

## **Immune Tolerance After Delivery of Dying Cells to Dendritic Cells In Situ**

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### **Abstract**

Peripheral immune tolerance is believed to be induced by the processing and presentation of self-tissues that die during physiologic tissue turnover. To examine the mechanism that mediates tolerance, we injected mice with dying syngeneic TAP<sup>-/-</sup> splenocytes loaded with small amounts of the protein antigen, ovalbumin (OVA). After ingestion and presentation of cell-associated OVA by the CD8<sup>+</sup> subset of dendritic cells in situ, large numbers of antigen-reactive, CD8<sup>+</sup> T cell receptor (TCR) transgenic T lymphocytes were driven into cell cycle, but then the T cells were deleted. The animals were also tolerant to challenge with OVA in complete Freund's adjuvant. An agonistic anti-CD40 monoclonal antibody was then administered together with the OVA-loaded splenocytes, so that the dendritic cells in the recipient mice would mature. In contrast to observations made in the steady state, the antigen-reactive T cells expanded in numbers for 1–2 wk and produced large amounts of interleukin 2 and interferon  $\gamma$ , while the animals retained responsiveness to antigen rechallenge. The specific tolerance that develops when dendritic cells process self tissues in the steady state should prevent or reduce the development of autoimmunity when dying cells are subsequently processed during infection.

Key words: dendritic cells • peripheral tolerance • CD8<sup>+</sup> T cells • deletion • DC subset

### **Introduction**

Infection is frequently accompanied by cell death, and dying cells are efficiently processed and presented to T lymphocytes by dendritic cells (DCs; for a review, see reference 1). As a result, during infection there will be simultaneous presentation of self and foreign microbial antigens, creating the potential for autoimmunity (2, 3). The risk of autoimmunity is lessened by central or thymic tolerance. In the thymus, autoreactive T cells are deleted or negatively selected upon encounter of self-antigens, including self-antigens presented by thymic DCs (4, 5). However, central tolerance is incomplete, and autoreactive T cells can escape negative selection (6). In addition, some self-antigens do not access the thymus (7) while others may be expressed later in life, after the T cell repertoire has formed. Therefore, central tolerance needs to be buttressed by peripheral mechanisms (8, 9). It has been proposed that DCs induce peripheral tolerance

by capturing cells that normally die during cell turnover (2, 3, 10, 11). Although it is known that DCs can capture dead cells in vivo (10, 12), it remains to be shown that tolerance ensues as a result of the ingestion of self-tissues.

To determine if antigens expressed in dying cells can induce tolerance in vivo, we loaded a test protein OVA into the cytoplasm of syngeneic cells under hyperosmotic conditions (13). This osmotic shock also induces cell death, and when the treated splenocytes are injected intravenously, they are captured and presented by host CD8<sup>+</sup> DCs (12). By injecting OVA loaded but TAP<sup>-/-</sup> cells, which could not directly present OVA, we aimed to limit the presentation of dying cells to recipient DCs in situ. In addition, we used a polyclonal anti-OVA reagent to quantify the levels of OVA protein within a single dose of the injected TAP<sup>-/-</sup> splenocytes. The amount of OVA was small, <150 ng, confirming the experience of others (14). Nevertheless we will show that, as long as one studies DCs and T cells in vivo in the steady state, without further manipulation, profound systemic tolerance develops after presentation of OVA from dying cells.

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## Materials and Methods

**Mice.** C57BL/6 (CD45.2) and TCR transgenic F5 mice, specific for hemagglutinin peptide presented on H-2K<sup>b</sup>, were from Taconic and TAP<sup>-/-</sup> mice from The Jackson Laboratory. OT-I TCR transgenic mice, specific for OVA peptide (SIINFEKL) in the context of H-2K<sup>b</sup>, were provided by Dr. F. Carbone (University of Melbourne, Parkville, Victoria, Australia). To obtain CD45.1<sup>+</sup> OT-I cells, OT-I mice were crossed to CD45.1<sup>+</sup> B6.SJL-*Ptprca*<sup>t</sup> (Taconic), and F<sub>1</sub> mice tested for expression of V<sub>β5.1/5.2</sub> and CD45.1. Mice were maintained in specific pathogen-free conditions and studied at 6–8 wk according to institutional guidelines.

**Abs and Reagents.** Rat mAbs for MHC class II (TIB120, M5/114.15.2), CD205 (HB290, DEC-205), granulocytes (RB6–8C5, Gr-1), B220 (TIB146, RA3–3A1), F4/80 (HB198), and CD8 (TIB211, 3.155) were from the American Type Culture Collection. FITC-anti-H-2K<sup>b</sup>, I-A<sup>b</sup>, CD25, CD40, CD80, CD86, PE-conjugated anti-CD8α, CD11b, CD11c, CD44, CD45.1, CD62L, CD69, Cy-Chrome<sup>®</sup>CD8, V<sub>β5.1+5.2</sub>, V<sub>α2</sub>, and V<sub>β11</sub>, biotinylated anti-CD119 and IFN-γ, and allophycocyanin (APC)-streptavidin were from BD Biosciences. Anti-CD11c, CD8 microbeads were from Miltenyi Biotec. Sheep anti-rat IgG conjugated to magnetic beads were from Dynal. Other reagents were RPMI 1640 (GIBCO BRL), FCS (GIBCO BRL), CFA (Difco Labs), carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes), ACK buffer (Biosource International), 30% BSA solution (Sigma-Aldrich), and OVA tested to have <20 pg endotoxin/mg protein (Seikagaku Corporation), and for ELISPOT and ELISA assays, ABC kit (Vector Laboratories) and DAB (Dako).

