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*J Immunol* 2010; 184:598-606; Prepublished online 7 December 2009;
doi: 10.4049/jimmunol.0900032
http://www.jimmunol.org/content/184/2/598

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

Tony J. Kenna,* Tanya Waldie,* Alice McNally,* Meagan Thomson,* Hideo Yagita,† Ranjeny Thomas,* and Raymond J. Steptoe*

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, naïve 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 (3–10%) survives to generate memory cells (2). This generates a population of cells that, compared with naïve 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 naïve 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).

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 naïve 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 naïve 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-Ptp{\(r}\) mice to generate mice bearing CD45.1\(^+\) OT-I cells. 11c.OVA mice express a membrane-bound OVA construct under the

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Received for publication January 7, 2009. Accepted for publication November 8, 2009.

This work was supported by the National Health and Medical Research Council and The Diamantina Institute, University of Queensland.

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Abbreviations used in this paper: BM, bone marrow; DC, dendritic cell; FL, Flt3 ligand; Treg, T regulatory cell.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900032

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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 (pDOL-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.OVA 

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 (XMGI.2), IL-2 (JES6-5H4), and TNF-α (MP6-XT22) were purchased from Biologend (San Diego, CA). Annexin V and mAbs against TCR Vv2 (B20.1) and Vβ5 (M9-4) were obtained from BD Pharmingen (San Diego, CA). Anti-granzyme B (16G6) and anti-FoxP3 were purchased from eBioscience (San Diego, CA). Anti–H2-K 

Generation of bone marrow chimeras Hind limb bones were collected from C02 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-2m 

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 effectormemory 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 CD44hiCD62Llo effector memory phenotype or CD44hiCD62Lhi 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 re trafficking of OT-I cells to nonlymphoid tissue sites where memory T cells normally accumulate. We confirmed these findings using a more physiologically prolonged 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 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...
in both recipient sets at this time point (Fig. 1A). In contrast, 5 d after transfer, OT-I T cells in MIL.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 MIL.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 MIL.OVA recipients**

To determine the possible mechanisms that led to truncated effector/memory OT-I proliferative responses in MIL.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 MIL.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 MIL.OVA recipients, whereas no change was seen in CD8 expression (Fig. 2A). Downregulation of TCR was also observed in MIL.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 MIL.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 Vy2 staining, was markedly downregulated only on OT-I T cells in MIL.OVA recipients, total TCR staining differed little among MIL.OVA, 11c.OVA, and nontransgenic recipients (Fig. 2C). This indicated that surface TCR from OT-I T cells in MIL.OVA recipients was lost primarily through internalization. TCRα- and β-chains remained downregulated on OT-I T cells in MIL.OVA recipients for ≥42 d after transfer (data not shown), indicating TCR downregulation developed as a stable phenotype of OT-I T cells in MIL.OVA 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 MIL.OVA recipients did (Fig. 2D, upper panel). Because the loss of OVA257–264-induced IFN-γ production in MIL.OVA 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 MIL.OVA, 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 MIL.OVA mice and contribute to OT-I memory T cell inactivation. To explore this, MIL.OVA 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 MIL.OVA 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 MILOVA 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 MILOVA mice, we compared effector function in MILOVA 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 MILOVA 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 MILOVA 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 MILOVA 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 MILOVA 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 MILOVA 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 MILOVA 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 MILOVA 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 QuiaL, demonstrated that population expansion and induction of IFN-γ production by effector/memory OT-I cells was blocked in MILOVA 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 diabeticogenic 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, C). 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 MILOVA 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 cells persisted in the spleen 7 d after OT-I effector/memory cell transfer, as in nonhematopoietic controls (Fig. 6B, C), in which OT-I effector/memory inactivation does not occur. Similarly, when non-BM–derived cells could express and present OVA, but

FIGURE 4. OVA-specific memory recall responses are rapidly inactivated in MILOVA mice. CD45.1+ OT-I effector/memory cells were transferred to 11c.OVA, MILOVA, or nontransgenic mice, and recipients were challenged or not with OVA/QuilA s.c. at 7 (A and B) or 21 (C and D) days posttransfer. A and C, The total number of OT-I (CD45.1+/CD8+/Vα2+) T cells in spleens of recipient mice was determined 7 d after OVA/QuilA challenge. B and D, IFN-γ production in response to OVA257–264 was determined by intracellular cytokine staining, and the total number of IFN-γ–producing OT-I cells per spleen was calculated. Data are pooled from two independent experiments.

