

# Efficient Targeting of Protein Antigen to the Dendritic Cell Receptor DEC-205 in the Steady State Leads to Antigen Presentation on Major Histocompatibility Complex Class I Products and Peripheral CD8<sup>+</sup> T Cell Tolerance

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## Abstract

To identify endocytic receptors that allow dendritic cells (DCs) to capture and present antigens on major histocompatibility complex (MHC) class I products *in vivo*, we evaluated DEC-205, which is abundant on DCs in lymphoid tissues. Ovalbumin (OVA) protein, when chemically coupled to monoclonal  $\alpha$ DEC-205 antibody, was presented by CD11c<sup>+</sup> lymph node DCs, but not by CD11c<sup>-</sup> cells, to OVA-specific, CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Receptor-mediated presentation was at least 400 times more efficient than unconjugated OVA and, for MHC class I, the DCs had to express transporter of antigenic peptides (TAP) transporters. When  $\alpha$ DEC-205:OVA was injected subcutaneously, OVA protein was identified over a 4–48 h period in DCs, primarily in the lymph nodes draining the injection site. *In vivo*, the OVA protein was selectively presented by DCs to TCR transgenic CD8<sup>+</sup> cells, again at least 400 times more effectively than soluble OVA and in a TAP-dependent fashion. Targeting of  $\alpha$ DEC-205:OVA to DCs in the steady state initially induced 4–7 cycles of T cell division, but the T cells were then deleted and the mice became specifically unresponsive to rechallenge with OVA in complete Freund's adjuvant. In contrast, simultaneous delivery of a DC maturation stimulus via CD40, together with  $\alpha$ DEC-205:OVA, induced strong immunity. The CD8<sup>+</sup> T cells responding in the presence of agonistic  $\alpha$ CD40 antibody produced large amounts of interleukin 2 and interferon  $\gamma$ , acquired cytolytic function *in vivo*, emigrated in large numbers to the lung, and responded vigorously to OVA rechallenge. Therefore, DEC-205 provides an efficient receptor-based mechanism for DCs to process proteins for MHC class I presentation *in vivo*, leading to tolerance in the steady state and immunity after DC maturation.

Key words: dendritic cells • DEC-205 receptor • tolerance • CD8 T cell • MHC class I

## Introduction

The immune system resists pathogens while remaining tolerant of self-antigens and innocuous proteins in the environment. Immune tolerance can be maintained by both central and peripheral mechanisms. Central tolerance for T cells is established in the thymus, where self-reactive T cells can be deleted (1–3), e.g., after an encounter with antigen

on the surface of thymic medullary dendritic cells (DCs)\* (4–6). Peripheral tolerance mechanisms are required in situations where central tolerance is incomplete and for antigens that do not access the thymus, e.g., environmental proteins typically found at body surfaces like the airway and intestine (for reviews, see references 7–10). DCs in the periphery continuously capture proteins from the airway and intestine (11, 12) and are anatomically positioned to present these antigens to T cells in lymphoid organs (13, 14). A

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\*Abbreviations used in this paper: CFSE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell.

well-studied model involves the presentation of antigens from insulin producing, pancreatic islet  $\beta$  cells on MHC class I and II products of DCs in pancreatic lymph nodes (15–18). However, the experimental induction of peripheral tolerance to proteins presented on MHC class I usually requires the injection of large amounts of preprocessed peptides rather than small amounts of intact proteins (19). Efficient molecular pathways for developing tolerance to environmental and self-proteins presented on MHC class I products remain to be defined.

DCs have a number of receptors for adsorptive uptake of antigens. Some are shared with other cells, such as Fc $\gamma$  receptors (20–23) and the macrophage mannose receptor (24). Other receptors are more DC restricted, e.g., Langerin or CD207 (25), DC-SIGN or CD209 (26, 27), an asialoglycoprotein receptor (28), and BDCA-2 (29), but at this time, these receptors have not been shown to mediate the selective presentation of antigens on the MHC class I and II products of DCs in vivo. In contrast, DEC-205, an endocytic receptor with 10 membrane-external, contiguous C-type lectin domains (30, 31), mediates the efficient processing and presentation of antigens on MHC class II products in vivo (32). DEC-205 is expressed at high levels on DCs in the T cell areas of lymphoid organs (13, 33), and  $\alpha$ DEC-205 antibodies are available that selectively target these DCs in mice (32). Here we chemically couple a protein to  $\alpha$ DEC-205 and demonstrate the capacity of this receptor to deliver endocytosed protein in the absence of infection to the TAP-dependent pathway for MHC class I peptide loading. Small amounts of injected antigen, targeted to DCs by the DEC-205 adsorptive pathway, are able to induce solid peripheral CD8<sup>+</sup> T cell tolerance. These results identify an efficient receptor-based mechanism for DCs to continually capture antigens for presentation on MHC class I products in the steady state and to block the development of CD8<sup>+</sup> T cell reactivity.

## Materials and Methods

**Antibodies.** Antibodies to CD45.1 (A20) and other cell surface markers (CD8 $\alpha$ /53–6.7, CD62L/MEL-14, CD80/16–10A1, CD86/GL1, MHC class II I-A<sup>b</sup>/AF6–120.1) including TCR specificities (V $\beta$ <sub>5.1/5.2</sub>/MR9–4; V $\alpha$ <sub>2</sub>/B20.1) were purchased from BD Biosciences. Hybridomas producing antibodies were obtained from the American Type Culture Collection including DEC-205/CD205 (NLDC-145 HB 290), GL-117 (a nonreactive rat IgG2a isotype match for DEC-205), CD107a/LAMP-1, MHC class II (M5/114, TIB120), F4/80 (HB 198), NK1.1, B220 (RA3–6B2), CD4 (GK1.5, TIB 207), CD8 (53.6.7 TIB 105), and CD11c (N418, HB224). Agonistic  $\alpha$ CD40 FGK45.5 mAb was provided by Dr. T. Rolink (Basel Institute for Immunology, Basel, Switzerland). Magnetic microbeads were from Miltenyi Biotec.

**Mice.** C57BL/6 (B6) mice were obtained from Taconic and TAP<sup>-/-</sup> mice from The Jackson Laboratory. OT-I mice were provided by Dr. F. Carbone (University of Melbourne, Parkville, Victoria, Australia) and OT-II were obtained from the Trudeau Institute. CD45.1<sup>+</sup> OT-I mice were produced by crossing OT-I to B6.SJL-Ptprc mice (The Jackson Laboratory).

**Conjugation of OVA to Monoclonal Antibodies.** Purified IgGs were conjugated to maleimide activated OVA (Pierce Chemical Co.) or LPS-free OVA (Seikagaku Corporation) activated with Sulfo-SMCC (sulfo-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate; Pierce Chemical Co.) using the manufacturer's protocol. Antibody:OVA conjugates prepared with OVA from both sources gave similar results in terms of DC targeting and T cell responses in vivo. Although it is difficult to exclude some contamination with LPS in our preparations, the DCs in mice injected with antibody conjugates did not show evidence for maturation, as occurs when LPS is administered to mice. For coupling, the antibodies were reduced using 2-mercapto-ethanolamine:HCl and separated from the reducing agent over a desalting column. Then maleimide-activated OVA was mixed with the reduced antibodies overnight at 4°C. The antibody:OVA conjugates were passed over a protein G column to remove unconjugated OVA, concentrated by spin columns (Millipore) and evaluated by spectrophotometry and SDS-PAGE.

