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Cognate CD4⁺ Help Elicited by Resting Dendritic Cells Does Not Impair the Induction of Peripheral Tolerance in CD8⁺ T Cells¹

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Peripheral tolerance is required to prevent autoimmune tissue destruction by self-reactive T cells that escape negative selection in the thymus. One mechanism of peripheral tolerance in CD8⁺ T cells is their activation by resting dendritic cells (DC). In contrast, DC can be “licensed” by CD4⁺ T cells to induce cytotoxic function in CD8⁺ T cells. The question that then arises, whether CD4⁺ T cell help could impair peripheral tolerance induction in self-reactive CD8⁺ T cells, has not been addressed. In this study we show that CD4⁺ T cell activation by resting DC results in helper function that transiently promotes the expansion and differentiation of cognate CD8⁺ T cells. However, both the CD4⁺ and CD8⁺ T cell populations ultimately undergo partial deletion and acquire Ag unresponsiveness, disabling their ability to destroy OVA-expressing pancreatic β cells and cause diabetes. Thus, effective peripheral tolerance can be induced by resting DC in the presence of CD4⁺ and CD8⁺ T cells with specificity for the same Ag. *The Journal of Immunology*, 2007, 178: 2094–2103.

The function of T cells is determined by the nature of their encounter with Ag-presenting dendritic cells (DC)³. Under steady-state conditions, the majority of lymphoid tissue DC types exist in a nonactivated or “resting” state (1). Resting DC process exogenous (2, 3) or endogenous (4–6) Ags for presentation on MHC class I and II molecules but have a limited capacity to stimulate T cells (7, 8). Microbial or inflammatory stimuli that signal through TLRs (9, 10) or CD40 (11) transform resting DC into activated DC with the capacity to efficiently stimulate Ag-specific effector T cell function. In the absence of these maturational signals, the encounter between DC and T cells may lead to one or more forms of T cell tolerance (2, 12). Peripheral tolerance induced in this manner would complement central tolerance by inactivating self-reactive T cells that had escaped thymic deletion.

Cognate help provided by CD4⁺ T cells to CD8⁺ T cells serves to enhance the priming of CD8⁺ T cell effector function, prevent tolerance induction, and enhance CD8⁺ memory cell generation (13). Although help may be exerted directly between T cells, the major contribution is via the APC (14). Cognate CD4⁺ T cells

license DC for full activation of CD8⁺ CTL effector function (15–17) through what may be both CD40–CD154-dependent (18–20) and -independent (14) pathways. Activation by cognate CD4⁺ T cells might therefore impede the tolerance-inducing potential of resting DC. Consistent with this notion, the deletion of self-reactive CD8⁺ T cells in response to cross-presented self-Ag is reported to be impaired, and development of autoimmune disease is promoted by cognate CD4⁺ T cell help (21), and tolerance is converted to effector immunity by ligation of CD40 on DC (7). Nevertheless, peripheral tolerance induction in CD8⁺ T cells by resting peripheral DC must occur in the presence of cognate CD4⁺ T cells, because CD4⁺ and CD8⁺ T cells with specificity for the same Ag can evade thymic deletion and escape into the periphery (22, 23).

In this study we aimed to resolve the effect of cognate CD4⁺ T cells on the induction of peripheral CD8⁺ T cell tolerance by resting DC. We first generated a mouse model in which OVA is constitutively expressed as a neo-self-Ag by DC. By adoptively transferring CD4⁺ and CD8⁺ OVA-specific TCR transgenic T cells into these mice, we then determined whether the induction of tolerance in CD8⁺ T cells was altered by “help” from coactivated cognate CD4⁺ T cells. Furthermore, by the addition of OVA expressed as a transgene in pancreatic β cells we could determine whether tolerance induction by resting DC in this context prevented “autoimmune” diabetes.

Materials and Methods

Animals

Mice were bred and maintained at the Walter and Eliza Hall Institute (Parkville, Australia) and the Centre for Immunology and Cancer Research at the University of Queensland (Woolloongabba, Australia). OT-I mice carry a transgenic TCR for the MHC class I-restricted peptide OVA_{257–264} (24) and OT-II mice carry a transgenic TCR for the MHC class II-restricted peptide OVA_{323–339} (25). Rat insulin promoter (RIP)-OVA^{low} transgenic mice (26) were kindly provided by Dr. W. R. Heath (Walter and Eliza Hall Institute, Parkville, Australia). OT-I and OT-II mice were crossed with C57BL/6.SJL Ptpcr^a mice congenic for CD45.1 to generate mice bearing CD45.1⁺ OT-I and OT-II cells, respectively. CD11c.OVA mice were backcrossed to C57BL/6.SJL Ptpcr^a mice to generate CD11c.OVA

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³ Abbreviations used in this paper: DC, dendritic cell; int, intermediate; LN, lymph node; MFI, mean fluorescence intensity; RIP, rat insulin promoter; Treg, regulatory T cell.

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mice congenic for CD45.1. OT-I. Rag1^{-/-} mice were purchased from Animal Resource Centre (Perth, Australia). Mice were sex-matched within experiments; OT-I and OT-II donor mice were used at 6–12 wk of age and recipient mice were used at 8–12 wk of age. Animal studies were approved by the institutional animal ethics committees of the Walter and Eliza Hall Institute of Medical Research and University of Queensland.

Generation and screening of CD11c membrane-bound OVA mice

Membrane-bound OVA (27) was inserted behind the CD11c promoter (28) and the DNA was injected into C57BL/6 oocytes. Transgenic founder mice were screened for functional expression of OVA by testing the ability of bone marrow-derived DC (29) to stimulate the proliferation of OT-I T cells *in vitro*.

Cell preparation and adoptive transfers

For OVA-specific T cell transfers, lymph node (LN) cells were prepared from OT-I and OT-II mice or B6.SJL × OT-I and B6.SJL × OT-II mice. Typically, 41% of LN cells from OT-I and 36% of LN cells from OT-II mice were TCR transgenic based on the expression of Vα2 and CD4 or CD8. OT-I and OT-II cells were labeled with CFSE as described (30) or left unlabeled and adoptively transferred by *i.v.* (lateral tail vein) injection. Unless stated otherwise, OT-I and OT-II cells were mixed and injected together. For cell tracking and ELISPOT analysis experiments, 5 × 10⁶ OT-I and 5 × 10⁶ OT-II cells were coinjected. For *in vivo* CTL assays, 1 × 10⁶ OT-I and 1 × 10⁶ OT-II cells were coinjected. For studies of T cell phenotype after transfer, 10 × 10⁶ OT-I and 10 × 10⁶ OT-II were coinjected. Anti-CD40 mAb (clone FGK-45) was administered *ip* (100 μg on days 0, 3, 6, 9, and 12 after injection) where indicated. Anti-CD154 mAb (MR-1) and isotype control hamster IgG (anti-human bcl-2; clone 6C8) were administered *ip* (250 μg on day 0).