FIGURE 5. Presentation of Ag by diverse APC types prevents autoimmune tissue destruction. CD45.1+ OT-I effector/memory cells were transferred to RIP-OVA mice crossed with MILOVA (RIP × MII), 11c. OVA (RIP × 11c), or nontransgenic controls (RIP × non-tg). A, Blood glucose was monitored prior to and after transfer. Data (mean ± SD; n = 8) were pooled from two independent experiments. Immune cell infiltration of islets was examined (B) and scored (C) in H&E stained pancreata collected 23 d after transfer of 2 × 107 OT-I cells. Photomicrographs show representative islets and islet infiltration scored as described in Materials and Methods from four individual mice per group taken from two separate experiments (original magnification ×100). D, Killing of CFSE-labeled OVA257–264-pulsed targets was determined 23 d after transfer of 2 × 107 OT-I cells. Data are from a single experiment with four mice per group.
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 non-hematopoietic cells did not contribute to rapid effector/memory OT-I T-cell 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 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

FIGURE 6. Rapid inactivation of CD8+ effector/memory T cells in MII.OVA mice is mediated by hematopoietic cells. BM chimeras were generated from MII.OVA and H-2Kb−mice (A–C) or MII.OVA and B6.SIL (D) mice. A, BM engraftment was determined by flow cytometry of splenocytes at experiment termination using allospecific mAbs, such that MII.OVA-derived cells stain positive for mAb, and H-2Kb−mice derived cells are single-positive only. Data are representative of two or three mice per group in each of two independent experiments. B, Six to seven weeks after BM transplantation, mice were injected with CD45.1+ OT-I effector/memory cells; 1 wk later, the proportion of OT-I (CD45.1+/CD8+) cells producing IFN-γ in spleens (B) or the total number of IFN-γ–producing OT-I T cells (C) was determined by flow cytometry. D, Chimeras were generated by transferring titrated proportions of MII.OVA and nontransgenic B6.SIL BM to B6.SIL mice. Recipients were injected with CD45.1+ OT-I effector/memory cells; 1 wk later, the proportion of OT-I (CD45.1+/CD8+) cells producing IFN-γ in spleens was determined by flow cytometry. The extent of MII.OVA engraftment was detected using anti-CD45.2 staining at the termination of the experiment. Data (mean ± SD) for all experiments are pooled from three independent experiments using two or three mice per group. E, OT-I OVA or nontransgenic mice were injected daily with FL or PBS, and OT-I effector/memory cells were transferred on the ninth day. Three days later, the total number of CD11c+ cells per spleen (E) and TCR expression on CD45.1+/CD8+ cells were determined by flow cytometry. Data are pooled from (E) or representative of (F) two independent experiments with two mice per group. 1) No significant difference among any group in these sets.
APCs and the level of Ag expression by individual APCs could be shown. Thus, increases in the absolute number of Ag-expressing level of expression restricted to DCs in 11c.OVA mice (data not expression in DCs and B cells of MII.OVA mice but a much lower possibilities exist. TaqMan analyses indicated a high level of OVA more diverse APCs alters memory T cell–APC interactions to in-T cells (17). The mechanisms by which targeting cognate Ags to DCs leads to the inactivation of fully differentiated CD8+ memory and mixed effector/memory CD8+ T cell responses through a mechanism that requires persistent Ag exposure (17). However, target- ing Ags to DCs alone results in a relatively slow process of in-activation 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 capa- ble 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 in-duction 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 re sponsiveness 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 de-leion 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-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 memory OT-I T cell inactivation in MII.OVA recipients. Characterization of the relative roles of these pathways in the contrasting tolerance set-tings 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 con-trolling 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 tar-geted 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 in-duce 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. Alterna-tively, 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 dis-pensable 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. Al-though 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 im-munotherapy of established autoimmune disease. Additionally, alloreactive memory T cells generated through heterologous acti-vation 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 im-munotherapy. 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.

Acknowledgments
We thank Francis Carbone and William Heath (University of Melbourne) and Leonard Harrison, Andreas Strasser, and Philippe Boullet (Walter and Eliza Hall Institute) for providing mice and Ken Shortman (Walter and Eliza Hall Institute) for assistance with mobilizing DCs in vivo. The authors also thank Michelle Kappler for performing animal procedures and Dr. Graham Leggatt and Salvatore Fiorenza for interesting discussions of the work.

Disclosures
The authors have no financial conflicts of interest.

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