**Isolation of DCs and Antigen-specific T Cells.** Popliteal, inguinal, axillary, and brachial LNs were removed from adult female B6 or TAP<sup>-/-</sup> mice. Single cell suspensions were prepared with 400 U/ml collagenase for 25 min (Roche). The cells were incubated with anti-mouse CD11c or CD19 MACS<sup>®</sup> microbeads for 30 min on ice. CD11c<sup>+</sup> (DC-enriched), CD19<sup>+</sup> (B cell-enriched), and double negative cells were separated by application of a magnetic field and dispensed into 48-well plates at  $5 \times 10^5$  cells/well. Cells were cultured with antigen overnight (16–20 h) at 37°C and washed twice in PBS before use as antigen presenting cells for naive TCR transgenic, OVA-specific T cells. Bone marrow DCs were prepared with GM-CSF as described previously (34). 6 d after bone marrow harvest, the DCs were dislodged, washed, and pulsed with antibody:OVA conjugate (10  $\mu$ g/ml) for 6 h before coculture with T cells. CD8<sup>+</sup> or CD4<sup>+</sup> T cells were prepared from OT-I or OT-II mice, respectively. OT-I and OT-II T cells were purified from single cell suspensions of lymph node or spleen cells by negative selection using hybridoma supernatants (above) directed against MHC-II, F4/80, B220, NK 1.1, and CD4 or CD8 and goat anti-rat Dynabeads<sup>®</sup> (Dyna) at a ratio of 4 beads to 1 target cell. Culture medium was RPMI, 7% FBS, 100 U/ml penicillin streptomycin mixture, 0.25 mg/ml fungizone, 10 mM HEPES, and 55  $\mu$ M  $\beta$ -mercaptoethanol. To identify OT-I cells in the lung, single cell suspensions were prepared by flushing the vascular system of the lung with 10 ml PBS and 10 U/ml heparin (Elkins-Sinn) through the right ventricle and dissociating the tissue in a 70  $\mu$ m Cell Strainer<sup>®</sup> (Becton Dickinson).

**In Vivo Capture of Antibody:OVA Conjugates.** B6 or TAP<sup>-/-</sup> mice were injected with graded doses of IgG:OVA conjugates or soluble OVA in the paws subcutaneously. Lymph nodes (popliteal, inguinal, axillary, and brachial) were harvested 1–4 d later and the DCs isolated (as above) to test for antigen presentation to OT-I cells in vitro.  $10^5$  T cells were added to graded doses of CD11c<sup>+</sup> DCs, CD11c<sup>-</sup> nonDCs, or CD11c<sup>-</sup>CD5<sup>-</sup> B cells in round bottom 96-well plates. [<sup>3</sup>H]thymidine (1  $\mu$ Ci; Amersham Biosciences) was added at 48–72 h to detect incorporation into DNA. Data shown are means of triplicates where the standard deviation was <10% of the mean cpm. To detect OVA, CD11c<sup>+</sup> or CD11c<sup>-</sup> cells were washed, lysed in SDS sample buffer for SDS-PAGE, and then aliquots corresponding to  $10^6$  cells transferred to PVDF membranes (Hybond-P; Amersham Biosciences). For immunoblotting, we used HRP-conjugated polyclonal rabbit anti-OVA (Research Diagnostics, Inc.)

diluted in Roti-Block® (Carl Roth) visualized by ECL® (Amersham Biosciences).

**Flow Cytometry.** Multicolor flow cytometry was used to monitor three functional responses as described in the following sections: proliferation of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled T cells as assessed by progressive halving of the amount of fluorescence per cell; maturation of DCs as assessed by up-regulation of surface antigens like CD80, CD86, and MHC II; and induction of T cell effector function, i.e., accumulation of intracellular cytokines and expression of CTL activity. We used a FACSCalibur™ (BD Biosciences) with subsequent analysis of data in CELLQuest™ (BD Biosciences) or FlowJo® (Tree Star).

**Labeling of T Cells with CFSE for In Vivo T Cell Proliferative Responses.** OT-I T cells at  $10^7$  cells/ml were incubated with CFSE (Molecular Probes; 5  $\mu$ M) for 10 min at 37°C. An equal volume of FCS was added, and the cells washed two times with PBS/0.1% BSA and once with PBS.  $2 \times 10^6$  labeled OT-I T cells were injected intravenously into B6 recipients. 24 h later, antibody:OVA conjugates were injected into all 4 paws with or without agonistic  $\alpha$ CD40 antibody, 100  $\mu$ g subcutaneously. After 3 d, lymph node suspensions were stained for V $\beta_{5.1/5.2}$ , V $\alpha_2$ , and CD8 and evaluated by multicolor flow cytometry.

**In Vivo DC Maturation.** 100  $\mu$ g of  $\alpha$ CD40 was injected subcutaneously into B6 mice that had or had not received antibody:OVA conjugates and OT-I T cells. 1–3 d later, lymph node CD11c<sup>+</sup> cells were isolated by staining with PE-conjugated  $\alpha$ CD11c and separation with anti-PE microbeads and MACS®. CD11c<sup>+</sup> cells were stained for CD80, CD86, or I-A<sup>b</sup> (MHC II) and evaluated by flow cytometry. In some experiments, the CD11c<sup>+</sup> cells were double labeled with biotin- $\alpha$ DEC-205 to follow DEC-205 high and low expressing subsets of DCs.

**In Vivo T Cell Effector Responses.**  $10^6$  CD45.1<sup>+</sup> OT-I T cells were purified (above) and injected intravenously into B6 mice. 24 h later, antibody:OVA conjugates with or without  $\alpha$ CD40 were injected. 3 and 12 d later, lymph node suspensions were stained for surface CD45.1, V $\beta_{5.1/5.2}$ , CD62L, intracellular IL-2 (JES6–5H4), or IFN- $\gamma$  (XMG1.2).  $5 \times 10^6$  lymph node cells were pulsed in 24-well dishes with the OT-I cognate peptide (SIINFELK) for 5 h in the presence of brefeldin A (Sigma-Aldrich). Cells were then harvested, washed twice with PBS/2% FBS, and stained for extracellular CD45.1 and V $\beta_{5.1/5.2}$ . These cells were then fixed and stained for cytokines with the BD Biosciences Intracellular Cytokine Staining Starter Kit as per the manufacturer's protocol.

In vivo CTL assays were performed as described previously (35) by injecting 1:1 mixtures of peptide-pulsed and unpulsed syngeneic splenocytes ( $3 \times 10^6$  each) labeled with 5  $\mu$ M (CFSE<sup>hi</sup>) and 0.5  $\mu$ M (CFSE<sup>lo</sup>) CFSE, respectively, as described above. 12 h later, single cell suspensions from lymph nodes were evaluated by flow cytometry. Specific killing was evaluated by the reduction of the CFSE<sup>hi</sup> population without any reduction of the CFSE<sup>lo</sup> population relative to control mice lacking OT-I T cells.

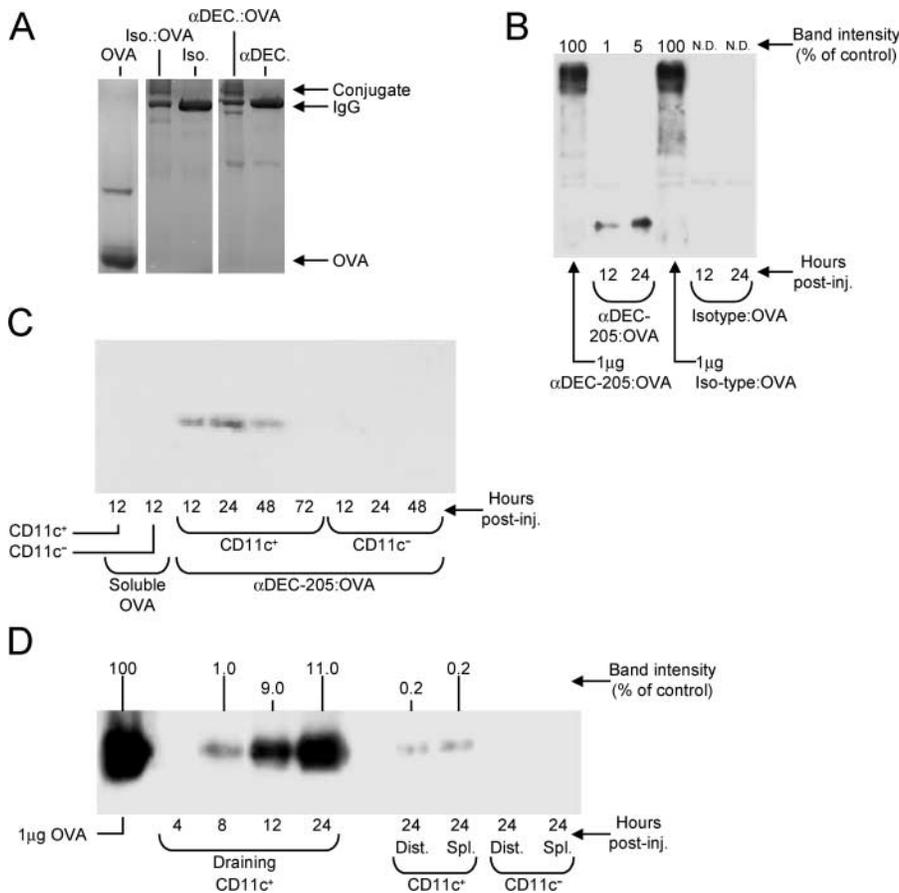
**Tests for Immune Tolerance.** Mice were given OT-I T cells and IgG:OVA conjugates with or without  $\alpha$ CD40 (see above). 12 d after antigen injection, mice were boosted with OVA protein (50  $\mu$ g subcutaneously; Calbiochem) suspended in complete Freund's adjuvant (Difco). 3 d later, mice were killed and lymph nodes harvested for either in vitro restimulation and intracellular IL-2 and IFN- $\gamma$  staining, or in vivo CTL assays (above).