**Cell Preparations.** Spleen DCs were positively selected by anti-CD11c microbeads<sup>®</sup> from collagenase-digested low-density cells (12). CD8<sup>+</sup> OT-I T cells were prepared from meshed cell suspensions of lymph nodes and spleen by depleting B220, CD4, F4/80, MHC class II, and NK1.1-expressing cells using sheep anti-rat IgG Dynabeads<sup>®</sup>. Splenocytes or T cells were suspended at 10<sup>7</sup>/ml in PBS and incubated with CFSE at concentrations of 5 μM for 10 min at 37°C, and washed once with PBS supplemented with 0.1% FCS and twice with PBS. Antigen-loaded cells were prepared by osmotic shock. In brief, 15 × 10<sup>7</sup> splenocytes were washed in RPMI twice, and resuspended in 1 ml of hypertonic medium (0.5 M sucrose, 10% wt/vol polyethylene glycol 1000, and 10 mM Hepes in RPMI 1640, pH 7.2) containing 10 mg/ml OVA, or HEL control protein, for 10 min at 37°C. 13 ml of prewarmed hypotonic medium (40% H<sub>2</sub>O, 60% RPMI 1640) was added, and the cells incubated for an additional 2 min at 37°C. Immediately after the incubation, the cells were spun, washed twice with ice-cold PBS, and injected into mice as a source of dying cells.

**Adoptive Transfer, Administration of Cell-associated Antigen, and Antigenic Challenge In Vivo.** 3 × 10<sup>6</sup> CFSE-labeled transgenic OT-I T cells (15) in PBS were injected intravenously in 0.2 ml. 1 d later, the mice received intravenously 25 × 10<sup>6</sup>/0.1 ml of antigen-loaded cells with or without 100 μg of agonistic anti-CD40 mAb intraperitoneally (FGK 45.5; a gift of Dr. T. Rolink, Basel Institute for Immunology, Basel, Switzerland). T cell activation was then monitored with flow cytometry. To test for tolerance of the injected TCR-transgenic T cells, mice were given 0.3 × 10<sup>6</sup> OT-I T cells 1–2 d before the injection of osmotically shocked splenocytes, followed by a challenge with OVA emulsified in CFA at 10 μg/25 μl into the front footpads 9 d later. Alternatively, mice were given two doses of splenocytes 6 d apart, and challenged 9 d after the second dose.

**Flow Cytometry.** To examine uptake of CFSE-labeled apoptotic cells (12), low-density splenocytes were stained with CD11c-PE and CD8α-Cy-Chrome<sup>™</sup>, and DCs monitored as CD11c<sup>+</sup> cells on a FACSCalibur<sup>™</sup>, analyzed with CELLQuest<sup>™</sup> program (Becton Dickinson) or FlowJo (Tree Star Software). To assess DC maturation, low-density splenocytes were stained with PE-CD11c, CD8-Cy-Chrome<sup>™</sup> in combination with FITC-labeled mAbs against costimulatory molecules (CD40, 80, and 86) or with biotinylated mAbs for CD119 followed by streptavidin-APC. Prior to staining, cells were incubated with 2.4G2 mAb on ice for 15 min to prevent nonspecific binding of mAbs. All procedures were performed using cold PBS containing 2% FCS and 5 mM EDTA. To monitor OT-I cell proliferation and activation, single cell suspensions of spleen or lymph nodes were prepared from mice injected with CFSE-labeled OT-I T cells. The latter were identified with CD8α-Cy-Chrome<sup>™</sup>, biotinylated anti-CD45.1 plus streptavidin-APC in combination with PE-mAbs. For intracellular cytokine staining, lymphocytes were stimulated *in vitro* with 500 ng/ml OVA peptide (SIINFEKL) in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich) at 37°C for 4 h. The cells were first stained with CD8α-Cy-Chrome<sup>™</sup> and biotinylated CD45.1 plus streptavidin-APC, fixed and permeabilized (cytofix/cytoperm buffer from BD Biosciences), and then stained with PE-conjugated anti-IL-2 or IFN-γ mAbs.

**Quantitation of Cell-associated OVA.** Aliquots of OVA-shocked splenocytes were lysed in SDS sample buffer and transferred to PVDF membranes (Immobilon P; Millipore) for staining with HRP-conjugated polyclonal rabbit anti-OVA (Research Diagnostics, Inc.) and ECL visualization (Hyperfilm ECL; Amersham Biotech). Signals were quantified relative to known amounts of OVA protein added to splenic cell lysates.

**Proliferation and ELISPOT Assays.** To assess presentation of the captured antigens, isolated APCs were γ-irradiated at 15 Gy, and mixed in graded dose with 2 × 10<sup>5</sup> TCR-transgenic T cells in 200 μl of RPMI supplemented with 5% FCS, 50 μM 2-mercaptoethanol, 100 μg/ml streptomycin, and 100 U/ml penicillin G (complete medium) in 96-well flat-bottom culture plates. <sup>3</sup>H-TdR was added at 38–48 h. For T cell proliferation and ELISPOT assays of systemic immunity in adoptive transfer recipients, syngeneic splenocytes pulsed for 1 h with 500 ng/ml SIINFEKL peptide were used as APCs after extensive washing with complete medium. Graded doses were then cultured with 2 × 10<sup>5</sup> CD8<sup>+</sup> T cells in 96-well culture plates or ELISPOT plates coated with anti-IFN-γ. Proliferation was determined as <sup>3</sup>H-TdR uptake between 40–50 h. For IFN-γ production, ELISPOT plates were incubated for 36 h and biotinylated anti-IFN-γ-specific mAb for detection was added followed by streptavidin-HRP after extensive washing. Then, the frequency of IFN-γ-producing cells was visualized by adding DAB as a HRP substrate.