In vitro assays

For ELISPOT and proliferation assays spleens or LN were collected, mechanically disrupted by pressing through 100-μm stainless steel mesh or 70-μm nylon mesh cell strainers (BD Biosciences), washed (PBS with 2.5% FCS), and, for spleen cells, erythrocytes were lysed with hypotonic NH₄Cl/Tris buffer. Cells were resuspended in complete medium (RPMI 1640 supplemented with 1 mM sodium pyruvate and 0.1 mM nonessential amino acids; all from Invitrogen Life Technologies), 50 μM 2-ME (Sigma-Aldrich), and 5% (v/v) FCS (JRH Biosciences). Cells were plated at 5 × 10⁵ cells/well in ELISPOT plates (Millipore Biotec) previously coated with 5 μg/ml capture mAb (clones R4-6A2 or 11B11; BD Biosciences) in PBS at 4°C overnight and blocked with 1% BSA (Calbiochem). OVA peptides (OVA_{257–264} at 0.5 μg/ml and OVA_{323–339} at 10 μg/ml; Auspep) or complete medium were added, plates were incubated for 48 h and washed, and a detection Ab (4 μg/ml; clones XMG1.2 or BVD6-24G2; BD Biosciences) in 1% BSA was added overnight at 4°C. After washing, a streptavidin-HRP complex (DakoCytomation) in 1% BSA was added for 2 h at room temperature, plates were washed again, and bound cytokine was visualized with 3-amino-9-ethylcarbazole (Calbiochem). Spots were counted with an ELISPOT reader (Autoimmun Diagnostika). Proliferation assays were as described for ELISPOT assays but using round-bottom wells. Proliferation was determined as [³H]TdR uptake between 48 and 56 h. Degranulation assays were performed *in vitro* as described (31) using anti-CD107a and anti-CD107b on spleen cells isolated 28 days after the transfer of OT-I and OT-II cells to nontransgenic recipients either left untreated or immunized with OVA/QuilA at the time of transfer and to 11c.OVA recipients either left untreated or administered anti-CD40 (clone FGK-45; 100 μg) on days 0, 3, 6, 9, and 12 after transfer. Results were expressed as the proportion of OT-I (CD45.1⁺/CD8⁺) cells staining for surface CD107a/CD107b or as the change in mean fluorescence intensity (ΔMFI; MFI_{stained} – MFI_{isotype}).

To assess the presentation of OVA-derived peptides by CD11c^{high} or CD11c^{intermediate} (CD11c^{int}) cells, spleens were digested using collagenase/DNase as described previously (29). Erythrocytes were lysed (NH₄Cl and Tris buffer) and the remaining cells were washed and stained with anti-CD11c and anti-B220 mAb. Cells were sorted (FACSDiva; BD Biosciences) to collect the CD11c^{high} (CD11c^{high}/B220⁻) and CD11c^{int} (CD11c^{int}/B220⁺) populations. To measure Ag presentation, [³H]TdR proliferation assays were performed as described above using OT-I LN cells as readouts. In some experiments CD11c⁺ cells were enriched using MACS beads (Miltenyi Biotec) from untreated 11c.OVA mice or nontransgenic and 11c.OVA OT-I and OT-II recipients for use in OT-I [³H]TdR incorporation assays. For the determination of MHC class I or MHC class

II presentation of OVA-derived peptides by DC subsets, DC were prepared and sorted as described (5). Ag presentation was measured using CFSE as described (5) with OT-I or OT-II cells as readouts.

In vivo assays

To determine the *in vivo* responsiveness of OVA-specific T cells, mice were immunized *s.c.* in the base of tail with OVA (100 μg) in CFA (Difco; BD Biosciences). CTL activity *in vivo* was determined as described (32). Briefly, syngeneic spleen cells were either pulsed or not pulsed with OVA_{257–264} (0.02 μg/ml) for 1 h at 37°C, washed, and labeled with 5 μM CFSE or 0.5 μM CFSE, respectively; 10⁷ cells from each population were then injected *i.v.* Three hours later, spleens were collected and single cell suspensions were prepared. After washing, cells were analyzed by flow cytometry (FACScan; BD Biosciences) using propidium iodide dead cell exclusion. Approximately 5,000 total CFSE-labeled events were collected. CTL activity for test mice relative to 11c.OVA mice that did not receive exogenous OVA-specific OT-I or OT-II cells (control mice) was determined by the following formula: percentage of killing = [1 – test (counts^{unpulsed}/counts^{pulsed}) ÷ control (counts^{unpulsed}/counts^{pulsed})] × 100.

Abs and flow cytometric analysis

The mAbs against CD11c (clone N418), CD40 (clone FGK-45), CD3 (clone 145-2C11), B220 (clone RA3-6B2), CD40 (clone FGK-45), CD154 (clone MR-1), and human bcl-2 (clone 6C8) were purified from hybridoma supernatants and used unlabeled or conjugated in house. Streptavidin-fluorochrome conjugates (streptavidin-FITC, streptavidin-PE, and streptavidin-allophycocyanin) were from Caltag Laboratories. Streptavidin-PerCP.Cy5.5 and mAb against CD4 (clone RM4-5), CD8 (clone 53-6.7), CD69 (clone H1.2F3), CD62L (clone MEL-14), CD44 (clone IM7), CD45.1 (clone A20), CD5 (clone 53-7.3), CD25 (clone 7D4), TCR Vα2 (clone B20.1), CD107a (clone 1D4B), and CD107b (clone ABL-93) were purchased from BD Biosciences or BioLegend.

For flow cytometric analysis of OVA-specific T cell frequency and phenotype, spleens and LN were passed through 70-μm nylon mesh cell strainers, washed in PBS with 2.5% FCS, and erythrocytes were lysed in NH₄Cl/Tris buffer as required. Livers and lungs were removed from PBS-perfused mice. Livers were pressed through 100-μm stainless steel mesh and parenchymal cells were sedimented by centrifugation at 75 × *g* for 1 min. Cells in the supernatant were collected, washed in PBS with 2.5% FCS, resuspended in 30% Percoll (Amersham Biosciences) in PBS, underlain with Lympholyte-M (Cedarlane), and centrifuged at 750 × *g* for 20 min. Mononuclear cells were collected from the interface and washed. Lungs were chopped finely and digested individually with collagenase (type II at 1 mg/ml; Worthington) and DNase (1000 U/ml; Roche Diagnostics) in 10 ml of RPMI 1640 with 2% FCS for 1 h at 37°C. The resulting cells were washed twice in PBS with 2.5% FCS, resuspended in RPMI 1640 plus FCS, and overlaid on Lympholyte-M. After centrifugation at 1000 × *g* for 20 min, interface cells were collected and washed. Immunofluorescence staining for flow cytometry was performed as described previously (29). Flow cytometry was performed with FACScan, FACScalibur, or LSR cytometers (BD Biosciences). Analyses were generally on viable cells gated for propidium iodide exclusion. Absolute cell numbers were determined by a bead-based procedure (33) using a defined portion of each tissue.

Induction of diabetes in RIP-OVA^{low} mice

RIP-OVA^{low} mice were crossed with 11c.OVA mice to generate offspring expressing OVA in pancreatic β-cells with or without concurrent OVA expression in DC, and offspring genotype was determined by PCR. OT-I and OT-II LN cells were harvested, pooled, and injected (2 × 10⁶ OT-I and 5 × 10⁶ OT-II) *i.v.* Twenty-eight days after transfer recipients were immunized with OVA/CFA as described and blood glucose was checked weekly (Accu-Chek; Roche).

Statistical analyses

Comparison of means was performed using a Student *t* test (Excel; Microsoft). Multiple groups were compared using one-way ANOVA followed by a Newman-Keuls posttest (GraphPad Prism; GraphPad Software). Kaplan-Meier survival analysis was used to compare diabetes incidence (GraphPad Prism).

Results

CD11c^{high} DC present transgenically expressed Ag

To examine Ag presentation by resting DC under steady-state physiologic conditions, OVA was targeted on the CD11c promoter transgenically to DC. This approach avoids the provision of any

exogenous signals to DC, for example those associated with the ligation of surface receptors by Ab-targeted Ag or contaminants carried with the transferred Ag. To determine whether DC homeostasis was altered in transgenic (11c.OVA) mice expressing DC-targeted OVA and whether a transgenically targeted Ag was presented by DC, we analyzed the phenotype and function of splenic DC. The total number of CD11c^{high} (conventional) DC in spleen did not differ between transgenic mice and littermate controls (Fig. 1A), and no differences were detected in the proportion of major splenic DC subtypes (CD8 α^+ and CD8 α^-) or in their MHC class II expression (Fig. 1, B and C). Similarly, no changes were detected in the proportion of splenic CD11c^{int} cells (which comprise plasmacytoid DC, NK cells, and B cells) between transgenic and nontransgenic mice, indicating that the homeostasis of these cells was also unaltered (not shown). In response to activation signals provided by the ligation of CD40 with the agonistic anti-CD40 mAb FGK-45, splenic DC in 11c.OVA and nontransgenic control mice up-regulated MHC class II and costimulatory molecule expression to similar levels (not shown).