## Results

**Targeting Protein to DCs In Situ via DEC-205.** To examine the potential role of the DEC-205 endocytic receptor to process proteins through the MHC class I pathway, we first chemically coupled whole OVA protein to  $\alpha$ DEC-205 antibody. OVA is known to be presented on the H-2K<sup>b</sup> MHC class I molecule to CD8<sup>+</sup> T cells, including TCR transgenic OT-I T cells (36). After OVA coupling to rat anti-mouse DEC-205 antibody, free OVA was removed by purifying the conjugate on protein G columns, yielding mixtures of  $\sim$ 1:1 IgG:OVA conjugates (MW $\sim$ 200 kD) and unconjugated antibody (Fig. 1 A). Based on the observed conjugation efficiency and the 4:1 mass ratio of IgG to OVA, we estimated that <10% of the conjugate consisted of OVA protein.

To verify that the  $\alpha$ DEC-205:OVA targeted DCs in mice, we injected 1  $\mu$ g of the conjugates subcutaneously and probed for OVA protein in isolated CD11c<sup>+</sup> DC-enriched (14, 37) and CD11c<sup>-</sup> DC-depleted, lymph node cells. Prior work had demonstrated the uptake of rat  $\alpha$ DEC-205 antibody by most DCs (32), but here we followed the capture of the OVA protein. Uptake by DCs plateaued in the draining node within 12–24 h of injection (Fig. 1, B–D). The 45 kD size of the immunoreactive OVA in the DCs was similar to native OVA, suggesting that most of the protein had been released from its conjugation to the injected antibody (Fig. 1 B). Injection of 10  $\mu$ g soluble OVA subcutaneously did not yield detectable antigen in the lymph nodes draining the site of injection (Fig. 1 C, left lane). With the  $\alpha$ DEC-205:OVA conjugate, OVA was only found in the CD11c<sup>+</sup> DC-enriched fraction and not the CD11c<sup>-</sup> DC-depleted cells (Fig. 1 C). Smaller amounts of OVA were also detected in DCs in distal sites like lymph nodes and spleen (Fig. 1 D). The amount of captured OVA corresponded to  $\sim$ 10 ng of protein per  $10^6$  DCs or  $10^5$  molecules/DC (Fig. 1 B). We conclude that protein coupled to  $\alpha$ DEC-205 antibody is efficiently delivered to DCs in situ.

**Isolated Lymph Node DCs Present  $\alpha$ DEC-205:OVA Conjugates on MHC Class I via TAPs.** To determine whether antigen delivered by  $\alpha$ DEC-205 to DCs can be processed for presentation by MHC class I, we first isolated DCs from lymph nodes and treated them with  $\alpha$ DEC-205:OVA in vitro. The antibody:OVA conjugates were incubated with CD11c<sup>+</sup> and CD11c<sup>-</sup> lymph node cells overnight, excess conjugate was removed by washing, and then the cells were cocultured for 2 d with OVA-specific TCR transgenic T cells (CD8<sup>+</sup> MHC class I-restricted OT-I or CD4<sup>+</sup> MHC class II-restricted OT-II T cells). We found that OVA was presented by CD11c<sup>+</sup> DCs on both MHC class I and class II products (Fig. 2 A). DCs exposed to 0.1  $\mu$ g/ml of  $\alpha$ DEC-205:OVA (with <10 ng/ml OVA protein) were more active in presenting to MHC class I restricted CD8<sup>+</sup> OT-I T cells than DCs that had been exposed to 30  $\mu$ g/ml of soluble OVA (Fig. 2 A). Uptake via DEC-205 increased the efficiency of presentation relative to unconjugated OVA at least 1,000-fold for MHC I and



**Figure 1.** Conjugation and delivery of OVA via monoclonal antibodies. (A) OVA conjugated and unconjugated IgGs resolved by SDS-PAGE and stained with Coomassie blue. (B) Immunoblotting with anti-OVA antibody to detect OVA in the Ig:OVA conjugates and in CD11c<sup>+</sup> lymph node DCs (10<sup>6</sup>/lane), the latter at 12 and 24 h after subcutaneous injection of 1 μg of αDEC-205:OVA or isotype control:OVA. (C) The subcutaneous injection of 1 μg of αDEC-205:OVA per footpad, but not 10 μg of soluble OVA, targets CD11c<sup>+</sup> lymph node DCs. 10<sup>6</sup> CD11c<sup>+</sup> cells from the draining nodes per lane were immunoblotted 12–72 h after injection. (D) αDEC-205:OVA uptake occurs primarily in CD11c<sup>+</sup> DCs in the draining lymph nodes. As in C, but cell fractions were prepared from draining lymph nodes, distal (Dist.) nodes and spleen (Spl.).

300-fold for MHC II (Fig. 2 A). In contrast, neither CD19<sup>+</sup> B cells nor APCs depleted of CD11c<sup>+</sup> and CD19<sup>+</sup> cells were able to stimulate T cell proliferation above background levels, even at cell doses 30-fold higher than those required to observe presentation by DCs (Fig. 2 B). Isotype control IgG:OVA conjugates were much less active than αDEC-205:OVA conjugates (Fig. 2 B).