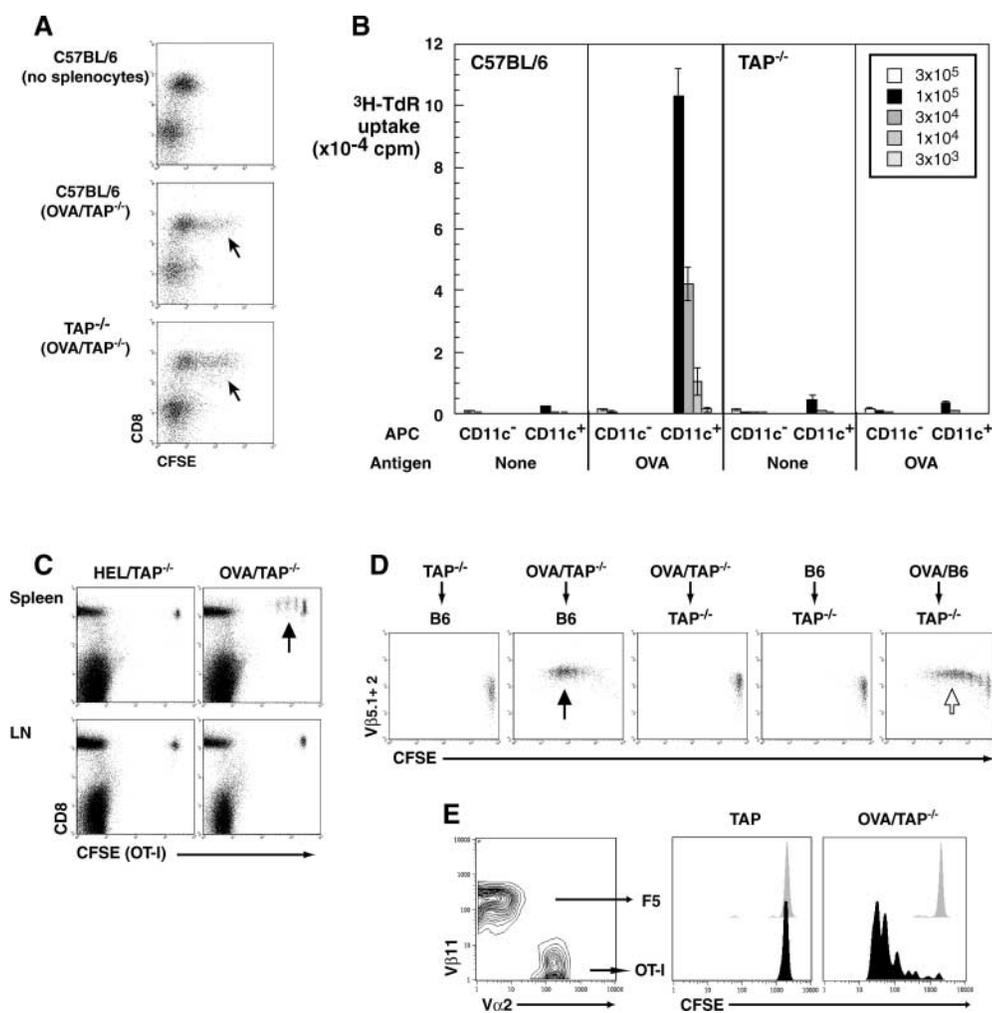
## Results

**Recipient Spleen DCs in the Steady State Present OVA Associated with Injected TAP<sup>-/-</sup> Dying Cells.** To verify that dying TAP<sup>-/-</sup> cells were captured by recipient CD8<sup>+</sup> DCs, the splenocytes were labeled with CFSE and injected into wild-type C57BL/6 and TAP<sup>-/-</sup> mice. DCs from both strains of mice were comparably active in ingesting the dying cells (Fig. 1 A, arrows), but only wild-type DCs were able to present antigen to OVA-specific, OT-I, TCR

transgenic T cells (Fig. 1 B). To monitor antigen presentation *in vivo*, CFSE-labeled OT-I T cells were adoptively transferred into C57BL/6 hosts that were then injected with OVA/TAP<sup>-/-</sup> shocked cells. Cell proliferation began at day 2, where it was restricted to the spleen rather than lymph node (Fig. 1 C, arrow), consistent with the fact that spleen but not lymph node DCs take up injected dying cells (12). The proliferation was much more extensive by day 3 (Fig. 1 D, black arrow). When we injected splenocytes from wild-type rather than TAP<sup>-/-</sup> mice, then direct presentation of OVA was observed even in TAP<sup>-/-</sup> recipients, but this direct presentation was less efficient (Fig. 1 D, white arrow). To restrict presentation of dying cells to the recipient DCs, rather than the injected cells, we always injected OVA/TAP<sup>-/-</sup> shocked cells into wild-type mice. The specificity of OT-I cell proliferation was evident when we injected mixtures of OT-I with F5 TCR transgenic T cells specific for an influenza peptide presented on H-2D<sup>b</sup>. The F5 T cells did not proliferate in mice responding to OVA (Fig. 1 E). Thus, injection of small amounts of OVA protein in TAP<sup>-/-</sup> shocked cells leads to efficient antigen presentation on MHC class I products of recipient DCs.