Because the CD11c promoter may drive low levels of transgene expression in CD11c^{int} cells (34) in addition to CD11c^{high} conventional DC, we next tested which cells presented OVA-derived peptides. CD11c^{high} conventional DC and CD11c^{int} cells were sorted from the spleens of 11c.OVA and littermate control mice and their ability to stimulate proliferation of OVA-specific T cells was tested in vitro using CD8⁺ OVA-specific (OT-I) T cells. Only conventional (CD11c^{high}) DC from 11c.OVA mice induced the proliferation of OT-I T cells (Fig. 1D); CD11c^{int} cells from 11c.OVA mice and CD11c^{high} DC from nontransgenic controls did not (Fig. 1D). Splenic DC were then sorted into their major subsets and the presentation of OVA-derived peptides by MHC class I (H-2K^b) or MHC class II (I-A^b) molecules was tested in vitro using CD8⁺ (OT-I) and CD4⁺ (OT-II) OVA-specific T cells, respectively. Although each of the three major splenic DC subsets (CD4⁺/CD8⁻, CD4⁻/CD8⁺, and CD4⁻/CD8⁻) stimulated proliferation of both OT-II and OT-I T cells (Fig. 1, E and F), the CD8⁺/CD4⁻ subset was consistently the most effective. Thus, only conventional (CD11c^{high}) DC present OVA-derived peptides in 11c.OVA mice.

Resting OVA-expressing DC activate OVA-specific CD8⁺ and CD4⁺ T cells

ELISPOT assays performed on OVA/CFA-immunized C57BL/6 mice revealed the frequency of OVA_{323–339} (CD4⁺)- or OVA_{257–264} (CD8⁺)-responsive IFN- γ producing T cells in the spleen to be ~1 per 60,000 cells. In contrast, in similarly immunized 11c.OVA mice, IFN- γ producing CD4⁺ or CD8⁺ OVA-responsive T cells were below the level of detection (<1 per 10⁶ spleen cells; data not shown). This result indicated that the response of adoptively transferred OVA-specific T cells could be determined in 11c.OVA mice in the absence of endogenous T cell responses to OVA.

OVA-specific CD8⁺ (OT-I) and CD4⁺ (OT-II) TCR-transgenic T cells were labeled with CFSE and transferred *i.v.*, alone or in combination, to 11c.OVA or nontransgenic control mice. Whereas OT-I and OT-II T cells recovered from unimmunized nontransgenic mice 3 days after transfer had not proliferated as indicated by CFSE dilution, those recovered from the LN (not shown) and spleens of 11c.OVA mice or from OVA/CFA immunized nontransgenic recipients had undergone extensive proliferation (Fig. 2A). The combined transfer of OT-I and OT-II T cells was not required for activation, as extensive proliferation was also observed when OT-I or OT-II cells were transferred separately (not shown). The presentation of OVA by resting DC led to the rapid accumulation of expanded OT-I and OT-II T cells. In the spleens

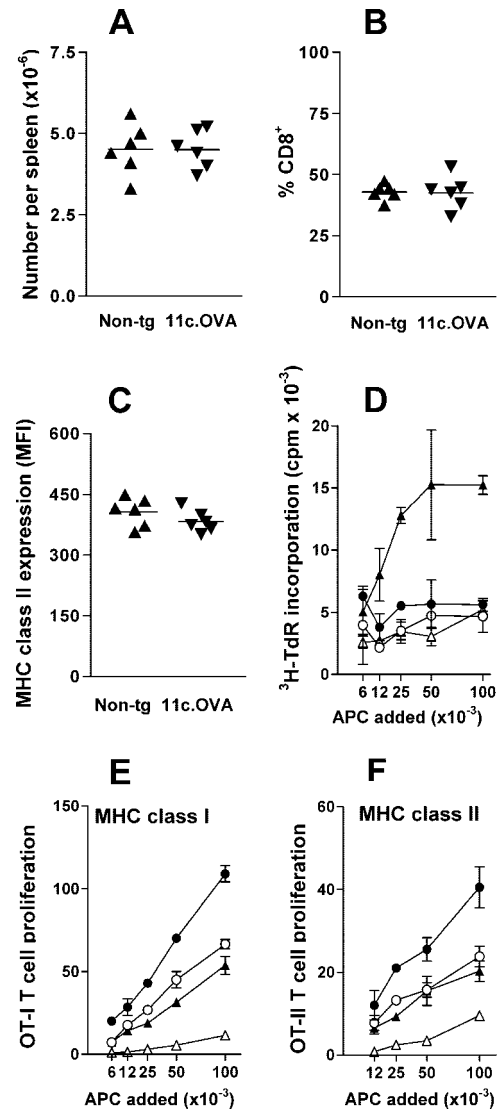


FIGURE 1. The CD11c promoter targets OVA to conventional DC without altering DC homeostasis. A–C, Spleens were harvested from nontransgenic (Non-tg; ▲) or 11c.OVA mice (▼) and digested with collagenase/DNase. Cell suspensions were stained and analyzed by flow cytometry. The total number of CD11c^{high} (conventional) DC (A), the proportion of CD8⁺ DC (B), and the expression of MHC class II (C) were compared. Data are compiled from two experiments. D, CD11c^{high} DC from 11c.OVA (▲) and littermate control (●) or CD11c^{int} cells from 11c.OVA (Δ) or littermate controls (○) were FACS sorted and graded numbers were added to OT-I LN cells. Proliferation was assessed by [³H]TdR incorporation during the last 18 h of 3-day cultures. Similar results were obtained in two separate experiments. E and F, The major splenic DC subsets, CD4⁺CD8⁻ (○), CD4⁻CD8⁺ (▲), and CD4⁻CD8⁻ (●) were FACS-sorted from 11c.OVA mice or nontransgenic controls (CD4⁺CD8⁺ only; Δ) and added to CFSE-labeled OT-II (F) or OT-I (E) T cells. Proliferation was determined by enumerating divided (CFSE^{low}) OVA-specific T cells 2.5 days later as detailed in *Materials and Methods*.

of 11c.OVA mice, OT-I and OT-II T cells were expanded ~40-fold ($p < 0.01$) and 20-fold ($p < 0.001$), respectively, relative to nontransgenic controls three days after transfer (Fig. 2B). The activation of DC *in vivo* through CD40 enhances the generation of effector T cells and prevents or breaks tolerance in several model systems (11, 18–20). The coadministration of agonistic anti-CD40 mAb at the time of OT-I and OT-II transfer increased the number of OT-I and OT-II T cells in the spleen of 11c.OVA mice a further

