CD8$^+$ but Not CD8$^-$ Dendritic Cells Cross-prime Cytotoxic T Cells In Vivo

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Abstract

Bone marrow–derived antigen-presenting cells (APCs) take up cell-associated antigens and present them in the context of major histocompatibility complex (MHC) class I molecules to CD8$^+$ T cells in a process referred to as cross-priming. Cross-priming is essential for the induction of CD8$^+$ T cell responses directed towards antigens not expressed in professional APCs. Although in vitro experiments have shown that dendritic cells (DCs) and macrophages are capable of presenting exogenous antigens in association with MHC class I, the cross-presenting cell in vivo has not been identified. We have isolated splenic DCs after in vivo priming with ovalbumin-loaded β2-microglobulin–deficient splenocytes and show that they indeed present cell-associated antigens in the context of MHC class I molecules. This process is transporter associated with antigen presentation (TAP) dependent, suggesting an endosome to cytosol transport. To determine whether a specific subset of splenic DCs is involved in this cross-presentation, we negatively and positively selected for CD8$^-$ and CD8$^+$ DCs. Only the CD8$^+$, and not the CD8$^-$, DC subset demonstrates cross-priming ability. FACS® studies after injection of splenocytes loaded with fluorescent beads showed that 1 and 0.6% of the CD8$^+$ and the CD8$^-$ DC subsets, respectively, had one or more associated beads. These results indicate that CD8$^+$ DCs play an important role in the generation of cytotoxic T lymphocyte responses specific for cell-associated antigens.

Key words: major histocompatibility complex class I • antigen presentation • antigen-presenting cell • cytotoxic T lymphocyte • cross-priming

Introduction

Naive CD8$^+$ T cells are stimulated to proliferate and to develop into cytotoxic effector T cells after recognition of short peptides associated with MHC class I molecules on professional APCs. "Endogenous" cytosolic proteins are generally the source of MHC class I–restricted antigens. These proteins are degraded by the proteasome, and peptides are transported to the endoplasmic reticulum by the transporter associated with antigen presentation (TAP)[1] transporter, where they can bind to newly synthesized MHC class I molecules. In contrast, membrane-associated proteins and endocytosed "exogenous" proteins are mainly presented by MHC class II molecules for recognition by CD4$^+$ T cells. However, the division of the endogenous and exogenous pathway is not absolute. 25 years ago, it was recognized that immunization with cells lacking host MHC alleles, but bearing foreign minor histocompatibility antigens, leads to minor specific CD8$^+$ T cell activation that is restricted to the host MHC allele (1, 2). This suggested that host APCs can process exogenous cell-associated antigens and present them in the context of MHC class I molecules, a process termed cross-priming or cross-presentation. Since then, cross-priming has been shown to be important in initiating MHC class I–restricted responses to tumors, peripheral self, viral, and bacterial antigens (3–6). This indicates that cross-presentation is a general mechanism for the induction of T cells specific for antigens not expressed by APCs themselves and is involved in a variety of T cell responses (7). In addition to T cell activation, cross-presentation has been shown to induce T cell tolerance (8, 9).

Bone marrow chimera studies demonstrated that the ability to cross-present cell-associated antigens is restricted to bone marrow–derived APCs (4, 10). Several groups have investigated the role of macrophages and dendritic cells (DCs) in presenting exogenously derived antigens in

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[1]*Abbreviations used in this paper:* B6, C57BL/6; β2m, β2-microglobulin; CFSE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; PALS, periarteriolar lymphatic sheaths; TAP, transporter associated with antigen presentation.

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MHC class I in vitro (for a review, see reference 7). Exogenous antigens in the form of soluble proteins, particulate antigens, and cell-associated antigens derived from apoptotic or necrotic cells have been shown to be taken up, processed, and presented to $CD8^+$ T cells by both cell types (11–17). Other reports have indicated that cross-presentation is a specific property of DCs (18–20). However, which bone marrow–derived APC is involved in MHC class I–restricted presentation of exogenous antigens in vivo has not been examined.