*An Injection of Dying Cells in the Steady State Does Not Lead to DC Maturation In Vivo.* To determine whether an injection of dying cells induces phenotypic changes in DCs, we examined a number of DC cell surface antigens (Table I). Neither the CD8<sup>+</sup> (that capture the injected dying cells) nor the CD8<sup>-</sup> DC subsets showed alterations in surface molecules known to be up-regulated upon terminal differentiation or maturation of DCs (16), i.e., MHC class II, CD40, 80, and 86. In contrast, each molecule was up-regulated if the injection of osmotically shocked cells was followed by FGK45, an agonistic anti-CD40 antibody (Table I). The response of the CD8<sup>+</sup> DCs to anti-CD40 was comparable whether or not the DCs were exposed to dying cells (data not depicted). Interestingly, the CD119 IFN- $\gamma$  receptor was down-regulated upon DC maturation with anti-CD40 (Table I). Thus, the uptake of dying cells is only accompanied by DC maturation if an additional stimulus like anti-CD40 is also administered.

*Presentation of Dying Cells in the Steady State Leads to CD8<sup>+</sup> T Cell Proliferation, but Anti-CD40 Stimulation Is Required for High Level Cytokine Formation and Homing Receptor Changes.* To assess the early physiologic consequences of presentation of dying cells in the absence or presence of a



**Figure 1.** Spleen DCs in the steady state capture apoptotic cells and present cell-associated OVA to CD8<sup>+</sup> OT-I T cells in a TAP-dependent manner. (A) Uptake (arrows) by the CD8<sup>+</sup> subset of CD11c<sup>+</sup> DCs, 3 h after injection of CFSE-labeled TAP<sup>-/-</sup> splenocytes, loaded with OVA during an osmotic shock. (B) As in A, but CD11c<sup>+</sup> and CD11c<sup>-</sup> fractions of spleen low density cells from B6 or TAP<sup>-/-</sup> were added in graded doses to  $2 \times 10^5$  purified CD8<sup>+</sup> OT-I T cells; T cell proliferation was measured by <sup>3</sup>H-TdR uptake at 40–50 h. (C) Proliferation  $3 \times 10^6$  CFSE-labeled, CD8<sup>+</sup> OT-I T cells (arrow) 2 d after intravenous injection of TAP<sup>-/-</sup> splenocytes loaded with either HEL or OVA protein in spleen and subcutaneous lymph nodes. (D) As in C, but proliferation was monitored 3 d after different splenocytes were injected into the indicated recipients (top labels). (E) In response to OVA-loaded splenocytes, CFSE-labeled, OVA-specific OT-I T cells (V $\alpha$ 2V $\beta$ 5) proliferate at day 3, but not 2F5 influenza-specific T cells (V $\beta$ 11). A–D are representative of at least three experiments and E, two experiments.

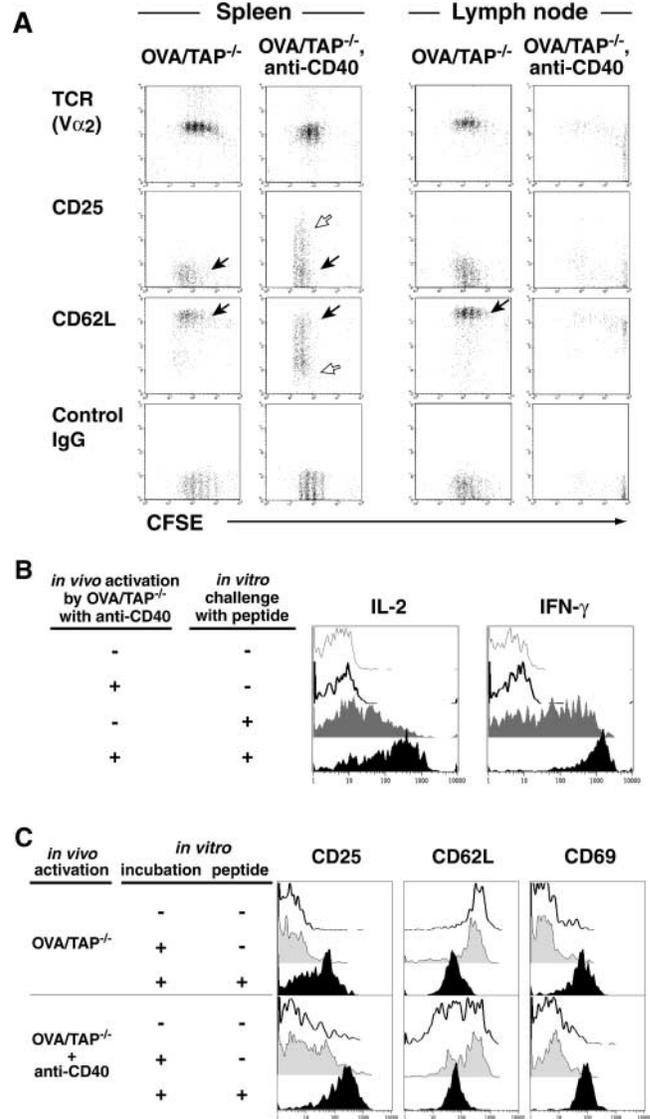
CD40 maturation stimulus, we examined OT-I responses in the spleen 3 d later. In the absence of CD40 stimulation, the proliferating T cells did not up-regulate CD25 IL-2 receptor nor down-regulate CD62L lymph node homing selectin (Fig. 2 A, black arrows). Both CD25 and CD62L changed in expression when OVA-loaded TAP<sup>-/-</sup> splenocytes were coadministered with anti-CD40 (Fig. 2 A, white arrows). Proliferating OT-I T cells were also found in lymph nodes of mice given splenocytes in the absence of anti-CD40, as these T cells could home there via CD62L (right side of Fig. 2 A, black arrow). Consistent with the down-regulation of CD62L, a homing receptor, proliferating OT-I cells were not found in the lymph nodes 3 d after anti-CD40 triggering (Fig. 2 A). Furthermore, T cells that had been stimulated in the presence of the agonistic anti-CD40 mAb in vivo were able to produce much more IFN- $\gamma$  and IL-2 upon brief stimulation with SIINFEKL peptide (Fig. 2 B, compare black and gray profiles). In vitro restimulation with antigen is usually used to establish that T cells have been primed (rather than tolerized as we will show below) by the administration of OVA-loaded splenocytes in vivo. Indeed, the explanted T cells rapidly down-regulated CD62L and up-regulated CD25 when rechallenged with antigen in culture (Fig. 2 C), as occurred when T cells were stimulated in vivo in the presence of the CD40 stimulus (Fig. 2 A). Therefore, restimulation in culture can change the properties of T cells from those observed during responses in vivo in the steady state.