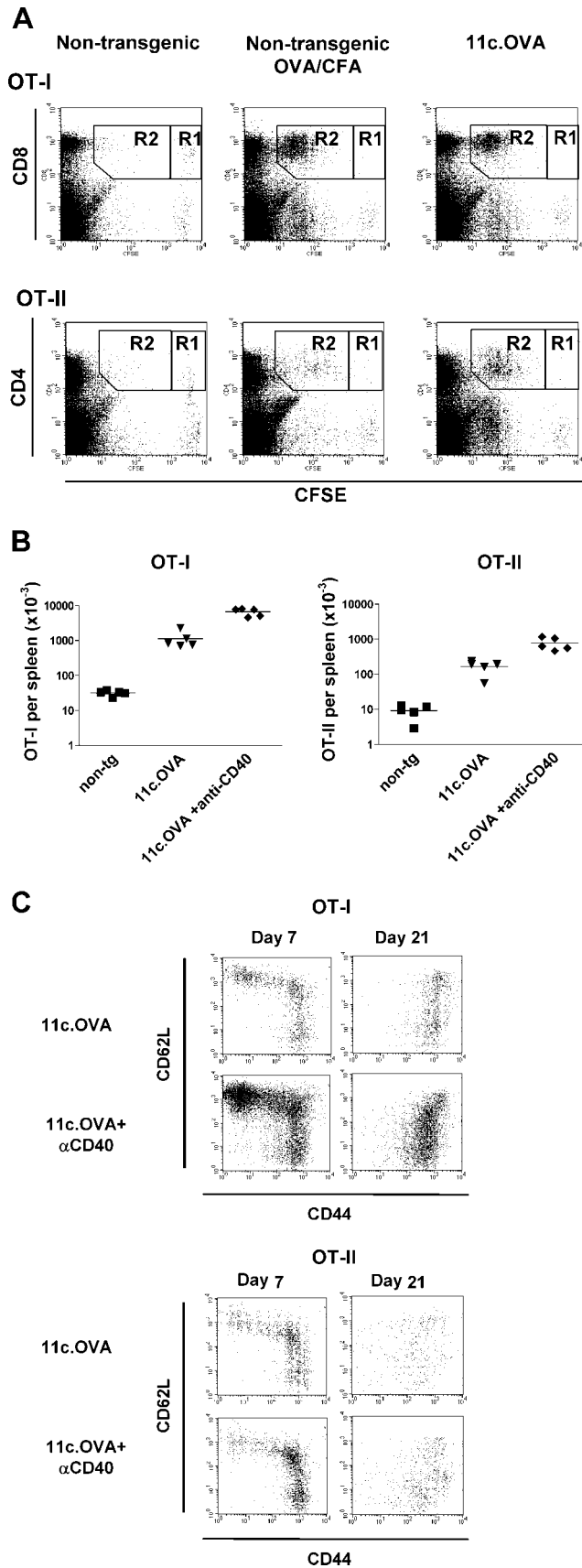


FIGURE 2. OVA-derived peptides are presented by resting 11c.OVA DC in vivo. *A*, Lymph node cells from OT-I and OT-II mice were labeled with CFSE, mixed, and transferred i.v. to 11c.OVA and OVA/CFA immunized or unimmunized nontransgenic control mice. Three days later spleen cell suspensions were stained with anti-CD8 or anti-CD4 mAb and

6-fold ($p < 0.001$) and 4-fold ($p < 0.01$), respectively, over those in untreated mice (Fig. 2*B*).

An analysis of CD44 and CD62L expression showed that OT-I and OT-II T cells in 11c.OVA mice, whether treated with anti-CD40 mAb or not, gradually acquired a CD44^{high} “memory” phenotype (Fig. 2*C*), and this was similar in the presence or absence of transferred OT-II T cells (not shown).

Resting DC elicit cognate CD4⁺ T cell help for concurrently activated CD8⁺ T cells

Cognate CD4⁺ T cell help serves to enhance the priming of CD8⁺ T cell effector function, prevent tolerance induction, and enhance CD8⁺ memory T cell generation (reviewed in Ref. 13). Concurrently activated cognate CD4⁺ T cells could influence CD8⁺ T cell responses in several ways. Therefore, we examined the effect of cotransferred cognate CD4⁺ T cells on the response of CD8⁺ T cells to activation by resting OVA-expressing DC.

Following an initial rapid increase, the total number OT-I T cells in the spleen of 11c.OVA recipients diminished substantially between 3 and 21 days after transfer (Fig. 3*A*) when transferred alone. The cotransfer of cognate OVA-specific CD4⁺ T cells, however, significantly ($p < 0.001$) increased the number of OT-I T cells in the spleens of 11c.OVA recipients 3 days after transfer (Fig. 3*A*). In contrast, by 21 days after transfer similar numbers of OT-I T cells were present in the spleens of 11c.OVA mice regardless of whether cognate OVA-specific CD4⁺ T cells were transferred or not and were ~4-fold more numerous than in nontransgenic recipients (Fig. 3*A*). The number of OT-II T cells in the spleen of 11c.OVA recipients followed a similar course of expansion and contraction, albeit with somewhat slower kinetics, and 21 days after transfer remained ~3-fold higher than in nontransgenic recipients (Fig. 3*A*). Residual transferred OT-I and OT-II T cells were long lived and persisted for at least 3 mo after transfer (not shown). To ensure that residual OT-I–derived T cells did not comprise OVA-unspecific T cells expressing endogenously rearranged TCR chains, OT-I cells from wild-type (CD45.2) donors or Rag1-deficient (CD45.2) donors (which cannot rearrange TCR α and β -chains) were adoptively transferred to CD45.1 congenic 11c.OVA recipients. No difference in the number of residual OT-I T cells was found 21 days after transfer (Fig. 3*B*), indicating that T cells expressing endogenously rearranged TCR chains did not contribute to the residual population of OT-I T cells. At 21 days after transfer the pattern of OT-I and OT-II accumulation in the lung and the liver was similar to that in the spleen (data not shown), indicating that the results obtained in the spleen were representative of those in peripheral nonlymphoid tissue sites to which postactivated T cells preferentially migrate and did not represent selective accumulation of postactivated T cells.

The effect of cognate CD4⁺ T cells on CD8⁺ T cell responses was also assessed by intracellular cytokine staining. Whereas 3 days

analyzed by flow cytometry. *Upper panels*, Transferred OT-I (CD8⁺) and OT-II (CD4⁺) (*lower panels*) T cells that remain undivided (R1) or have divided cells (R2). *B*, Lymph node cells from CD45.1⁺ OT-I and CD45.1⁺ OT-II mice were mixed and transferred i.v. to anti-CD40 mAb-treated or untreated 11c.OVA mice and nontransgenic controls. Three days later spleens were collected and the total number of OT-I (CD45.1⁺ CD8⁺V α 2⁺) or OT-II (CD45.1⁺ CD4⁺V α 2⁺) T cells in the spleen was determined using a counting bead FACS assay. *C*, Lymph node cells from CD45.1⁺ OT-I and CD45.1⁺ OT-II mice were mixed and transferred i.v. to anti-CD40 (α CD40) mAb or untreated 11c.OVA mice. Seven or 21 days later, spleens were harvested and the phenotype of transferred OT-I and OT-II cells (gated as CD45.1⁺CD8⁺ or CD45.1⁺ CD4⁺) was determined.