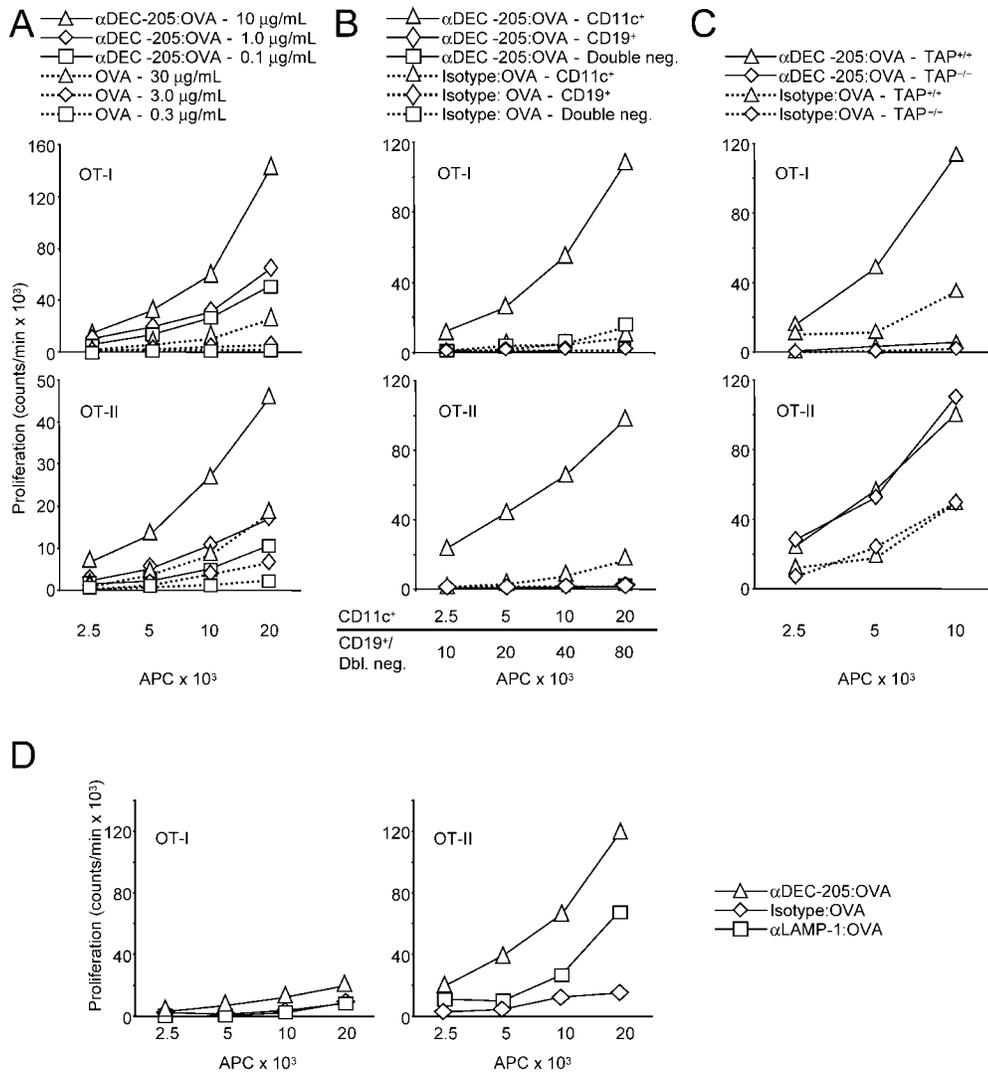
Several additional controls (data not depicted) were performed to verify the function of the DEC-205 receptor in the presentation of protein conjugated to the corresponding monoclonal antibody. Because DCs can utilize Fcγ receptors to present immune complexes on MHC class I products (20, 21), we showed that blocking these receptors (at least CD16 and CD32) with 2.4G2 αFcγR monoclonal antibody (38) did not reduce presentation of αDEC-205:OVA. To evaluate another antibody:OVA conjugate that targets the endocytic system, we used LAMP-1, a lysosome-associated membrane protein expressed in DCs and other cells. The αLAMP-1:OVA conjugate was only comparable to isotype control antibody:OVA in bringing about the presentation of OVA peptides in the context of either MHC-I or -II by CD11c<sup>+</sup> DCs. Conjugation was essential for efficient OVA targeting since mixtures of unconjugated αDEC-205 and soluble OVA were only presented with a comparable efficiency to equivalent doses of soluble OVA, ruling out the possibility that αDEC-205 was simply enhancing presentation of nonconjugated OVA. In summary, the DEC-205 re-

ceptor mediates efficient presentation of protein antigens on both MHC class I and II products of lymph node DCs.

To establish that MHC class I presentation by αDEC-205:OVA was TAP dependent, we performed similar experiments with DCs obtained from TAP-deficient mice (TAP<sup>-/-</sup>; Fig. 2 C). In the absence of TAP, proteasome-processed peptides in DCs fail to move into the endoplasmic reticulum for association with MHC I molecules. We found that TAP<sup>-/-</sup> DCs were unable to present OVA on MHC class I after uptake of αDEC-205:OVA, but the same cells presented OVA on MHC class II (Fig. 2 C). Also, the TAP<sup>-/-</sup> DCs could present preprocessed OVA peptide to MHC class I restricted OT-I T cells (data not depicted). Thus, antigens endocytosed via DEC-205 are routed to the MHC class I processing machinery by a pathway that requires transport of peptides from the cytoplasm to the endoplasmic reticulum.

In contrast to DCs obtained from the lymph node, DEC-205 expressing DCs generated from bone marrow progenitors with GM-CSF were only able to present the αDEC-205:OVA conjugates to OVA-specific CD4<sup>+</sup> OT-II T cells but not CD8<sup>+</sup> OT-I T cells (Fig. 2 D). Therefore, the type of DC influences the capacity of DEC-205 to deliver antigens for TAP dependent MHC class I presentation.

*DEC-205-mediated MHC Class I Presentation In Vivo.* To determine if DCs targeted with αDEC-205:OVA also present OVA peptides on MHC class I in vivo, we next

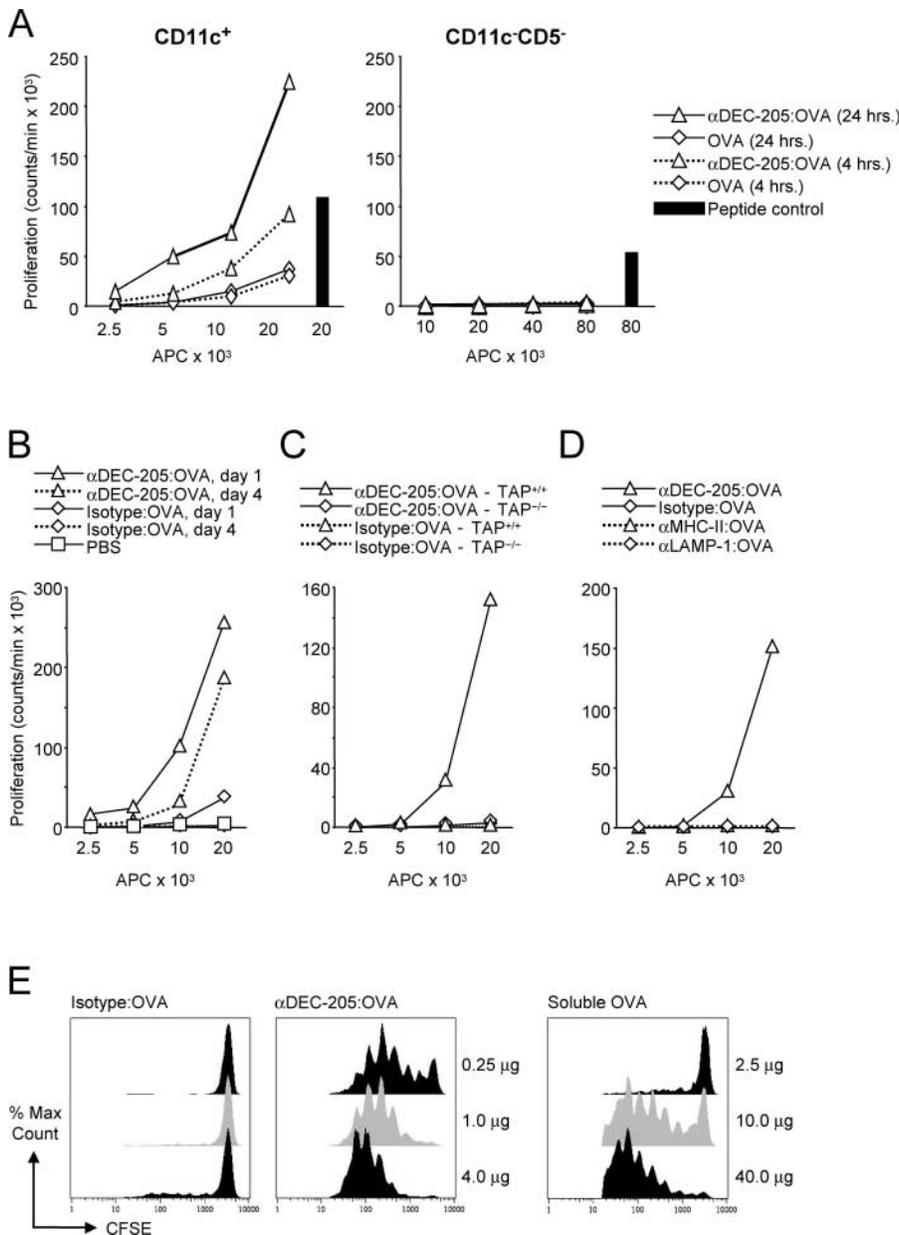


**Figure 2.** Targeting of  $\alpha$ DEC-205:OVA to CD11c<sup>+</sup> lymph node DCs in vitro. (A)  $\alpha$ DEC-205:OVA elicits stronger presentation than OVA alone in a dose-dependent manner. CD11c<sup>+</sup> cells from C57BL/6 lymph nodes were cultured 18 h in graded doses of  $\alpha$ DEC-205:OVA or OVA alone, washed, and cocultured with OT-I or OT-II T cells before measuring uptake of [<sup>3</sup>H]thymidine at 48–72 h to assess T cell proliferation. (B) Presentation of peptides derived from  $\alpha$ DEC-205:OVA is restricted to CD11c<sup>+</sup> lymph node cells, and not the CD19<sup>+</sup> or CD11c<sup>-</sup>CD19<sup>-</sup> (double negative) fractions. As in A, but with  $\alpha$ DEC-205:OVA or the isotype:OVA conjugate at 10  $\mu$ g/ml. (C) Presentation of peptides derived from  $\alpha$ DEC-205:OVA is TAP dependent. As in B, but CD11c<sup>+</sup> cells were prepared from C57BL/6 or TAP<sup>-/-</sup> mice. (D) Bone marrow DCs are unable to present  $\alpha$ DEC-205:OVA on MHC class I products. Cells from d6 cultures were harvested and cultured with antibody:OVA conjugates for 6 h at 10  $\mu$ g/ml, washed, and cocultured with T cells as in panel A. Data are representative of three experiments.