**Table I.** Maturation of DCs by Anti-CD40

	Geometric mean fluorescence intensity							
	CD8 <sup>+</sup> DCs			CD8 <sup>-</sup> DCs				
	TAP <sup>-/-</sup> plus PBS		TAP <sup>-/-</sup> plus anti-CD40		TAP <sup>-/-</sup> plus PBS		TAP <sup>-/-</sup> plus anti-CD40	
FITC-isotype	6	7	7	2	2	3		
FITC CD80	26	25	46	21	21	29		
FITC CD86	17	18	<b>51</b>	6	6	<b>21</b>		
FITC CD25	8	8	10	3	3	4		
FITC CD40	32	35	<b>104</b>	24	25	<b>50</b>		
FITC MHC II	88	98	<b>182</b>	84	93	118		
Biotin-isotype	3	3	3	2	2	3		
Biotin CD119	46	42	<b>7</b>	114	115	<b>43</b>		

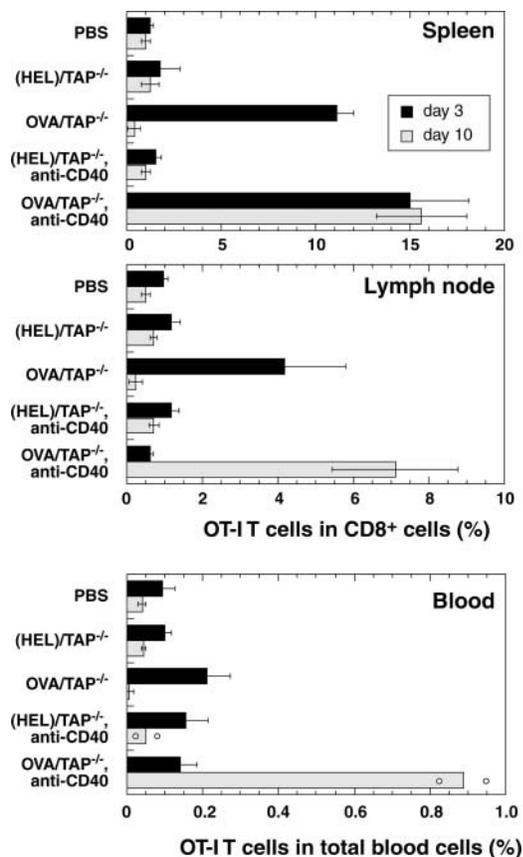
The state of maturation was monitored by surface markers of splenic DC subsets in mice given PBS or osmotically shocked cells 20 h earlier, in the absence or presence of agonistic anti-CD40 antibody. Spleen low density cells were prepared from the mice and stained with CD11c-PE and CD8-Cy-Chrome<sup>TM</sup> followed by either FITC-conjugated mAbs (MHC II, CD25, 40, 80, and 86) or biotinylated CD119 and avidin-APC. Data are geometric mean fluorescence intensity for each mAb from three similar experiments. The data with anti-CD40 (>2-fold changes in bold) were identical in the presence (shown here) or absence (not shown) of injected dying cells.

*Deletion of T Cells Responding to Cell-associated Antigens Presented by DCs in the Steady State.* The fate of the OT-I T cells responding to OVA presented by DCs in vivo changed significantly when mice were examined at 9–14 d. Although T cells initially (days 2 and 3) proliferated actively in response to presentation of dying cells by DCs, deletion was observed at later time points (Fig. 3 A, compare black and gray bars). In contrast, when OVA-loaded