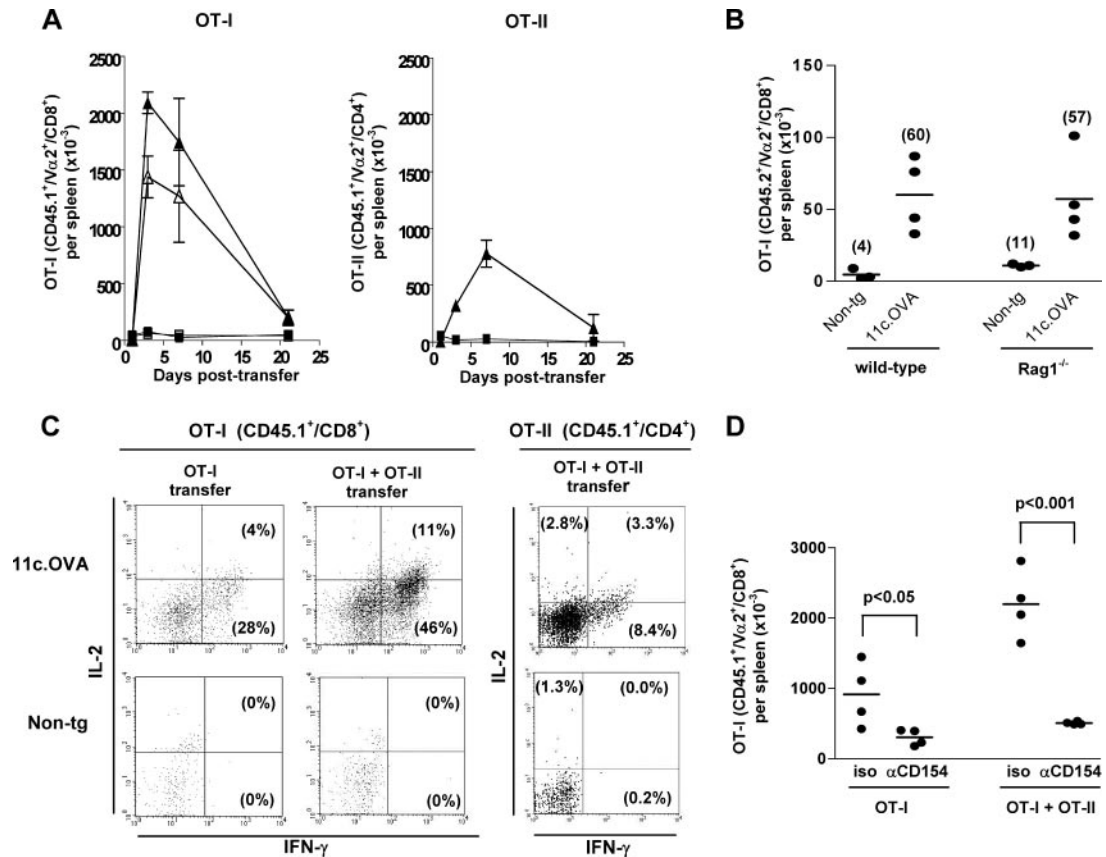


FIGURE 3. Cotransferred OVA-specific CD4⁺ T cells provide help for concurrently activated OVA-specific CD8⁺ T cells. **A**, Lymph node cells from CD45.1⁺ OT-I mice were transferred i.v. to 11c.OVA mice and nontransgenic controls either alone (Δ and \square , respectively) or together with LN cells from CD45.1⁺ OT-II mice (\blacktriangle and \blacksquare , respectively). One, 3, 7, and 21 days later, spleens were collected and the total number of OT-I (CD45.1⁺CD8⁺Vα2⁺) or OT-II (CD45.1⁺CD4⁺Vα2⁺) T cells in spleen was determined using a counting bead FACS assay. **B**, Lymph node cells from wild-type (CD45.2) or RAG1^{-/-} (CD45.2) OT-I mice were transferred i.v. to 11c.OVA mice and nontransgenic (Non-tg) controls (both CD45.1), and 21 days later spleens were collected and the total number of OT-I (CD45.2⁺CD8⁺Vα2⁺) T cells in spleen was determined using a counting bead FACS assay. Mean values for each group are shown in brackets. **C**, Lymph node cells from CD45.1⁺ OT-I alone (OT-I transfer) or together with LN cells from CD45.1⁺ OT-II mice (OT-I+II transfer) were transferred i.v. to 11c.OVA mice and nontransgenic controls. Three days later, spleen cells were recovered and cultured for 3 h with OVA_{257–264} and OVA_{323–339}, stained for intracellular cytokines, and gated on CD45.1⁺CD8⁺ (OT-I) cells (*left panels*) or CD45.1⁺CD4⁺ (OT-II) cells (*right panel*) for analysis. **D**, Lymph node cells from CD45.1⁺ OT-I mice were transferred i.v. alone or together with LN cells from CD45.1⁺ OT-II mice to 11c.OVA mice treated either with isotype control (iso) or anti-CD154 (αCD154) blocking mAb. Three days later, spleens were collected and the total number of OT-I (CD45.1⁺CD8⁺Vα2⁺) T cells in a spleen was determined using a counting bead FACS assay.

after transfer the OT-I T cells recovered from nontransgenic recipients produced no detectable IFN- γ or IL-2 (Fig. 3C), approximately one-quarter of the OT-I T cells recovered from 11c.OVA mice that received OT-I cells alone produced IFN- γ in response to the cognate peptide (Fig. 3C). In contrast, the frequency of OT-I T cells that produced IFN- γ was almost doubled by the cotransfer of OT-II T cells (Fig. 3C). IL-2 production by OT-I T cells was also increased. These data indicated that cognate OVA-specific CD4⁺ T cells activated by resting DC provided help for both expansion and cytokine production by OVA-specific CD8⁺ T cells. Consistent with the provision of helper function, OT-II T cells recovered from 11c.OVA mice, but not from nontransgenic controls, produced IL-2 and IFN- γ in response to OVA peptide stimulation (Fig. 3C). Because signaling provided by CD40-CD154 interaction has been described as a key pathway of CD4⁺ T cell help (reviewed in Ref. 13) and CD154 was transiently expressed on OT-II cells recovered from 11c.OVA mice (not shown), we next sought to determine whether the help for OT-I T cells provided by cotransferred OT-II cells was mediated by this pathway. A blockade of CD40-CD154 interactions completely prevented the increased accumulation of OT-I T cells normally observed in 11c.OVA mice

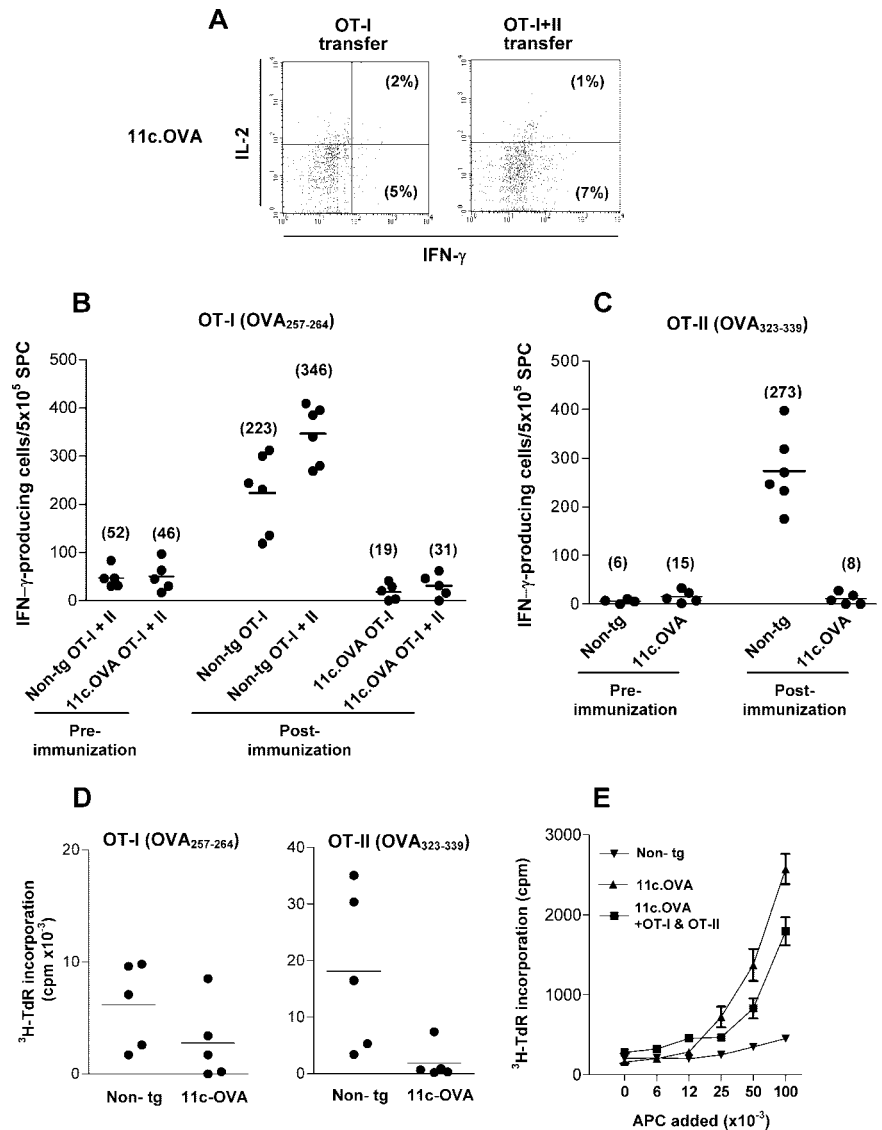
receiving both OT-I and OT-II T cells (Fig. 3D). Likewise, the cognate CD4⁺ T cell-dependent augmentation of IFN- γ production by OT-I T cells was inhibited by the CD40-CD154 blockade (not shown). The administration of anti-CD154 had a small effect on the accumulation of OT-I T cells in 11c.OVA mice, possibly via either a blockade of the direct effects of CD40 ligation on OT-I T cells (35) or a blockade of CD40-dependent OT-I-mediated activation of DC (36).

