of unresponsiveness over deletion (34–36) in naive T cells. Alternatively, the expression of Ags by B cells in addition to DCs in MII.OVA mice could imply that B cells are inherently more effective for the induction of unresponsiveness or anergy. Recent reports indicate that resting, but not activated, B cells inactivate memory CD4+ T cells through anergy induction (37) and that DCs may be dispensable for this process (38). However, our data indicate that increasing the frequency of Ag-expressing DCs in 11c.OVA mice is sufficient to promote rapid effector/memory OT-I inactivation. Other investigators reported increased tolerogenicity by increasing resting DC numbers through FL administration (39, 40). Alterations in the relationship between TCR affinity and Ag “dose” could be important determinants of the program of tolerance induced. Although this was not tested in the present study, it is likely that this would be fruitful ground for further study. Overall, our data favor the interpretation that increasing the frequency of cognate Ag-expressing APCs, rather than the specific APC type targeted or the Ag expression level of individual APCs, is a crucial determinant in promoting rapid CD8+ memory cell inactivation.

Because of their fully differentiated nature, their relative lack of reliance on costimulation, and their resistance to suppression by Tregs, memory T cells are considered a significant hurdle to immunotherapy of established autoimmune disease. Additionally, alloreactive memory T cells generated through heterologous activation provide a significant barrier to organ transplantation. Therefore, the development of methods for cell intrinsic inactivation of memory T cells could provide attractive opportunities for immunotherapy. In this study, we showed that targeting Ags to a variety of MHC class II+ APC types leads to the rapid loss of functional Ag responsiveness in cognate Ag-specific CD8+ memory and effector T cells. This provides a direction for future research in autoimmune disease immunotherapy.

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Disclosures

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