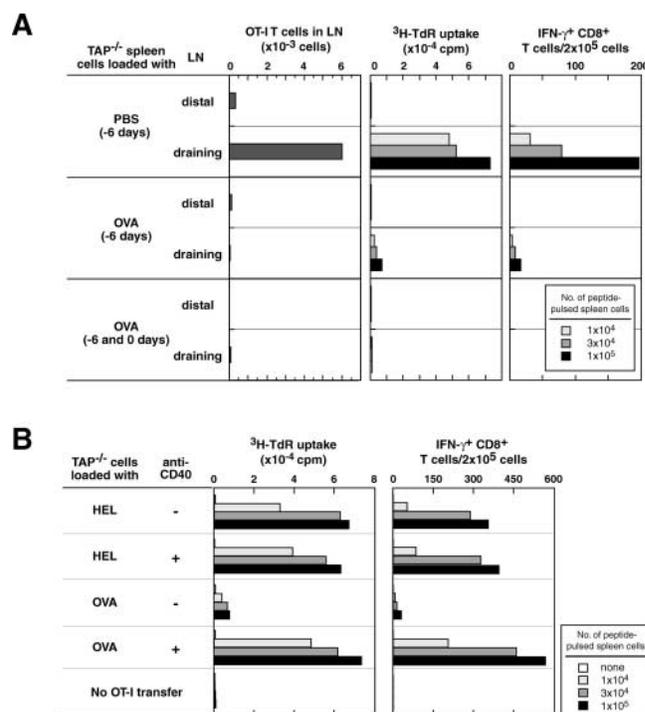
**Figure 2.** The early response, by surface phenotype and cytokine production, of OT-I T cells proliferating in response to dying cells in the absence or presence of anti-CD40 maturation. (A) Proliferation and surface phenotype of  $3 \times 10^6$  CFSE-labeled, CD45.1<sup>+</sup> OT-I T cells 3 d after injection of OVA-loaded TAP<sup>-/-</sup> splenocytes  $\pm$  100  $\mu$ g anti-CD40 mAb intraperitoneally. Black and white arrows represent CD62L<sup>high</sup> and CD62L<sup>low</sup> populations, respectively. (B) As in A, but the day 3 responding splenocytes were restimulated with OVA peptide (SIINFEKL) for 4 h at 37°C with 5  $\mu$ g/ml BFA. The cells were stained for CD8 and CD45.1 as in A, and intracellular IFN- $\gamma$  and IL-2 identified with mAb after fixation and permeabilization. (C) Activation of OT-I cells by tissue culture in the absence or presence of restimulation by SIINFEKL pulsed splenocytes for 4 h. The data shown are representative of three experiments.

TAP<sup>-/-</sup> splenocytes were coadministered with anti-CD40, there was a prolonged expansion of the injected CD45.1 bearing OT-I T cells in spleen, lymph node, and blood (Fig. 3, gray bars). At day 10, the majority of the T cells, expanded by the combination of OVA/TAP<sup>-/-</sup> splenocytes and anti-CD40, were expressing high levels of the CD62L homing receptor, but some cells in the spleen and blood were still CD62L<sup>low</sup> (data not depicted). As a control, the response of OT-I to an injection of TAP<sup>-/-</sup> cells loaded with nonspecific hen egg lysozyme protein (HEL) was monitored. There was no expansion of the OVA-specific OT-I cells relative to the PBS control, even with anti-CD40 maturation, but the OT-I cells persisted 10 d (Fig. 3). As a second control for the specificity of OT-I T cell deletion, we cotransferred F5 T cells specific for an influenza peptide. The cotransferred F5 T cells remained in blood, LN, and spleen while the OT-I T cells were deleted in mice that received TAP/OVA (data not depicted). Therefore, in the absence of maturation stimuli, there is an initial vigorous T cell response to OVA presented by DCs and this is followed by antigen-specific T cell deletion.



**Figure 3.** Contrasting survival of OT-I T cells responding to DCs presenting OVA-loaded splenocytes in the absence or presence of anti-CD40 maturation. As in Fig. 2, the proportion of responding OT-I T cells (CD45.1<sup>+</sup> CD8<sup>+</sup>) was measured at 3 d (black) or 10 d (gray) as a percentage of CD8<sup>+</sup> cells in spleen and subcutaneous lymph nodes (means of 4–5 experiments) or as a proportion of total blood cells (2–3 experiments).

**DC Presentation of Dying Cells Leads to Systemic Unresponsiveness to OVA Challenge with CFA.** To determine if systemic tolerance had been induced by DCs presenting cell-associated antigen in the steady-state, we rechallenge the mice with OVA in CFA. Mice were injected with OVA-charged splenocytes either once (day –6) or twice (day –6 and 0), and 9 d later challenged with OVA in CFA. In mice given TAP<sup>-/-</sup> splenocytes without OVA protein, a strong immune response was evident 3 d after the challenge with OVA in CFA, by three criteria (Fig. 4 A, top row). These were (a) increased OT-I T cell numbers in the lymph nodes draining the site of OVA/CFA injection, (b) strong proliferation to OVA challenge in culture, and (c) the appearance of many IFN- $\gamma$ -secreting cells in ELISPOT assays. In contrast, mice exposed to one or two doses of OVA-bearing splenocytes became unresponsive to challenge with CFA/OVA (Fig. 4 A). This tolerance was antigen specific, as coadministered F5 TCR transgenic cells



**Figure 4.** Tolerance of mice given OVA-loaded TAP<sup>-/-</sup> splenocytes to OVA challenge with CFA. (A) 2 d after injection of  $0.3 \times 10^6$  OT-I T cells, B6 mice were injected with  $25 \times 10^6$  OVA-loaded or unloaded TAP<sup>-/-</sup> splenocytes intravenously. Some mice were given OVA-loaded TAP<sup>-/-</sup> splenocytes once (day –6) and others twice (days –6 and 0). 9 d after the last injection of OVA-loaded TAP<sup>-/-</sup> splenocytes, the mice were challenged with OVA in CFA, and total numbers of CD8<sup>+</sup> CD45.1<sup>+</sup> OT-I cells in draining (brachial) and distal (inguinal) nodes were measured by FACS<sup>®</sup> (left) as in Fig. 3. Enriched CD8<sup>+</sup> T cells using magnetic microbeads<sup>®</sup> also were cocultured with the graded doses (inset) of SIINFEKL-pulsed spleen cells after  $\gamma$ -irradiation at 15 Gy. T cell proliferation was measured by <sup>3</sup>H-TdR uptake at 38–48 h (middle), and IFN- $\gamma$ -secreting cells by ELISPOT at 36 h (right). (B) Immunity develops if OVA-loaded splenocytes are presented along with anti-CD40 agonistic antibody, assessed as in B with ELISPOT (right panel) and T cell proliferation (38–48 h; left panel). The data in A and B are representative of three experiments.

specific for a viral peptide (see Fig. 1 E) were not deleted (data not depicted).