The help provided to OT-I T cells by cotransferred OT-II T cells was transient, as increased expansion (Fig. 3A) along with increased IFN- γ production by OT-I cells cotransferred with OT-II T cells were not evident by 21 days after transfer (Fig. 4A). This was mirrored by IL-2 and IFN- γ production by OT-II T cells (not shown).

Cognate CD4⁺ T cells do not prevent tolerance induction in CD8⁺ T cells

Partial deletion of the Ag-specific T cell pool following an initial phase of Ag-driven expansion is a normal event in T cell homeostasis and appears to be one component of peripheral tolerance induction. Our data were consistent with the partial deletion of

FIGURE 4. Cognate CD4⁺ T cells do not prevent CD8⁺ T cell tolerance induction by resting DC. *A*, Lymph node cells from CD45.1⁺ OT-I mice were transferred i.v. alone (OT-I transfer) or together with LN cells from CD45.1⁺ OT-II mice (OT-I+II transfer) to 11c.OVA mice. Twenty-one days later, spleen cells were recovered and cultured for 3 h with OVA_{257–264} and OVA_{323–339}, stained for intracellular cytokines, and gated on OT-I (CD45.1⁺CD8⁺) cells for analysis. *B* and *C*, Lymph node cells from OT-I mice were transferred i.v. alone (OT-I) or together with LN cells from OT-II mice (OT-I+II) to 11c.OVA mice and nontransgenic (Non-tg) controls. Twenty-one or 28 days after transfer, spleens were harvested and the frequency of OT-I or OT-II T cells secreting IFN- γ in response to OVA_{257–264} (*B*) or OVA_{323–339} (*C*), respectively, was determined by an ELISPOT assay. Preimmunization data are from mice 21 days after transfer and postimmunization data are from mice at day 28 that were immunized with OVA/CFA at day 21. Mean frequency for each group is shown in brackets. *D*, Lymph node cells from OT-I and OT-II mice were transferred to 11c.OVA mice and nontransgenic controls. After 21 days, recipients were immunized with OVA/CFA and after a further 7 days the proliferative response of splenocytes to OVA_{257–264} (OT-I) or OVA_{323–339} (OT-II) was determined. *E*, CD11c⁺ DC were enriched from spleens of nontransgenic (\blacktriangledown) and 11c.OVA (\blacksquare) mice 21 days after transfer of OT-I and OT-II LN cells or from untreated 11c.OVA mice (\blacktriangle) and added to OT-I LN cells, and proliferation was assessed by [³H]TdR incorporation. Data are representative of two separate experiments.



both CD4⁺ and CD8⁺ OVA-specific T cells following activation by resting OVA-expressing DC, although a substantial number of residual postactivated OVA-specific T cells remained in 11c.OVA mice 21 days after transfer. The limited ability of residual OT-I T cells recovered from 11c.OVA mice to produce the effector cytokine IFN- γ (Fig. 4A) indicated that, rather than acquiring memory status, these cells had been inactivated. To test this possibility, OVA peptide-specific cytokine production was measured by ELISPOT assay. Despite the 3-fold greater number of OVA-specific T cells in spleens of 11c.OVA mice 21 days after transfer, the frequency of OVA-specific CD8⁺ and CD4⁺ T cells producing IFN- γ in response to cognate peptide was similar in 11c.OVA mice and nontransgenic controls whether OT-I cells were transferred together with OT-II T cells (Fig. 4B; preimmunization) or alone (not shown). Similar results were obtained for IL-4 production (data not shown), indicating that despite their postactivated phenotype OT-I and OT-II cells in the spleen of 11c.OVA mice had limited capacity to produce either Th1 or Th2 cytokines.

We next tested the ability of OT-I and OT-II cells in 11c.OVA mice to respond to immunization in vivo. At 21 days after transfer 11c.OVA and nontransgenic control mice that received OT-I cells alone or OT-I and OT-II cells were immunized with OVA/CFA. One week later OVA peptide-specific cytokine production was

measured by ELISPOT assay. Immunization with OVA/CFA significantly increased the frequency of IFN- γ -producing OVA-specific CD8⁺ (Fig. 4B; preimmunization vs postimmunization, $p < 0.001$) and CD4⁺ (Fig. 4C preimmunization vs postimmunization, $p < 0.001$) T cells in nontransgenic recipients only and not in 11c.OVA recipients. The presence of cotransferred OVA-specific CD4⁺ T cells boosted priming in nontransgenic controls (Fig. 4B; postimmunization nontransgenic (non-tg) OT-I vs nontransgenic OT-I plus OT-II, $p < 0.01$), consistent with their ability to provide help, but did not restore responsiveness to immunization in 11c.OVA mice. In a similar fashion, OVA/CFA immunization increased the frequency of IL-4-producing OVA-specific CD8⁺ and CD4⁺ T cells only in nontransgenic recipients, even when both cognate CD8⁺ and CD4⁺ OVA-specific T cells were transferred (data not shown). In addition to priming cytokine production in nontransgenic but not 11c.OVA mice, OVA/CFA immunization expanded OT-I and OT-II T cells in nontransgenic recipients whereas the number of OT-I and OT-II T cells in the spleen of 11c.OVA mice continued to decline despite OVA/CFA immunization (not shown). Splenic OT-I and OT-II T cells recovered from OVA/CFA-immunized 11c.OVA mice exhibited not only impaired production of the effector cytokines IFN- γ and IL-4 in vitro

Table I. CD5 expression is increased on CD8⁺ OVA-specific T cells in 11c.OVA mice^a

	Nontransgenic	11c.OVA	11c.OVA/ Anti-CD40
	167	314 (1.9)	147 (0.9)
	92	392 (4.3)	230 (2.5)
	44	113 (2.6)	36 (0.8)
	70	215 (3.1)	35 (0.5)
MFI (mean ± SD)	93 ± 53	259 ± 121 ^b	92 ± 85
Fold increase ^c (mean ± SD)		3.0 ± 1.0 ^d	1.2 ± 0.9

^a Spleens were harvested from recipients of CD45.1⁺ OT-I and CD45.1⁺ OT-II T cells 21 days after transfer and the expression of CD5 was determined by flow cytometry. Data were collected from histograms gated on CD45.1⁺CD8⁺ (OT-I) cells and pooled from two separate experiments each with two mice per group.

^b Significantly greater than 11c.OVA plus anti-CD40 ($p < 0.01$).

^c Relative to cells transferred to nontransgenic controls.

^d Significantly greater than 11c.OVA plus anti-CD40 ($p < 0.05$).

but also impaired proliferative responses to cognate peptide compared with cells from OVA/CFA-immunized nontransgenic mice (Fig. 4D). Combined, these data indicate that OT-I T cells remaining after partial deletion were rendered unresponsive to Ag stimulation and incapable of exerting effector function regardless of whether the cognate OVA-specific CD4⁺ T cells that provide help had been cotransferred or not.