DCs comprise populations of cells with heterogeneous phenotypes (21, 22). Murine splenic DCs can be divided into two subsets, both of which express high levels of CD11c. One DC population is further characterized by high expression of the myeloid marker CD11b and by absence of expression of CD8α and DEC-205, whereas the other is $CD11b^{low}CD8\alpha^+$DEC-205^+ (23–27). The $CD8^-CD11b^{high}$ DCs can be further divided into a $CD4^+$ and $CD4^-$ population (28). Some evidence suggests that the $CD8^+$ and $CD8^-$ DC subsets belong to different lineages. Several transcription factor knockout and mutant mice exhibited differential effects on the development of each subset in vivo (29–32). In addition, $CD8^+$ DCs were shown to be derived from a thymic progenitor that could not generate myeloid cells, whereas $CD8^-$ DCs can be grown from bone marrow using GM-CSF (33–35). Therefore, $CD8^+$ DCs are thought to relate to the lymphoid lineage, whereas $CD8^-$ DCs are considered myeloid related. For convenience, in this paper we will use the terms lymphoid DC and myeloid DC to describe the $CD8^+$ and $CD8^-$ DC subsets.

In addition to phenotypic differences, the lymphoid and myeloid DCs reside in different areas of the spleen. Whereas lymphoid DCs are localized in the T cell–rich areas of the periarteriolar lymphatic sheaths (PALS), myeloid DCs can be found in the marginal zone (21, 36). Functional distinctions between lymphoid and myeloid DCs have also been reported. Initial in vitro studies indicated that lymphoid DCs could suppress T cell responses by causing apoptosis of $CD4^+$ T cells and by limiting IL-2 production by $CD8^+$ T cells, whereas myeloid DCs were shown to be strong stimulators of primary T cell responses (37, 38). These findings led to the suggestion that myeloid and lymphoid DCs function as T cell stimulators and tolerizers, respectively. However, in a recent study, peptide-coated DCs of both subsets were shown to induce strong $CD8^+$ T cell responses, indicating no inherent tolerizing function for lymphoid DCs (39). Both DC subsets do appear to differ in their ability to activate $CD4^+$ cells, as myeloid DCs preferentially stimulate Th2 responses, whereas lymphoid DCs preferentially induce Th1 responses (40–42). The role of both DC subsets in cross-priming of $CD8^+$ T cells in vivo has not been investigated.

To determine the cell types responsible for cross-priming in vivo, we primed C57BL/6 (B6) mice with irradiated, β2-microglobulin$^{-/-}$ ($\beta2m^{-/-}$) splenocytes loaded with OVA in their cytoplasm. Such $\beta2m^{-/-}$ splenocytes are unable to present the OVA epitope in association with MHC class I molecules, but they elicit, when injected in B6 mice, OVA-specific, MHC class I–restricted CTL responses. We show that host splenic DCs take up and present cell-associated OVA to $CD8^+$ T cells. Analysis of the OVA-presenting DCs indicated that only lymphoid DCs, and not myeloid DCs, cross-present antigens in vivo. This result points to an important and distinct role for lymphoid DCs in the generation of CD8 T cell responses directed towards cell-associated antigens.

Materials and Methods

Mice. $\beta2m^{-/-}$ and B6 mice were purchased from Taconic Farms. OT-I recombination-activating gene (RAG)$2^{-/-}$ and Thy1.1^+ OT-I mice were bred in our specific pathogen-free facility and have a transgenic Vα2Vβ5 TCR specific for the $OVA_{257–264}$ epitope in the context of H2-K^b (43).

Antibodies. CD11c-, CD11b-, CD8α-, CD8β-, K^b/D^b-, and I-A^b–specific antibodies were purchased from BD PharMingen. Biotinylated DEC-205–specific antibodies were a gift from A. Rudensky (University of Washington, Seattle, WA). Flow cytometry was conducted on a FACSCalibur™ and analyzed using CELLQuest™ software (Becton Dickinson). Cell sorting was performed in HBSS with 25 mM Hepes using a FACS Vantage™ (Becton Dickinson).

