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 memory CD8+ T cells to exert tissue-destructive effector function. In this study, we compared different Ag-targeting strategies and show, using an MHC class II promoter to drive Ag expression in a diverse range of APCs, that CD8+ memory T cells can be 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: 598–606.

Following Ag stimulation in the presence of costimulatory signals, naive T cells undergo a program of expansion and terminal differentiation, leading to the generation of effector and memory T cells. In a primary immune response, the bulk of the clonally expanded T cells, primarily short-lived effector T cells, die (1, 2); however, a small proportion (5–10%) survives to generate memory cells (2). This generates a population of cells that, compared with naive T cells, exhibits faster response kinetics (3), increased avidity (4), and little or no dependence on costimulation (3, 5). Although memory T cells have relatively little inherent effector function, they are long-lived and provide a pool of cells from which effector T cells can rapidly be generated.

In T cell-mediated autoimmune diseases, the priming of target-specific naive T cells leads to the differentiation of effector and memory T cells that can be detected early in the preclinical phase of disease progression (6). As disease develops, populations of these cells become established and drive target tissue destruction (7, 8). In some of these diseases, exemplified by type 1 diabetes, target tissues are completely destroyed; although target-specific effector T cells eventually wane, evidence suggests that in the absence of Ag, long-lived Ag-specific memory T cells persist. These can rapidly expand and differentiate into effector cells if, for example, target tissue replacement therapy is attempted (9).

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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+ conventional DCs (13). MILOVA mice expressing a membrane-bound OVA construct under control of an MHC class II promoter (pDOOl-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 MILOVA mice were C57BL/6. RIP-OLA+ mice expressing OVA directed to pancreatic β-cells by a rat insulin promoter; they were described previously (21). RIP-OLA+ x 11c.OVA (RIP x 11c), RIP-OLA+ x MILOVA (RIP x MIL), and RIP-OLA+ x C57BL/6 (RIP x non-tg) mice were F1 offspring of RIP-OLA+ mice with 11c.OVA, MILOVA, 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+ OT-I mice were cultured in six-well plates (2 x 10^6/ml) in 3 ml complete RPMI (RPMI 1640 supplemented with l 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 μg/ml OVA323–339 (Mimotopes, Melbourne, Australia) and 10 ng/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 10^6/ml in the absence of Ag but with 10 ng/ml IL-15 (PeproTech) for an additional 2 d. Cells were harvested and washed in PBS prior to transfer to experimental mice. For some experiments, memory CD8+ T cells were generated in vivo as described (17). Unless stated otherwise, 2 x 10^6 OT-I T cells were transferred i.v. in all experiments. Where indicated, Flt3 ligand (FL) (Agen, Thousand Oaks, CA) or PBS was injected i.p. (10 μg/day) for 11 d, and effector/memory OT-I cells were injected i.v. on the ninth day of treatment and recovered 3 d later for analysis. For in vivo depletion of CD25+ cells, mice were injected with anti-CD25 mAb (PC61, 1 mg i.p.).

Abs and flow cytometry

Abs against CD8 (53–6.7), CD44 (IM7), CD45.1 (A20), CD62L (MEL-14), CD69 (H1.2F3), H-2K (AF6-88.5), IFN-γ (XMG1.2), IL-2 (JES6-5H4), and TNF-α (MP6-XT22) were purchased from Biologend (San Diego, CA). Annexin V and mAbs against TCR Vα2 (B20.1) and Vβ5 (M93–4) were obtained from BD Pharmingen (San Diego, CA). Anti-granzyme B (16G6) and anti-FoxP3 were purchased from eBioscience (San Diego, CA). Anti–H-2-K+ (5F1) was produced, purified, and conjugated in house. Anti–Fas-L (MFL4) was produced and purified as described (22). mAb against CD25 (PC61, rat IgG1) was purified from hybridoma supernatants generated in-house.

For cytotoxic assay, cells were prepared from lymphoid tissues, liver, and lung and stained as described (17). Annexin V staining was performed according to the manufacturer’s instructions after staining for CD8 and CD45.1. For detection of intracellular TCR or granzyme B, cells were surface stained with CD8 and CD45.1, fixed (Fixation Buffer, Biolegend), permeabilized (Perm/Wash buffer, BD Pharmingen), and stained for Vα2, Vβ5, or granzyme B. FoxP3 staining was performed according to the manufacturer’s instructions (eBioscience). Cytometric data were acquired on a FACScalibur or FACScanto (BD Biosciences) cytometer. Absolute OT-I cell numbers were determined by a bead-based procedure (23). Total IFN-γ–producing OT-I cell number was calculated based on intracellular cytotoxic staining and absolute OT-I cell numbers.