To determine if the systemic tolerance induced by dying cells could be converted to immunity, we compared mice injected with OVA-loaded splenocytes in the absence or presence of anti-CD40 and then challenged with OVA in CFA. If the mice had been exposed to OVA/TAP<sup>-/-</sup> splenocytes in the presence of anti-CD40, they responded vigorously to challenge with antigen in strong adjuvant, i.e., the T cells would proliferate and form IFN- $\gamma$ -producing ELISPOTS (Fig. 4 B). In summary, DC presentation of OVA from dead cells to MHC class I-restricted OT-I cells leads to tolerance in the steady state, but immunity if the DCs are matured with anti-CD40.

## Discussion

In the absence of acute inflammation and infection, most DCs in vivo are functionally immature, capable of capturing antigens but lacking many specializations needed to induce immunity (for a review, see reference 17). Maturation, a term designed to describe DC differentiation that results in immunity (18, 19), takes place under several situations, particularly exposure to microbial pathogens that signal DCs through Toll-like receptors (20). During maturation, DCs produce large amounts of cytokines like IL-12 and up-regulate several B7 and TNF/TNF-R family molecules. However, a frequent consequence of infection is the death of infected cells as well as adjacent self-tissues. Therefore, when DCs are capturing pathogens, they are also capturing and presenting antigens from dying cells and the presentation is particularly efficient. This dilemma, that DCs will efficiently present self-antigens during infection, has given rise to a new proposal for the maintenance of self-tolerance (2, 3) evaluated here. We find that the capture of dying self-cells by DCs in the steady state leads to tolerance, thereby allowing subsequent responses to focus on microbial versus self antigens.

It may seem surprising that dying cells induce tolerance since similar cells have been used previously to study immune priming. However, prior studies differed in two potentially important ways. First, the injected OVA-laden splenocytes contained DCs that rapidly mature upon removal from an animal (21); our use of dying TAP<sup>-/-</sup> splenocytes allowed presentation to take place primarily on recipient nonmaturing DCs in the steady state (Fig. 1, Table I). Second, prior studies documented priming using in vitro restimulation assays performed shortly after the injection of OVA-charged splenocytes. We found that proliferating T cells early on in the peripheral tolerance process could be activated upon removal from the animal (Fig. 2 C), e.g., because of exposure to mature DCs during the culture assay. This in vitro assay could then give a false impression of priming, whereas if the T cells remained in vivo, they eventually would be deleted and the mice would become tolerant.

The induction of tolerance by DCs capturing dying splenocytes is functionally similar to the peripheral tolerance

observed in mice expressing antigens as transgenes on pancreatic islet  $\beta$  cells (22, 23). In these elegant studies, antigens were presented in the draining pancreatic lymph node by "tolerogenic" DCs (11, 24, 25). One of the ways in which this cross presentation could begin would be for DCs to capture  $\beta$  cells dying during normal islet turnover. DCs can capture dying intestinal epithelial cells in the lamina propria (10), while Hugues et al. induced the limited death of  $\beta$  cells with streptozotocin and observed decreased responses to  $\beta$  cells (10). In studies submitted during the course of this work, animals became tolerant when injected with DCs from mice exposed to hapten-modified splenocytes (26). Our findings provide direct evidence that the capture of dying cells by DCs results in antigen-specific deletion of tolerance in situ, and demonstrate that a single injection with low levels of a cell-associated protein, <150 ng/mouse, can delete >10<sup>6</sup> CD8<sup>+</sup> T cells. Likewise, small amounts of protein, when delivered via the DEC-205 receptor to DCs in vivo, result in deletion of CD4<sup>+</sup> T cells (27). In prior studies on the induction of peripheral tolerance, in which antigens were not targeted to DCs, it was necessary to use much larger doses of antigen (~100  $\mu$ g/mouse) and in the form of preprocessed antigenic peptides (28–30). The induction of peripheral tolerance extends prior evidence from the thymus (4, 5) that DCs help to define immunologic self.