isolated CD11c<sup>+</sup> and CD11c<sup>-</sup> cells (or B cells enriched from the CD11c<sup>-</sup> population by depleting CD5<sup>+</sup> T cells) from mice injected subcutaneously with conjugates or with soluble OVA, and then we assayed for antigen presentation to OT-I cells in vitro without further addition of antigen. After injection of  $\alpha$ DEC-205:OVA, we detected strong presentation at 4 and 24 h after injection, but only by DCs (Fig. 3 A, left) and not by CD11c<sup>-</sup> or enriched B cells (Fig. 3 A, right). The B cells also were inactive when the animals were given  $\alpha$ CD40 antibody (together with antibody:OVA) to enhance their costimulatory properties (data not depicted). 100-fold higher doses of soluble OVA relative to  $\alpha$ DEC-205:OVA (which is <10% OVA) were required to detect presentation, but again the DCs selectively presented the antigen. This presentation was greater when the cells were isolated 24 h rather than 4 h after injection (Fig. 3 A). After injection of  $\alpha$ DEC-205:OVA, presentation was readily detected for as long as 4 d (Fig. 3 B). The presentation by DCs in vivo was TAP dependent (Fig. 3 C). In contrast, antibody:OVA conjugates directed toward other

DC markers, e.g., MHC-II and LAMP-1, did not enhance OVA-presentation (Fig. 3 D). In all experiments, there was little or no capture of the isotype-control:OVA conjugate (Figs. 3, B–D).

To verify that DCs targeted with  $\alpha$ DEC-205:OVA could also present antigen to T cells in situ, we transferred  $2 \times 10^6$  CFSE-labeled, antigen-specific T cells (CD8<sup>+</sup> OT-I cells) 1 d before injection with  $\alpha$ DEC-205:OVA, isotype:OVA, or soluble OVA. After 3 d, the lymph nodes (Fig. 3 E), spleen, and blood (data not depicted) were evaluated for OT-I proliferation as assayed by CFSE dilution. Virtually all of the OT-I cells in lymph node entered cell cycle and underwent 3–7 cell divisions after a dose of just 1.0  $\mu$ g of  $\alpha$ DEC-205:OVA conjugate (<100 ng OVA) per mouse (Fig. 3 E). For soluble OVA, at least 400-fold more protein was required to induce comparable proliferative responses, and, again, the isotype-control:OVA conjugate elicited little or no proliferation (Fig. 3 E). To prove that DEC-205 but not Fc $\gamma$  receptors were mediating presentation, we verified that presentation was abol-



**Figure 3.** Targeting of  $\alpha$ DEC-205:OVA to lymph node CD11c<sup>+</sup> DCs in vivo. (A) Only CD11c<sup>+</sup> lymph node DCs efficiently present exogenous  $\alpha$ DEC-205:OVA, and to a lesser extent OVA, to OT-I T cells. C57BL/6 mice were injected with 4.0  $\mu$ g (1.0  $\mu$ g/footpad) of  $\alpha$ DEC-205:OVA conjugate or 400  $\mu$ g (100  $\mu$ g/footpad) of soluble OVA subcutaneously 4 and 24 h before sacrifice. The CD11c<sup>+</sup> and CD11c<sup>-</sup>CD5<sup>-</sup> (B cell) fractions were MACS<sup>®</sup> sorted from lymph nodes and evaluated for presentation to OT-I T cells as in Fig. 2 A. Peptide controls were performed with the highest titration of APCs (DCs, left; B cells, right) for each group. (B) As in panel A but CD11c<sup>+</sup> DC's were studied 1 and 4 d after injection of 4.0  $\mu$ g (1.0  $\mu$ g/footpad) of antibody:OVA conjugates subcutaneously. (C) Presentation by DCs of OVA peptides from C57BL/6 but not TAP<sup>-/-</sup> mice given 4.0  $\mu$ g (1.0  $\mu$ g/footpad) of IgG:OVA conjugates subcutaneously 4 d earlier. (D)  $\alpha$ DEC-205:OVA elicits better presentation of OVA derived peptides than other DC-targeted conjugates, each injected with 4.0  $\mu$ g (1.0  $\mu$ g/footpad) of IgG:OVA conjugates subcutaneously 4 d earlier. (E)  $\alpha$ DEC-205:OVA induces stronger in vivo proliferation of OT-I T cells than OVA alone. C57BL/6 mice were injected intravenously with  $2 \times 10^6$  CFSE-labeled OT-I T cells and then graded doses of IgG:OVA conjugates or OVA subcutaneously 24 h later. 3 d after conjugate injection, lymph nodes were harvested and the expansion of CD8<sup>+</sup>V $\alpha$ <sub>2</sub>V $\beta$ <sub>5.1/5.2</sub> cells evaluated by flow cytometry for CFSE dilution. Each panel represents two or more experiments.

ished with DCs from DEC-205<sup>-/-</sup> mice (data not depicted). In summary,  $\alpha$ DEC-205 efficiently targets antigens for presentation by the exogenous pathway to MHC class I in vivo.

**$\alpha$ DEC-205:OVA Does Not Mature DCs In Vivo.** To examine whether  $\alpha$ DEC-205:OVA treatment results in DC maturation in the presence or absence of OVA-specific OT-I T cells, we did FACS<sup>®</sup> studies of DCs from mice injected with conjugates 1 or 3 d earlier under a variety of conditions. As illustrated in Fig. 4 A, surface expression of CD80, CD86, as well as MHC class II products were unchanged in  $\alpha$ DEC-205:OVA-injected mice, whether or not they received OT-I T cells. The number of DCs also did not change in mice given  $\alpha$ DEC-205:OVA. However, coadministration of an agonistic  $\alpha$ CD40 antibody (FGK-

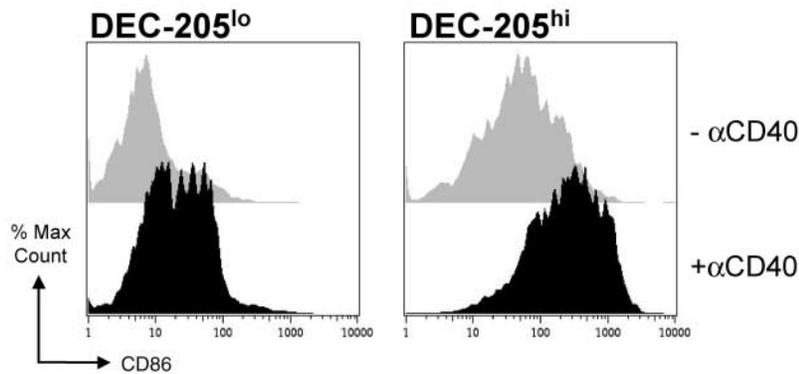
45.5) as an adjuvant activated the DCs in situ over a 3 d period and increased their numbers about twofold. The extent of maturation with  $\alpha$ CD40 was similar in the absence or presence of antigen ( $\alpha$ DEC-205:OVA) or OT-I T cells (Fig. 4 A). Maturation was detected in CD11c<sup>+</sup> DCs that had low and high levels of DEC-205, but the levels of CD86 were higher in the DEC-205 high fraction (Fig. 4 B). In summary, although lymph node DCs in the steady state express molecules used in T cell activation like CD86, these DCs do not seem to differentiate further when exposed to  $\alpha$ DEC-205:OVA but do differentiate in response to agonistic  $\alpha$ CD40 antibody.