In vitro and in vivo assays

Intracellular cytotoxic staining was performed as described (13) in cells stimulated with 0.5 μg/ml SIINFEKL (Apeus, 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 vitro, 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+CDS+ 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 [Sof speros 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 μg) prior to OT-I cell transfer and every other day thereafter. 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 C57/B10 euthanized donor mice, and bone marrow (BM) cells were harvested by flushing with cold PBS/2.5% FCS. Erythrocytes were lysed (NH4Cl/Tris buffer), and for H-2b/H-2bm1 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 10^6) i.v. to irradiated (2 x 550 cGy 3 h apart, [137Cs] source) 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 allotype-specific anti–H-2-K Abs for H-2b/H-2bm1 or CD45 allotype-specific Abs for mixed MILOVA chimeras.

Iset infiltration analysis

Pancreata were collected from C57/B10 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 cells and <50% islet cells destroyed, and 4 = >50% islet cells destroyed (25).

Statistical analysis

Comparison of means was performed using the Student 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+ effector/memory T cells

Previously, we showed that central memory or mixed populations of effector and memory CD8+ 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+ T cells when Ag expression was more widespread, we compared the CD8+ 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 (MILOVA). For transfer, a mixed population of effector/memory cells was generated by culturing OT-I lymph node cells with IL-2 and OVA257–264 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 portions of CD44+/CD62L+ effector memory phenotype or CD44hi/CD62Llo 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 MILOVA 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 MILOVA lymphoid tissues mice was not due to retrafficking of OT-I cells to nonlymphoid tissue sites where memory T cells normally accumulate.

We confirmed these findings using a more physiological long-lived in vivo-generated memory T cell population. Seven days after transfer, the pattern of OT-I in vivo-generated OT-I memory T cell...
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 of 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
in both recipient sets at this time point (Fig. 1A). In contrast, 5 d after transfer, OT-I T cells in MILOVA 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 MILOVA 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 MILOVA recipients**

To determine the possible mechanisms that led to truncated effector/memory OT-I proliferative responses in MILOVA 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 MILOVA 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 MILOVA recipients, whereas no change was seen in CD8 expression (Fig. 2A). Downregulation of TCR was also observed in MILOVA, but not 11c.OVA, recipients when long-lived OT-I T 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 MILOVA 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 Vy2 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 T 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.
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, MII.OVA, and nontransgenic control mice (data not shown). Together, these data indicate that effector/memory OT-I T cells transferred to MII.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 MII.OVA mice, we compared effector function in MII.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 MII.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 MII.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 T 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 MII.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 MII.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 MII.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 MII.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 MII.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 T cells was blocked in MII.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 T 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 × 10⁷) 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 OVA expressed in β cells; in vivo CTL assays performed at the termination of the experiments showed, as expected, little CTL activity in RIP × MII recipients but incomplete damping of CTL activity in RIP × 11c recipients relative to RIP × MII recipients (Fig. 5D). These data indicate that widespread expression of Ag in diverse MHC class II APC types inactivates CD8+ effector/memory T cells without eliciting tissue-destructive effector function, whereas the expression 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 non-hematopoietic 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 MII.OVA mice. BM chimeras of MII.OVA and H-2K bm1 (bm1) mice, which have an H-2K unable to present OVA257-264, were generated so that the effect of restricting the expression and presentation of OVA257-264 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 (Fig. 6A). In recipient mice in which BM- and non-BM–derived cells (bm1→bm1 or bm1) were unable to express OVA or present OVA257-264, substantial numbers of IFN-γ-producing OT-I T cells persisted in the spleen 7 d after OT-I effector/memory cell transfer, as in nontransgenic controls (Fig. 6B, 6C), in which OT-I effector/memory inactivation does not occur. Similarly, 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 cell 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 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 in a trinologically 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 autoaggressive 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 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, itch, c-ebi, 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-ebi and cbl-b are implicated (31), and these could be key controllers of rapid memory OT-I T cell inactivation in MILOVA 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 MILOVA 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 MI: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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