Residual OVA-specific CD8⁺ T cells in 11c.OVA mice are unresponsive

The expression of CD5, an inhibitory signaling molecule, is a marker of the “anergic” state induced in CD8⁺ T cells exposed to persistent antigenic stimulation (37). We found a significantly higher ($p < 0.01$) level of CD5 expression on OT-I cells in 11c.OVA compared with nontransgenic control mice or in anti-CD40 mAb-treated 11c.OVA mice (containing effector OT-I cells) measured either 7 (not shown) or 21 days after transfer (Table I). A similar trend was seen for OT-II cells, but this was not statistically significant (not shown). The kinetics of the early activation marker CD69 expression were consistent with the gradual acquisition of an Ag nonresponsive state. CD69 was expressed by almost all OT-I and OT-II T cells 3 days after the transfer to 11c.OVA, but by 7 days after the transfer the proportion had fallen by half and by 21 days CD69 was no longer expressed (data not shown). This inability to maintain activation to antigenic stimulation was not due to down-regulation of the TCR, because the surface expression of TCR V α 2 chain remained unchanged (not shown). We found no evidence that CD4⁺CD25⁺ regulatory T cells (Treg) were generated from OT-II cells transferred into 11c.OVA mice (data not shown). The killing of OVA-expressing DC and the loss of OVA presentation was not responsible for the contraction of OVA-specific T cells, because the number of CD11c^{high} DC 21 days after OT-II and OT-I T cell transfer was similar in the spleens of 11c.OVA and nontransgenic recipients (not shown), and CD11c⁺ DC enriched from the spleens of 11c.OVA OT-I and OT-II recipients continued to present OVA-derived peptides (Fig. 4E).

Presentation of “self-Ag” by resting DC prevents “autoimmune” tissue destruction in vivo

Having determined with in vitro surrogates of CD8⁺ T cell effector function that cognate CD4⁺ T cells did not prevent DC-induced CD8⁺ T cell tolerance, we then used in vivo assays to test whether CD8⁺ T cell tolerance occurred in the presence of cognate CD4⁺ T cells. We first used an in vivo CTL assay performed 21 days

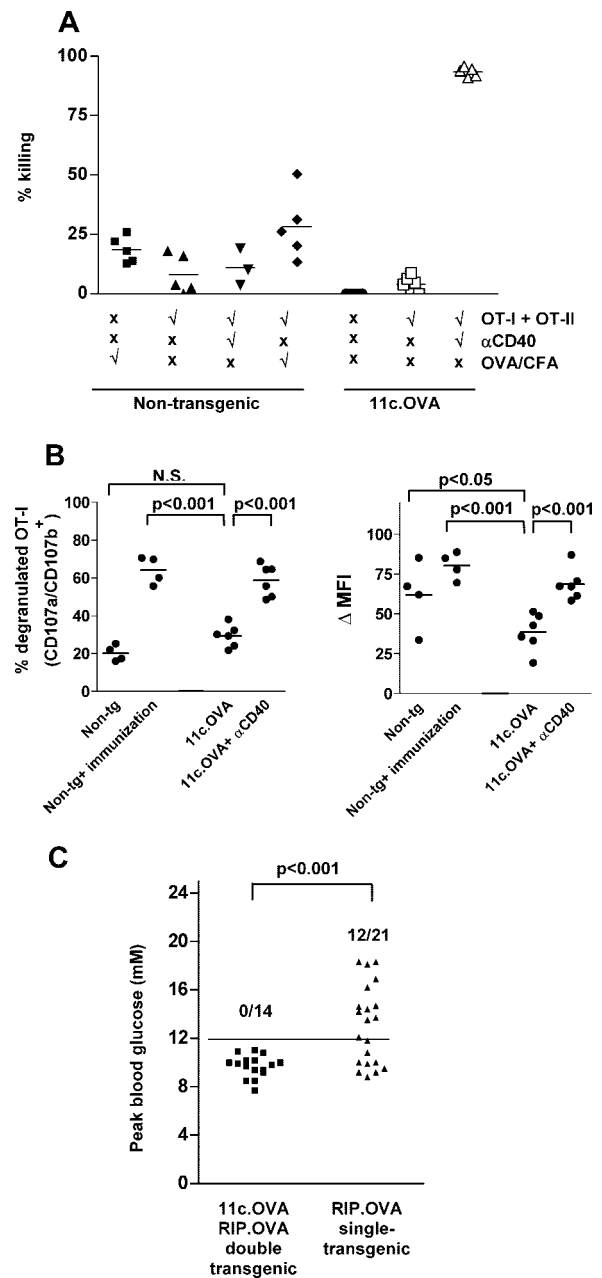


FIGURE 5. Presentation of self-Ag by resting DC prevents autoimmune disease. **A**, Lymph node cells from OT-I and OT-II were mixed and transferred i.v. to nontransgenic control and 11c.OVA mice. Mice were left untreated, immunized with OVA/CFA at the time of T cell transfer, or treated with anti-CD40 (αCD40) mAb. Killing of CFSE-labeled OVA_{257–264} pulsed targets was determined 21 days after T cell transfer. Data are pooled from three separate experiments. **B**, LN cells from OT-I and OT-II were mixed and transferred i.v. to nontransgenic recipients that were left untreated or immunized with OVA/QuilA at the time of transfer and 11c.OVA recipients that were left untreated or administered anti-CD40 (FGK-45; 100 μg) on days 0, 3, 6, 9, and 12 after transfer. Twenty-eight days after transfer, degranulation of OT-I cells in response to stimulation with OVA_{257–264} was measured. **C**, OT-I and OT-II cells were transferred i.v. into offspring of 11c.OVA^{+/-} × RIP-OVA^{low/+} mice. Four weeks after T cell transfer, mice were immunized with OVA/CFA and blood glucose was measured weekly for the next 7 wk. Mice carrying both the RIP-OVA^{low} and 11c.OVA transgene (■) are on the left and mice carrying only the RIP-OVA^{low} transgene (▲) on the right. Shown are peak blood glucose values for individual mice pooled from three experiments, and the proportion of mice in each group in which the peak blood glucose exceeded the threshold (12 mM) for diabetes. Statistical significance was tested using Kaplan-Meier survival analysis.

after the transfer of OT-I and OT-II cells in which OVA_{257–264}-pulsed or unpulsed syngeneic spleen cell targets were injected i.v. and the extent of target killing was determined. OVA/CFA immunization at the time of combined OT-I and OT-II T cell transfer elicited moderate killing of pulsed targets in nontransgenic control mice and was required ($p < 0.05$, compared with unimmunized mice) to induce OVA-specific CTL activity in nontransgenic OT-I and OT-II cell recipients (Fig. 5A). In 11c.OVA mice, no pulsed target killing was observed consistent with an absence of endogenous OVA-specific T cells. Substantial CTL activity was induced by OVA-expressing DC in 11c.OVA mice only when agonistic anti-CD40 mAb was administered (days 0, 3, 6, and 9 after transfer), with almost complete killing of pulsed targets under these conditions (Fig. 5A). This result indicates that persistent exogenous “help” provided by CD40 ligation reverses tolerance. In contrast, the presentation of OVA by resting DC in 11c.OVA mice almost completely abrogated OT-I CTL activity (Fig. 5A), and the CTL activity did not differ from that in nontransgenic recipients in which OT-I T cells remain Ag inexperienced.

Because CTL activity may reflect the number of OVA-specific CTL, as a measure of CTL activity itself we tested OT-I T cell degranulation in response to cognate peptide in vitro. The proportion of OT-I T cells that degranulated in response to OVA_{257–264} stimulation did not differ in 11c.OVA and nontransgenic recipients but was significantly lower than that in immunized nontransgenic recipients or 11c.OVA recipients treated with anti-CD40 to prevent tolerance induction (Fig. 5B). Furthermore, the extent of granule exocytosis, as indicated by CD107a/b staining, was significantly less in OT-I T cells from 11c.OVA mice than from untreated nontransgenic recipients (in which OT-I T cells remain in a naive state) or from 11c.OVA mice treated with anti-CD40 mAb (Fig. 5B). Taken together, the data indicate that in 11c.OVA mice OT-I T cells exert weak CTL activity, similar to that of naive OT-I T cells. We conclude that OT-I CTL activity is reduced in 11c.OVA mice by both deletion and inactivation.