Cross-priming with OVA-loaded $\beta2m^{-/-}$ Cells. Single cell suspensions were prepared in serum-free medium from spleen and cervical, axillary, brachial, inguinal, and mesenteric lymph nodes from female $\beta2m^{-/-}$ mice. Cells were loaded with OVA by osmotic shock as described previously (44). In short, $\sim15 \times 10^7$ cells were incubated 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 (Calbiochem) for 10 min at 37°C. 13 ml of prewarmed hypotonic medium (40% H_2O, 60% RPMI 1640) was added and the cells were incubated for an additional 2 min at 37°C. The cells were centrifuged, washed twice with cold PBS, and irradiated (1,350 rads). $20–35 \times 10^6$ OVA-loaded cells were injected in 200 μl of PBS into the tail vein. In one experiment, $\beta2m^{-/-}$ cells were shocked with both OVA and yellow/green fluorescent 0.2 μm beads (Molecular Probes). The beads were washed two times in PBS by centrifugation for 10 min at 1,400 rpm and sonication for 10 min. The beads were resuspended to 0.004% (wt/vol) in hypertonic solution containing OVA before shocking. After osmotic shocking of the cells, free beads were removed by three washes in PBS.

CFSE Labeling of OT-I Cells. OT-I cells from spleen and lymph nodes were washed twice in PBS containing 0.1% BSA. To label cells with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) the cells were resuspended at 5×10^7 cells/ml in PBS/0.1% BSA with 10 μM CFSE for 10 min at 37°C. Cells were washed twice with cold RPMI 1640/10% FCS (RP10) followed by two washes in PBS. 10^6 $CD8^+V\alpha2^+CFSE^+$ OT-I cells in 200 μl of PBS were injected into the tail vein.

Low Density Cell Preparation. 14 h after injection with irradiated OVA-loaded $\beta2m^{-/-}$ cells, spleens of 5–35 mice were cut into grain size pieces and incubated in 1 ml per spleen of 1 mg/ml collagenase/dispase (Sigma-Aldrich) and 50 μg/ml DNase I (Boehringer) in PBS with continuous stirring at 37°C for 30 min or until digested. EDTA was added to a 10 mM final concentration, and the cell suspension was incubated for an additional 5

min at room temperature. RP10/10 mM EDTA/20 mM Hepes (RP10/HE) was added and the cells were pelleted. Red blood cells were lysed with ACK lysis buffer. Cells were washed once with RP10 and the cells were resuspended in 10 ml/5 spleens of RP10/HE. Undigested material was removed by filtration through a wire mesh screen and the cell suspension was loaded on 14.5% Accudenz (Accurate Chemical & Scientific Corporation) gradients in RP10/HE and centrifuged at 530 *g* for 20 min at room temperature (25). The low density fraction was recovered and washed once in RP10/HE and resuspended in RP10.

Magnetic Bead Depletion. 5×10^6 low density cells were incubated with 300 μl 2.4G2 supernatant (anti-Fc receptor) for 15 min on ice. Cells were then incubated with 200–250 μl of biotinylated antibody specific for CD11c, CD11b, CD8α, or CD8β in PBS/0.1% BSA for 30 min at 4°C under slow rotation. Depletion of antibody-bound cells with streptavidin-coated magnetic beads (Dynal) was performed in PBS/0.1% BSA according to the manufacturer's instructions.

Proliferation Assay. To detect OVA cross-priming, different DC preparations from injected mice were used as stimulators for naive OT-I cells in a [^{3}H]thymidine incorporation assay. Indicated numbers of irradiated (2,250 rads) DCs were incubated with 10^5 OT-I RAG2$^{-/-}$ cells in flat-bottomed plates in 200 μl RP10. As a positive control, stimulator cells were coated with 1 μM $OVA_{257–264}$ peptide for 1 h and washed three times. After 48 h, the plates were pulsed for 16 h with 1 μCi/well of [^{3}H]thymidine and harvested.