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## References

1. Heath, W.R., and F.R. Carbone. 2001. Cross-presentation in viral immunity and self tolerance. *Nat. Rev. Immunol.* 1:126–134.
2. Steinman, R.M., S. Turley, I. Mellman, and K. Inaba. 2000. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J. Exp. Med.* 191:411–416.
3. Steinman, R.M., and M.C. Nussenzweig. 2002. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc. Natl. Acad. Sci. USA.* 99:351–358.
4. Matzinger, P., and S. Guerder. 1989. Does T-cell tolerance require a dedicated antigen-presenting cell? *Nature.* 338:74–76.
5. Zal, T., A. Volkman, and B. Stockinger. 1994. Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen. *J. Exp. Med.* 180:2089–2099.
6. Bouneaud, C., P. Kourilsky, and P. Bousso. 2000. Impact of negative selection on the T cell repertoire reactive to a self-

- peptide: a large fraction of T cell clones escapes clonal detection. *Immunity*. 13:829–840.
7. Lo, D., L.C. Burkly, R.A. Flavell, R.D. Palmiter, and R.L. Brinster. 1989. Tolerance in transgenic mice expressing class II major histocompatibility complex on pancreatic acinar cells. *J. Exp. Med.* 170:87–104.
  8. Stockinger, B. 1999. T lymphocyte tolerance: from thymic deletion to peripheral control mechanisms. *Adv. Immunol.* 71: 229–265.
  9. Walker, L.S.K., and A.K. Abbas. 2002. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat. Rev. Immunol.* 2:11–19.
  10. Huang, F.-P., N. Platt, M. Wykes, J.R. Major, T.J. Powell, C.D. Jenkins, and G.G. MacPherson. 2000. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J. Exp. Med.* 191:435–442.
  11. Hugues, S., E. Mougneau, W. Ferlin, D. Jeske, P. Hofman, D. Homann, L. Beaudoin, C. Schrike, M. Von Herrath, A. Lehuen, and N. Glaichenhaus. 2002. Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic  $\beta$  cells. *Immunity*. 16:169–181.
  12. Iyoda, T., S. Shimoyama, K. Liu, Y. Omatsu, Y. Maeda, K. Takahara, Y. Akiyama, R.M. Steinman, and K. Inaba. 2002. The CD8<sup>+</sup> dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J. Exp. Med.* 195:1289–1302.
  13. Moore, M.W., F.R. Carbone, and M.J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell*. 54:777–785.
  14. Li, M., G.M. Davey, R.M. Sutherland, C. Kurts, A.M. Lew, C. Hirst, F.R. Carbone, and W.R. Heath. 2001. Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin in vivo. *J. Immunol.* 166:6099–6103.
  15. Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell*. 76:17–27.
  16. De Smedt, T., B. Pajak, E. Muraille, L. Lespagnard, E. Heinen, P. De Baetselier, J. Urbain, O. Leo, and M. Moser. 1996. Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo. *J. Exp. Med.* 184:1413–1424.
  17. Rescigno, M., C. Wenzler, D. Delia, C. Mutini, M. Lutz, and P. Ricciardi-Castagnoli. 1997. Dendritic cell maturation is required for initiation of the immune response. *J. Leukoc. Biol.* 61:415–426.
  18. Schuler, G., and R.M. Steinman. 1985. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J. Exp. Med.* 161:526–546.
  19. Inaba, K., G. Schuler, M.D. Witmer, J. Valinsky, B. Atassi, and R.M. Steinman. 1986. The immunologic properties of purified Langerhans cells: distinct requirements for the stimulation of unprimed and sensitized T lymphocytes. *J. Exp. Med.* 164:605–613.
  20. Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* 1:135–145.
  21. Inaba, K., J.P. Metlay, M.T. Crowley, and R.M. Steinman. 1990. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. *J. Exp. Med.* 172:631–640.
  22. Kurts, C., H. Kosaka, F.R. Carbone, J.F.A.P. Miller, and W.R. Heath. 1997. Class I-restricted cross-presentation of exogenous self antigens leads to deletion of autoreactive CD8<sup>+</sup> T cells. *J. Exp. Med.* 186:239–245.
  23. Hernandez, J., S. Aung, W.L. Redmond, and L.A. Sherman. 2001. Phenotypic and functional analysis of CD8<sup>+</sup> T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. *J. Exp. Med.* 194:707–718.
  24. Kurts, C., M. Cannarile, I. Klebba, and T. Brocker. 2001. Dendritic cells are sufficient to cross-present self-antigens to CD8 T cells in vivo. *J. Immunol.* 166:1439–1442.
  25. Belz, G.T., G.M.N. Behrens, C.M. Smith, J.F.A.P. Miller, C. Jones, K. Lejon, C.G. Fathman, S.N. Mueller, K. Shortman, F.R. Carbone, and W.R. Heath. 2002. The CD8 $\alpha$ <sup>+</sup> dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. *J. Exp. Med.* 196:1099–1104.
  26. Ferguson, T.A., J. Herndon, B. Elzey, T.S. Griffith, S. Schoenberger, and D.R. Green. 2002. Uptake of apoptotic antigen-coupled cells by lymphoid dendritic cells and cross-priming of CD8<sup>+</sup> T cells produce active immune unresponsiveness. *J. Immunol.* 168:5589–5595.
  27. Hawiger, D., K. Inaba, Y. Dorsett, K. Guo, K. Mahnke, M. Rivera, J.V. Ravetch, R.M. Steinman, and M.C. Nussenzweig. 2001. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J. Exp. Med.* 194:769–780.
  28. Kearney, E.R., K.A. Pape, D.Y. Loh, and M.K. Jenkins. 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity*. 1:327–339.
  29. Aichele, P., K. Brduscha-Riem, R.M. Zinkernagel, H. Hengartner, and H. Pircher. 1995. T cell priming versus T cell tolerance induced by synthetic peptides. *J. Exp. Med.* 182: 261–266.
  30. Liblau, R.S., R. Tisch, K. Shokat, X.-D. Yang, N. Dumont, C.C. Goodnow, and H.O. McDevitt. 1996. Intravenous injection of soluble antigen induces thymic and peripheral T-cell apoptosis. *Proc. Natl. Acad. Sci. USA.* 93:3031–3036.