**Distinct T Cell Responses In Vivo to Antigen Presented in the Steady State and CD40-based DC Maturation.** To follow the fate of the OT-I T cells that proliferated in response to

A

$\alpha$ CD40	-	-	+	+	-	-	+	+
$\alpha$ DEC-205:OVA	-	+	-	+	-	+	-	+
OT-I	-	-	-	-	+	+	+	+
CD86	80.1	67.5	<b>108</b>	<b>90.6</b>	60.5	72	<b>83.5</b>	<b>84.7</b>
CD80	124	138	<b>198</b>	<b>165</b>	147	166	<b>178</b>	<b>153</b>
MHC-II	470	488	<b>571</b>	<b>471</b>	470	561	<b>545</b>	<b>556</b>
CD86	59.8	66.3	<b>316</b>	<b>293</b>	53.1	84.3	<b>220</b>	<b>258</b>
CD80	134	104	<b>316</b>	<b>300</b>	132	185	<b>255</b>	<b>277</b>
MHC-II	319	445	<b>599</b>	<b>567</b>	374	497	<b>536</b>	<b>610</b>

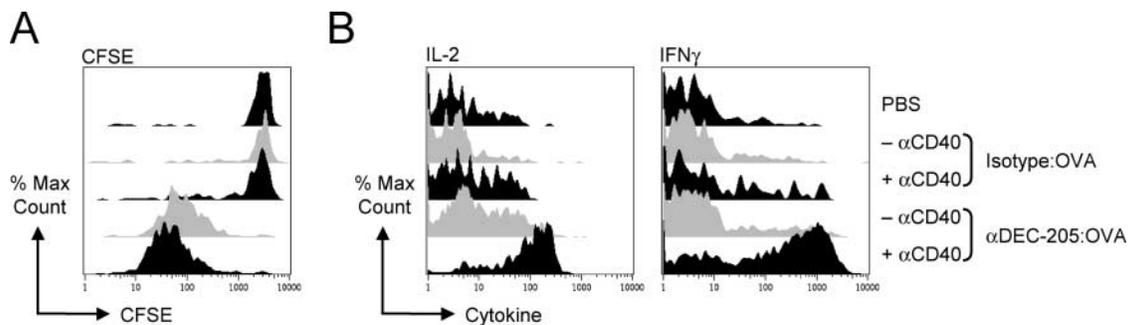
B



**Figure 4.** Maturation of DCs in vivo by agonistic  $\alpha$ CD40 but not by  $\alpha$ DEC-205:OVA. (A) C57BL/6 mice were injected subcutaneously with PBS or 4.0  $\mu$ g (1.0  $\mu$ g/footpad) of  $\alpha$ DEC-205:OVA conjugate with or without  $\alpha$ CD40 (100  $\mu$ g FGK45.5 subcutaneously), 1 and 3 d before sacrifice. CD11c<sup>+</sup> cells were sorted by MACS<sup>®</sup> from lymph nodes and evaluated by flow cytometry for expression of CD80, CD86, and MHC class II. Prior to injection of the OVA conjugate and  $\alpha$ CD40, the mice were given PBS (-) or OT-I (+) cells. The bold symbols are mean fluorescence indices of the CD11c<sup>+</sup> cells in the presence of a maturation stimulus, while the gray-bold at day 3 indicate a significant increase, consistent with maturation. (B) Illustrative FACS<sup>®</sup> data showing the maturation of the DEC-205<sup>hi</sup> CD11c<sup>+</sup> cells and DEC-205<sup>lo</sup>CD11c<sup>+</sup> cells, in mice treated 3 d before with PBS and  $\alpha$ CD40 as in panel A.

antigen targeted to DCs in vivo, we tracked the transferred T cells by flow cytometry using a combination of CD45.1 and V $\beta$ <sub>5.1/5.2</sub> markers, and we compared responses in the steady state to those following  $\alpha$ CD40-induced DC maturation. At 3 d after  $\alpha$ DEC-205:OVA injection, we found strong proliferative responses in the presence or absence DC maturation (Fig. 5 A). However,  $\alpha$ CD40 treated mice also showed greatly enhanced T cell production of IL-2 and IFN- $\gamma$  (Fig. 5 B, bottom row).

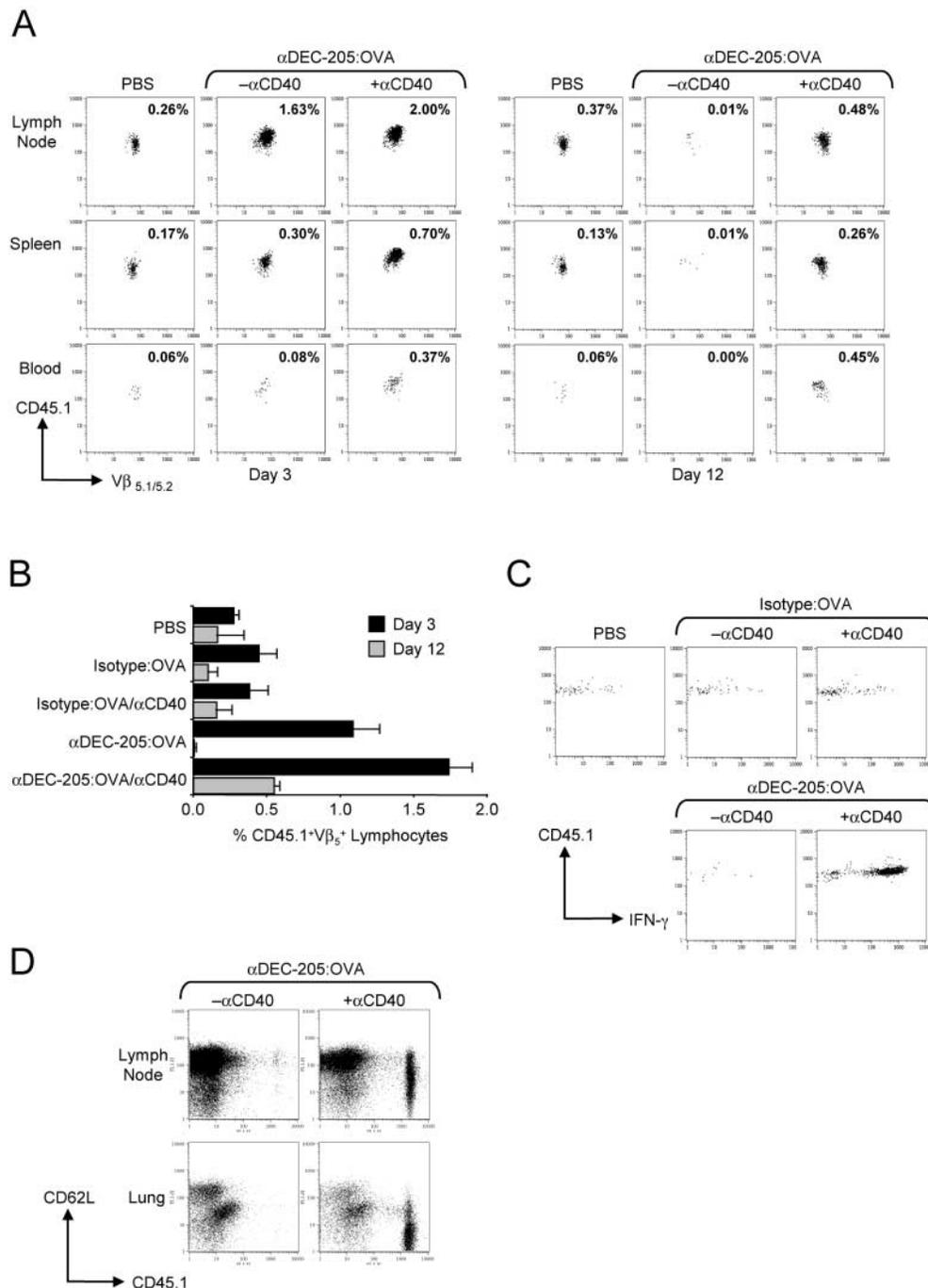
When we followed the numbers of injected OT-I T cells at 3 d and at 12–14 d in several lymphoid tissues (Fig. 6, A and B), we found that  $\alpha$ DEC-205:OVA in the steady state first expanded the OT-I cells, but by 12–14 d, the T cells were virtually entirely absent from lymph nodes, spleen, or blood (Fig. 6 A, compare left and right panels, and Fig. 6 B). However, if the  $\alpha$ DEC-205:OVA conjugate was given with  $\alpha$ CD40, the OT-I cells expanded relative to the PBS control (Fig. 6, A and B) or isotype-control:



**Figure 5.** Contrasting responses of OT-I T cells to  $\alpha$ DEC-205:OVA in the presence or absence of  $\alpha$ CD40-induced DC maturation. (A)  $\alpha$ CD40 has little impact on  $\alpha$ DEC-205:OVA induced proliferation of OT-I T cells. As in Fig. 3 E, but mice were or were not given  $\alpha$ CD40 (100  $\mu$ g FGK45.5 subcutaneously). (B) Differential IL-2 and IFN- $\gamma$  production by OT-I T cells in response to isotype:OVA or  $\alpha$ DEC-205:OVA with or without  $\alpha$ CD40. As in panel A, but lymph node suspensions were restimulated in vitro with the cognate OT-I peptide for 5 h in the presence of brefeldin A (5  $\mu$ g/ml) before staining for intracellular cytokine. Data are representative of three experiments.

OVA conjugate (data not depicted). Similarly, when IFN- $\gamma$  production was monitored by FACS<sup>®</sup> (Fig. 6 C) or by ELISPOT assays (data not depicted), the combination of  $\alpha$ DEC-205:OVA and  $\alpha$ CD40 induced a strong expansion of cytokine producing effectors, whereas cytokine producing OT-I cells were virtually deleted from the lymph node when mice had received  $\alpha$ DEC-205:OVA (Fig. 6 C). As it was possible that T cells were being redistributed to tissues in the steady state rather than undergoing true deletion, we isolated cells from one large peripheral organ, the lung. In mice exposed to  $\alpha$ DEC-205:OVA, we could not find any

OT-I T cells in the lung at day 10, but many OT-I cells were found in the lungs of mice given  $\alpha$ DEC-205:OVA plus  $\alpha$ CD40 (Fig. 6 D). The expanded numbers of cells in the lymph nodes and lungs in response to  $\alpha$ DEC-205:OVA plus  $\alpha$ CD40 included a large fraction of cells lacking the CD62L selectin for lymph node homing, a typical feature of activated T cells (Fig. 6 D). In summary, delivery of protein antigens to DCs leads to the deletion of MHC class I-restricted T cells, but antigen delivered together with a maturation stimulus, leads to T cell expansion, production of IFN- $\gamma$ , and export of T cells to peripheral tissues.



**Figure 6.** Deletion of OT-I T cells in response to  $\alpha$ DEC-205:OVA in steady state. (A) C57BL/6 mice were given CD45.1<sup>+</sup> OT-I T cells and antigen as described in 3E with or without  $\alpha$ CD40. 3 and 12 d later, lymph nodes, spleen, and blood were harvested and evaluated for OT-I T cells (CD45.1<sup>+</sup>Vβ<sub>5.1/5.2</sub><sup>+</sup>) by flow cytometry. (B) Data on the number of OT-I cells, expressed as the mean percentage of CD8<sup>+</sup> T cells from three experiments of the type shown in panel A. (C)  $\alpha$ CD40-rescued OT-I T cells are primed and secrete IFN- $\gamma$ . C57BL/6 mice were given OT-I T cells and antigen as described in panel A. 12 d after antigen administration, lymph nodes were harvested and OT-I T cells evaluated for IFN- $\gamma$  secretion as described in Fig. 5 B. (D) OT-I T cells are not present in a peripheral non-lymphoid tissue, after presentation of  $\alpha$ DEC-205:OVA by DCs in the steady state, in the absence of  $\alpha$ CD40 stimulation. As in panel A, but the lung was harvested 10 d after antigen administration and the OT-I cells were evaluated for expression of CD62L and CD45.1.

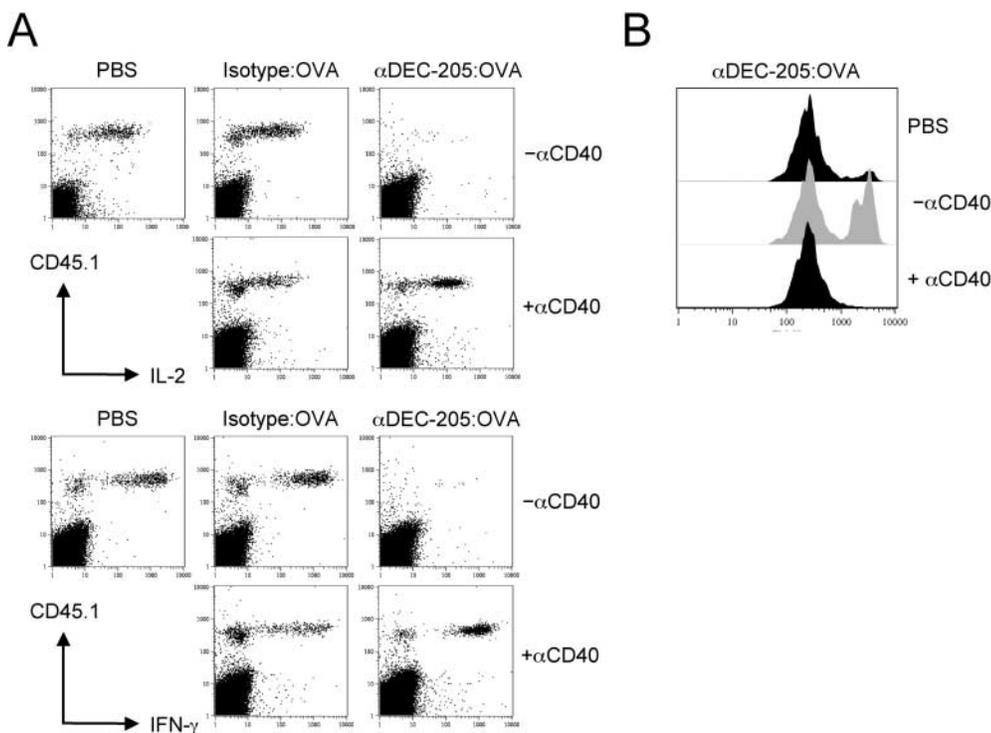
**Antigen Targeting to DCs Induces Peripheral CD8<sup>+</sup> T Cell Tolerance.** A critical criterion for the induction of peripheral tolerance is the inability to respond to rechallenge with antigen delivered together with a strong adjuvant. To determine whether  $\alpha$ DEC-205:OVA treated mice become tolerant to OVA, we immunized with 50  $\mu$ g of OVA protein suspended in CFA 12 d after administration of the conjugate. 3 d after OVA in CFA immunization, mice were killed and the immune response was evaluated using either ELISPOT assays (data not depicted) or FACS<sup>®</sup> assays for intracellular cytokine production (Fig. 7 A). Control mice pretreated with PBS or isotype-control:OVA were able to be primed to OVA in CFA (Fig. 7 A). In contrast, mice pretreated with  $\alpha$ DEC-205:OVA were unable to respond to OVA challenge (Fig. 7 A, top right panel in each group). These same mice could be immunized to non-OVA proteins, e.g., the PPD antigen in the CFA (data not depicted). We further assessed formation of effector CTL using a recently described in vivo assay for CTL function (35). Mice tolerized 12 d earlier with  $\alpha$ DEC-205:OVA could not form CTL, but mice given PBS or the combination of  $\alpha$ DEC-205:OVA with  $\alpha$ CD40 produced strong CTL responses (Fig. 7 B). We conclude that targeting antigens to DCs via DEC-205 in the steady state results in the induction of peripheral tolerance, with no effector T cell formation and memory, whereas targeting in the presence of a DC maturation stimulus leads to immunization.