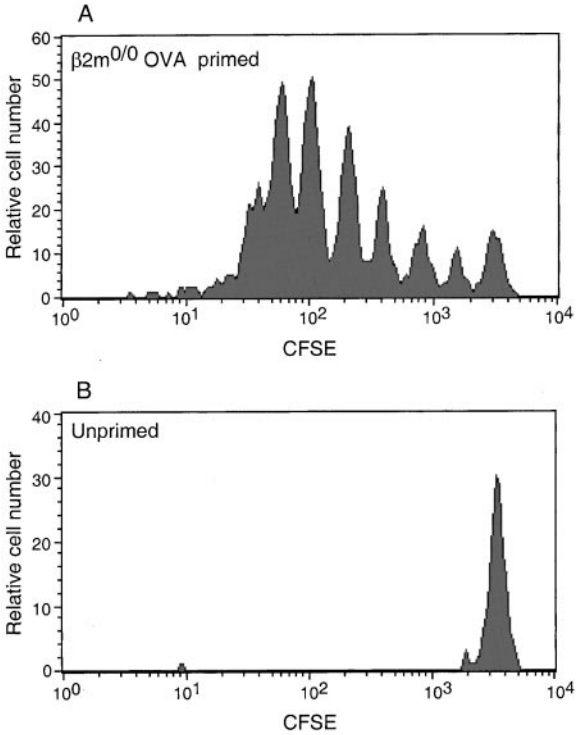

Figure 1. CD8$^+$ T cell proliferation in vivo after priming with OVA-loaded β2m$^{-/-}$ (β2m$^{0/0}$) cells. Thy1.1$^+$ CFSE-labeled OT-I cells were transferred into B6 mice. 3 d later, mice were primed by injection with irradiated OVA-loaded β2m$^{-/-}$ cells. Spleen cells were isolated 3 d after priming and stained for Thy1.1, Vα2, and CD8. (A) CFSE profile of Thy1.1$^+$Vα2$^+$CD8$^+$ OT-I cells from mice primed with OVA-loaded β2m$^{-/-}$ cells. (B) The same profile from a mouse that received no OVA-loaded β2m$^{-/-}$ cells.

Results

Cross-presentation to CD8$^+$ T Cells In Vivo. To study the MHC class I–restricted cross-presentation of cell-associated antigens, we injected B6 mice intravenously with β2m$^{-/-}$ cells that had been osmotically loaded with OVA. As expected, the injected cells are efficiently taken up and processed for MHC class I presentation by host APCs. This is demonstrated by the proliferative response of adoptively transferred, CFSE-labeled H2-K^b–restricted, OVA-specific TCR transgenic OT-I cells that serve as an indicator for efficient presentation in vivo. 3 d after priming, the transferred OT-I cells had divided up to seven times, as revealed by the sequential loss of CFSE intensity (Fig. 1 A). In contrast, OT-I cells did not proliferate in mice that did not receive OVA-loaded cells (Fig. 1 B).

Low Density Cells Cross-present Cell-associated Antigens in a TAP-dependent Manner. Because we immunized mice via the intravenous route, we focussed our attention on the