It has been proposed that presentation of self-Ags by resting DC is an important mechanism of self-tolerance that prevents autoimmune disease. To test this hypothesis, we exploited a CD8⁺ T cell-dependent model of OVA-specific “autoimmune” pancreatic β -cell destruction. In this model, OVA is expressed at a low level in pancreatic β cells of transgenic mice (RIP-OVA^{low} mice) and does not normally result in cross-tolerance of OVA-specific CD8⁺ T cells (26). RIP-OVA^{low} mice were crossed with 11c.OVA mice to generate mice expressing OVA either in β cells and DC (double transgenic) or in β cells alone (single transgenic). OT-I and OT-II T cells were transferred i.v. into these mice, which were then immunized with OVA/CFA 4 wk later to prime the remaining OVA-specific T cells and determine their capacity to induce diabetes. Diabetes was triggered by OVA/CFA immunization in slightly $>50\%$ of RIP-OVA^{low} single transgenic mice (Fig. 5C). In marked contrast, 11c.OVA \times RIP-OVA^{low} double transgenic mice expressing OVA in both DC and β cells were completely resistant to the induction of diabetes by OVA/CFA immunization ($p < 0.001$). Thus, the activation of self-reactive CD8⁺ T cells by resting DC in the presence of cognate self-reactive CD4⁺ T cells results in effective tolerance induction and prevention of “autoimmune” disease.

Discussion

Studies of tolerance induction by DC in vivo have used the adoptive transfer of DC purified ex vivo (12), the analysis of DC function after isolation (2), or the use of Abs to target Ag delivery (7, 8). Each of these approaches introduces potential exogenous activation signals to DC. To study the outcome of Ag-presentation by

resting DC in vivo while avoiding such exogenous influences, we genetically targeted Ag expression to DC. This approach was also recently used by Probst et al. (6, 38), who found that the presentation of Ag by resting DC impaired the effector function of CD8⁺ T cells. However, the mechanism(s) of tolerance induction was not described and the effect of cognate CD4⁺ T cells could not be examined. Because CD4⁺ and CD8⁺ T cells specific for the same autoantigen can escape thymic deletion (23) and cognate T cell help has been reported to convert tolerance to immunity (21), it was important to determine whether cognate CD4⁺ T cells prevented tolerance induction by resting DC. Although it may have been predicted, based on previous reports (11, 18–21), that the presence of cognate CD4⁺ T cells would prevent CD8⁺ T cell tolerance induction by DC, this was not the case. We found that the presentation of peptides derived from a model “self-Ag” by resting DC elicited transient effector function in CD4⁺ T cells but did not impair tolerance induction in cognate CD8⁺ T cells concomitantly activated by resting DC. This transient CD4⁺ T cell effector function differs from a recent report that no effector function is elicited in CD4⁺ T cells by DC unless the DC are activated by a microbial stimulus (10). However, in that report CD4⁺ T cell effector function was defined as help for Ab production by B cells and effector cytokine production, and the provision of help to CD8⁺ T cells was not studied.

Tolerance induction occurred in both CD4⁺ and CD8⁺ T cells despite their concurrent activation. It was evident that CD4⁺ and CD8⁺ T cells transiently expressed effector function as observed in other settings (39, 40), but this was insufficient to counteract tolerance induction. It is during this phase that the conditioning of DC by activated CD4⁺ (15, 17) or CD8⁺ (36) T cells could prevent tolerance. Our data show that, in contrast to CD4⁺ T cells activated ex vivo (15), CD4⁺ T cells activated by resting DC and undergoing tolerance induction, while able to provide transient help to CD8⁺ T cells, cannot effectively “license” DC to program full CD8⁺ effector T cell differentiation. In contrast, in accord with previous studies (11) exogenous licensing by anti-CD40 mAb can. It is possible that, when CD4⁺ help was shown to prevent tolerance induction (21), the transient exacerbation of CD8⁺ T cell effector function was sufficient to cause target tissue damage but eventual tolerance induction was not impaired. We cannot, however, rule out a contribution of different DC subsets to the alternate outcomes in response to cross-presented vs endogenously expressed Ag. In our study, Ag was presented by all major DC subsets whereas in cross-tolerance Ag appears to be presented only by CD8⁺ DC (2), which may respond differently to transient CD4⁺ T cell help.

In response to Ag presented by resting DC, CD4⁺ and CD8⁺ T cells underwent transient proliferation and expansion followed by population contraction. The remaining Ag-specific T cells, both CD4⁺ and CD8⁺, were then unresponsive to further Ag challenge and exhibited impaired effector function. This was similar to the course of the T cell response described in some other models of tolerance induction (41, 42). Whereas others have attributed peripheral tolerance induced by DC, particularly for CD8⁺ T cells, primarily to deletion (6, 8, 43), we find a key role for unresponsiveness induced by resting DC in vivo in limiting reactivity to endogenous self-Ags. Furthermore, while contraction of the clonal population is contributory, the acquisition of unresponsiveness is an essential component of peripheral tolerance induced by resting DC not only for CD4⁺ T cells as described previously (7, 44) but, as we show here, also for CD8⁺ T cells. Importantly, while the presence of concurrently activated cognate CD4⁺ T cells provides transient help to CD8⁺ T cells, the induction of Ag-specific CD8⁺

T cell unresponsiveness is not prevented. It is possible that unresponsiveness plays a more substantial role here due to the continuous presentation of Ag constitutively expressed by resting DC, in contrast to the transient presentation that occurs in other models (6–8). This is consistent with the finding that the induction of unresponsiveness at the expense of deletion is promoted by the widespread high-level expression of Ag (45).

The molecular mechanisms leading to the unresponsiveness of T cells activated *in vivo* by resting DC are yet to be defined, but it is likely they will be common to other models of “adaptive” tolerance in which the threshold for TCR-mediated signal transduction is modulated. The lack of T cell activation indicated by the decrease in CD69 expression despite the persistence of a cognate ligand is consistent with this suggestion. Likewise, so is the finding, similar to others (37), that expression of the inhibitory signaling molecule CD5 (46, 47) was increased on residual CD8⁺ T cells in 11c.OVA mice. Further studies will be required to determine the fate of unresponsive T cells in the face of persistent constitutive expression of cognate Ag by resting DC and whether these cells survive for long periods of time as reported in other models (48, 49).

Peripheral DC appear to play an important role in expanding CD4⁺CD25⁺ Treg *in vivo* (50). However, we found no enrichment of CD4⁺CD25⁺ OT-II-derived T cells in tolerant 11c.OVA mice. Our findings and those of others (48) do not support a role for “resting” DC in expanding the CD4⁺CD25⁺ Treg to persistently expressed Ags and are consistent with the idea that more “activated” DC are required to induce effector function in CD4⁺CD25⁺ Treg (51, 52). It is possible that the induction of Ag-specific CD4⁺CD25⁺ Treg *in vivo* after Ab-mediated targeting of Ag to “immature” DC (53) results from exogenous signals provided to the DC by the targeting conjugate. Alternatively, CD4⁺CD25⁺ Treg may be generated only by DC that present low levels of Ag (54). Whether T cells rendered unresponsive through constitutive Ag presentation by “resting” DC possess Treg activity, either through competition for APC (55) or other means (56) as described for “anergic” T cells, is yet to be determined.

The demonstration that peripheral tolerance can be induced concurrently in both CD4⁺ and CD8⁺ T cells has important clinical implications. Both T cell subsets are necessary for the development of most cell-mediated autoimmune diseases, including type 1 diabetes (57, 58), and in a therapeutic setting would need to be concurrently tolerized. The prevention of autoimmune diabetes in a model that is dependent on both CD4⁺ and CD8⁺ T cells (59) reinforces the relevance of these findings and highlights the protective effect of tolerance induction by resting DC.

In summary, we show that resting DC present constitutively expressed Ag *in vivo* and induce peripheral tolerance in both cognate CD4⁺ and CD8⁺ T cells. Cognate CD4⁺ T cell help is therefore not necessarily an impediment to CD8⁺ T cell tolerance induction. In addition to inducing tolerance that prevents autoimmune disease, resting DC could also contribute to the maintenance of pathological unresponsive states, such as in neoplasia (60) in which tumor Ags may be chronically presented by resting DC. Our findings therefore highlight the need to understand not only how to optimize peripheral tolerance induction by resting DC *in vivo* but also how to prevent or break it when necessary.

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Disclosures

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