## Discussion

These findings extend the evidence that DCs in peripheral lymphoid organs are constitutively processing antigens

in the steady state, and that the consequences of this presentation is peripheral tolerance. With the identification of an efficient receptor mediated pathway, DEC-205, it is clear that small amounts of a soluble protein can lead to presentation on MHC class I and tolerance of CD8<sup>+</sup> T cells. Prior studies with soluble proteins have emphasized the need for high doses of antigen to tolerize the immune system, and primarily on MHC class I products.

The DEC-205 endocytic receptor has several valuable roles in antigen uptake and processing by DCs. First, the receptor targets to MHC class II<sup>+</sup>, late endosomal/lysosomal compartments in cultured bone marrow-derived DCs (31). In contrast, most recycling endocytosis receptors traffic through peripheral or early endosomes. The late endosomal/lysosomal targeting of DEC-205 is associated with much more efficient antigen processing and presentation via the MHC class II pathway. These properties are mediated by the cytosolic domain of DEC-205, particularly an acidic EDE sequence (31). Second,  $\alpha$ DEC-205 antibodies in small amounts (<1  $\mu$ g/mouse) can be used to target antigens for presentation by DCs in vivo. When an antigenic peptide from hen egg lysozyme is engineered into the heavy chain of the  $\alpha$ DEC-205 antibody, the antibody and peptide is selectively delivered to DCs (32). Here,  $\alpha$ DEC-205 has been chemically conjugated to full length OVA and delivers the protein selectively to DCs in vivo as well. Third, we now find that DEC-205 mediates presentation of protein antigens via the exogenous but TAP-dependent MHC class I pathway. Uptake via DEC-205 corroborates that lymph node DCs in the steady state are capable of processing antigens onto MHC class I (35, 39–42) as well as MHC class II (18, 32) products. In contrast, we do not ob-



**Figure 7.**  $\alpha$ DEC-205:OVA induces peripheral tolerance to OVA in the steady state. (A) C57BL/6 mice were given CD45.1<sup>+</sup> OT-I T cells and antigen as described in 3E with or without  $\alpha$ CD40. 12 d after antigen administration, mice were boosted with 50  $\mu$ g of OVA protein in complete CFA. After 3 d, lymph nodes were harvested and OT-I T cells evaluated for secretion of IL-2 (top) or IFN- $\gamma$  (bottom) as in Fig. 5 B. (B) C57BL/6 mice were treated as in panel A, but 3 d after administration of OVA in CFA, mice were injected intravenously with a mixture of CFSE-labeled syngeneic splenocytes pulsed with (CFSE<sup>hi</sup>) or without (CFSE<sup>lo</sup>) the OT-I cognate peptide ( $3 \times 10^6$  of each). 12 h later the loss of CFSE<sup>hi</sup> cells in lymph nodes was evaluated as a measure of specific CTL activity. Naive mice do not exhibit any loss of CFSE labeled cells (not shown). The results are representative of three experiments.

serve presentation of  $\alpha$ DEC-205:OVA on MHC class I using DCs derived from bone marrow, although this DC population presents exogenous proteins on MHC class II products (43). In contrast, DCs from lymph node seem competent in both MHC class I and II presentation in the steady state in situ, without addition of maturation stimuli.

In the steady state, DEC-205 represents a specific receptor for DCs to induce peripheral tolerance to soluble antigens for both CD4<sup>+</sup> (32) and CD8<sup>+</sup> T cells (this paper). DCs also induce tolerance to cell associated antigens in vivo (35, 39–41), but the responsible receptors have not been identified to date. While DEC-205 greatly enhances presentation of protein antigens, it is possible that the receptor simply functions to enhance antigen uptake and not subsequent events required for presentation on MHC class I. Also, once suitable reagents become available, it will be possible to assess if other receptors on DCs (see Introduction) function to enhance antigen presentation and peripheral tolerance in vivo. Currently, monoclonal antibodies are primarily available for the DEC-205 receptor and, as mentioned above, have been previously shown to target DCs efficiently and selectively in vivo and enhance MHC class II presentation. The key new points in this paper are first that DCs have efficient receptor based mechanisms to enhance presentation on MHC class I products in vivo, second that these operate in the steady state, and third, the consequence of presentation is peripheral tolerance in the CD8<sup>+</sup> compartment by a deletional mechanism.

The existence of receptor-mediated uptake mechanisms should allow DCs to play a valuable role in silencing reactivity to harmless self-antigens and environmental proteins. A single dose of <0.1  $\mu$ g of OVA conjugated to  $\alpha$ DEC-205 antibody can delete and tolerize sizable numbers (>10<sup>6</sup>) of injected OVA-specific CD8<sup>+</sup> T cells, corresponding to >1% of the  $\sim$ 10<sup>8</sup> T cells in a mouse. This number is large, as <1/10,000 naive T cells are typically able to respond to a specific antigen. As a result, the capacity of DCs to present antigens by the exogenous pathway greatly exceeds the repertoire of T cells to be activated, at least with respect to an antigen that is recognized efficiently, i.e., 0.1 nM peptide, as studied here. It will be important to test other protein antigens and a broader repertoire of T cells, as our studies have at this point focused on the OT-I T cell, to obtain direct evidence on the number and function of antigen-specific T cells. Nonetheless, our findings indicate that CD8<sup>+</sup> T cell tolerance (as well as immunity) can be achieved in situ with low doses of intact protein antigens.

DCs in lymph nodes and spleen express many features suggesting that they are “immature” i.e., able to capture antigens but unable to stimulate immunity (44, 45). The DCs are active in endocytosis (40, 46) and also respond in vivo to microbial, inflammatory, and T cell stimuli by producing cytokines (47) and up-regulating several costimulatory molecules (48–50). The studies with  $\alpha$ DEC-205:OVA corroborate that DCs in vivo in the steady state do not stimulate an immune response even when they are effectively presenting antigen and inducing extensive T cell

proliferation. However, the T cells fail to differentiate and are deleted unless a maturation stimulus also is provided. To date, we have concentrated on CD40-based DC maturation. Nevertheless, the findings are consistent with the view that the immunogenic function of DCs, i.e., the induction of effector T cells and the development of memory, requires that at least two sets of events take place. One involves antigen capture and successful processing to form MHC-peptide complexes; this occurs in lymph node DCs in the steady state. The other requirement entails the intricate process of maturation, which changes DCs in many ways, e.g., increasing T cell costimulatory molecules, inducing cytokines like IL-12 and IL-2, and altering the expression of chemokine receptors.

Two pathways have been defined for the presentation of antigens on MHC class I products. The classical or “endogenous” pathway originates from newly synthesized proteins especially those derived from defective ribosomal initiation products (51). This pathway provides an elegant and established mechanism for protective immunity, guiding MHC class I-restricted cytotoxic T lymphocytes to peptides produced in infected and malignant cells. By recognizing MHC-peptide complexes displayed at the cell surface, the CD8<sup>+</sup> cytolytic response is focused on cells harboring pathogens and not on innocent bystander cells (52). A second “exogenous” pathway also exists, allowing endocytosed nonreplicating proteins to be presented on MHC class I. The exogenous pathway a priori could allow noninfected cells to take up protein and become targets for MHC class I-specific, CD8<sup>+</sup> cytolytic T lymphocytes. This potentially serious problem would be averted if the exogenous pathway were primarily expressed in DCs. During infection, the processing of dying infected cells by the exogenous pathway provides a means for DCs to initiate CD8<sup>+</sup> T cell immunity to pathogens that do not productively infect them. Likewise, during the steady state, DCs in lymphoid organs can use the exogenous pathway to present self-peptides on MHC class I (16, 40, 53; and this paper) and thereby induce tolerance. Therefore the induction of tolerance does not require that all cells be capable of processing antigens by the exogenous pathway; rather, the presence of efficient receptors like DEC-205 for exogenous presentation in vivo provides a specific mechanism for DCs to continually delete the peripheral T cell repertoire of reactivity to low levels of self and environmental proteins.

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