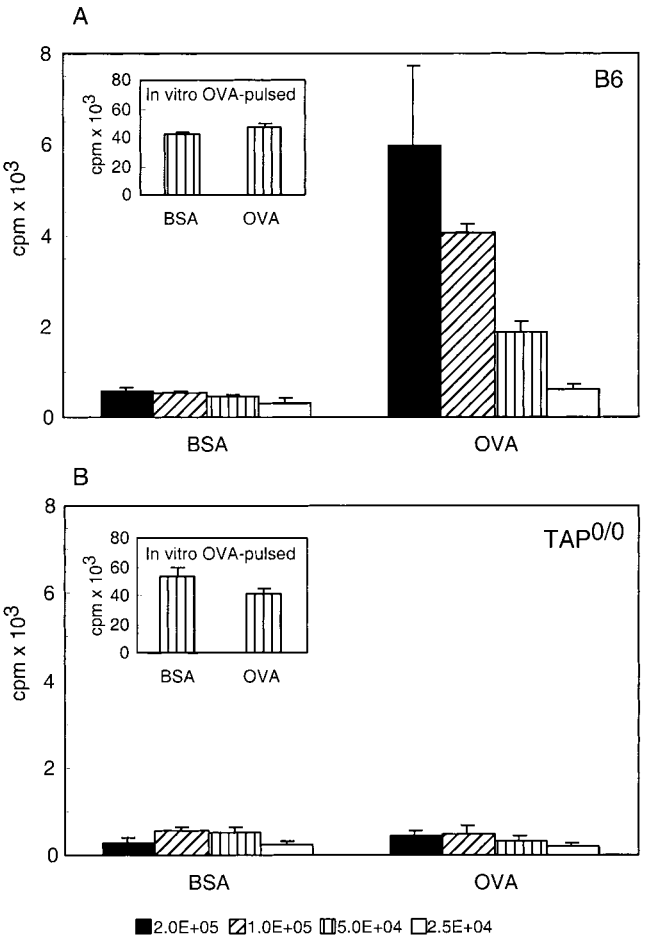

Figure 2. Low density spleen cells cross-present OVA in a TAP-dependent fashion. B6 (A) and TAP$^{-/-}$ (TAP$^{0/0}$) mice (B) were primed with OVA- or BSA-loaded β2m$^{-/-}$ cells. Low density spleen cells were isolated 14 h after injection and analyzed for their ability to stimulate OT-I cells in vitro. As indicated, the different bars represent titered numbers of low density cells added per well. Error bars indicate SEM of triplicate wells. The stimulatory capacity of low density cells after pulsing with $OVA_{257–264}$ peptide in vitro is shown in the insets.

spleen as the source of the cross-presenting APC. To identify the cross-presenting cell, we isolated low density spleen cells from mice 14 h after injection of $\beta 2m^{-/-}$ cells loaded with BSA or OVA. These cells were then used to stimulate naive OT-I cells in an in vitro proliferation assay in the absence of additional antigen. The low density cell preparation was enriched for DCs and generally contained <1% D^b/K^b-negative cells, indicating the paucity of injected OVA-loaded $\beta 2m^{-/-}$ cells (data not shown). Low density spleen cells from mice injected with OVA/$\beta 2m^{-/-}$ cells, but not from mice injected with BSA/$\beta 2m^{-/-}$ cells, were able to stimulate naive OT-I cells in vitro (Fig. 2 A). Low density cells from both types of mice were able to stimulate proliferation after peptide pulsing in vitro (Fig. 2 A, inset).

Processing of exogenous antigens can involve an endosome to cytosol pathway in which the endocytosed antigen is transported to the cytosol and further processed and presented via the "classical" endogenous pathway (20). The TAP transporter is essential in this pathway, in that it transports peptides that are generated by the proteasome from the cytosol to the endoplasmic reticulum where they bind nascent MHC class I molecules. TAP has been shown to be essential for cross-priming MHC class I–restricted responses in vivo (3, 45). Alternatively, a TAP-independent pathway of class I MHC cross-presentation has also been described (7, 46, 47). To determine which pathway is used for the cross-presentation of cell-associated antigens in vivo, BSA- or OVA-loaded $\beta 2m^{-/-}$ cells were injected into $TAP^{-/-}$ mice and low density spleen cells from these mice were evaluated for their capacity to stimulate OT-I in vitro. Low density cells from $TAP^{-/-}$ mice could not stimulate OT-I cells, indicating that the endosome to cytosol pathway is employed for cross-presentation of cell-associated antigens in this system (Fig. 2 B). This result also shows that contaminating APCs in the OT-I cell preparation did not contribute to the proliferation in vitro. The various low density spleen cell preparations did not differ in their presentation capacity, as exogenous OVA peptide pulsing of all preparations showed similarly strong stimulatory capacity for OT-I T cells.

Only Lymphoid DCs Cross-present OVA Antigen to $CD8^+$ T Cells. To investigate further which cell population within the low density cell preparation was responsible for cross-presentation, we first evaluated which cell types were present. Flow cytometric analysis of the low density cells revealed four cell populations as defined by CD11c and CD11b staining (Fig. 3). A population of $CD11c^-CD11b^-$ cells that consisted mainly of T and B cells made up 10–20% of the preparation. Besides these residual T and B cells, a population of $CD11c^-$ and $CD11b^-$ cells were detected that were autofluorescent in the FL-1, FL-2, and FL-3 channels, which could be misinterpreted as low level

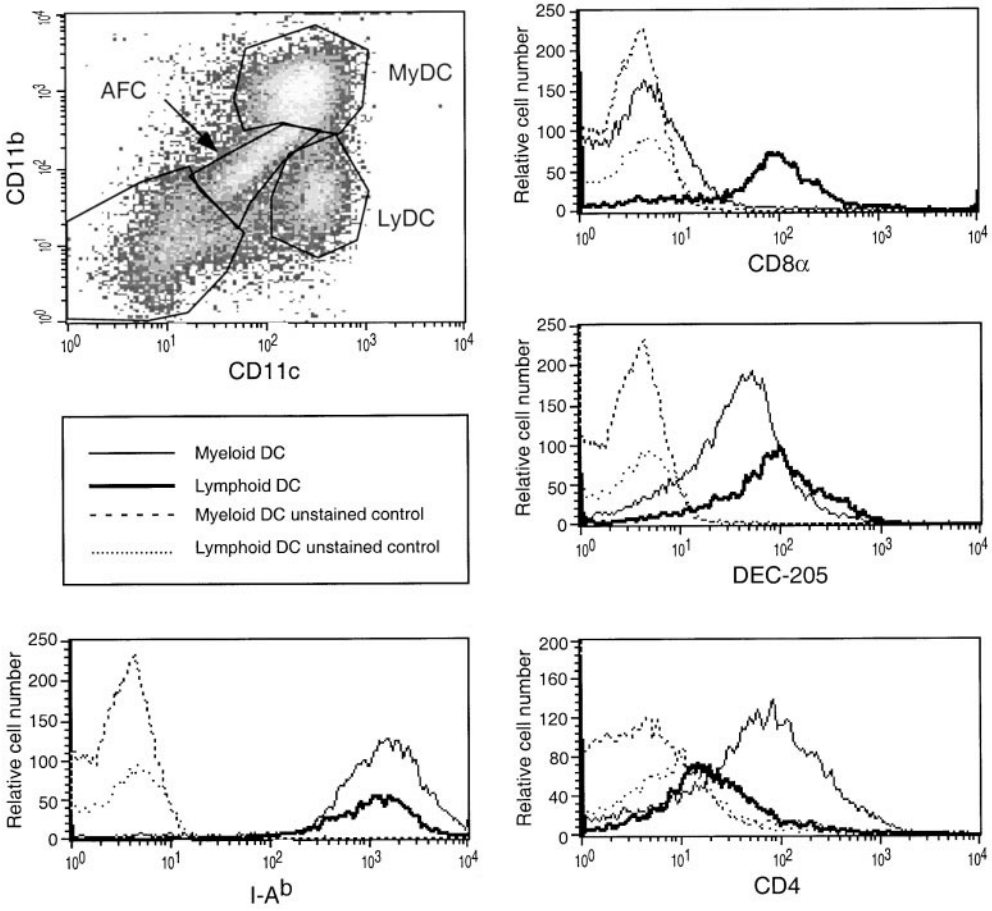

Figure 3. Phenotypic analysis of low density cell preparation. Low density spleen cell preparations were isolated from B6 mice 14 h after priming with OVA-loaded $\beta 2m^{-/-}$ cells. The cells were stained for CD11c, CD11b, and the indicated third reagent, and analyzed by FACS®. Gated myeloid DCs ($CD11b^{high}CD11c^{high}$) and lymphoid DCs ($CD11b^{low}CD11c^{high}$) were further analyzed for CD8α, DEC-205, CD4, and MHC class II expression. MyDC, myeloid DCs; LyDC, lymphoid DCs; AFC, autofluorescent cells, mostly macrophages.

expression of CD11c and CD11b. The autofluorescent cells expressed MHC class I, heterogeneous levels of MHC class II, and low levels of the macrophage marker F4/80, but no CD4, CD8α, or DEC205 (data not shown). This phenotype is consistent with recently described autofluorescent cells present in DC cell preparations that were identified as macrophages (28). Our low density cell preparation contained ~30–40% myeloid and 10–30% lymphoid DCs, both expressing high levels of CD11c but different levels of CD11b (Fig. 3). Analysis of the myeloid and lymphoid DC subsets revealed high MHC class II expression by both subsets, high CD8α and DEC205 expression by the lymphoid DCs, and heterogenous CD4 and low DEC-205 expression by the myeloid DC subset, consistent with previously published studies (25, 26, 28).

We asked whether cross-presentation is a specific characteristic of DCs and, if so, whether it is a feature of a particular DC subset. Therefore, we depleted the low density cell preparation from OVA/β2m$^{-/-}$-primed mice of specific cell subsets using antibodies and magnetic beads and the remaining cells were evaluated for their capacity to stimulate OT-I cells in vitro (Fig. 4). Depletion of CD11c$^+$ cells in the DC preparation resulted in the loss of both the myeloid and lymphoid DC subsets (66% CD11c$^+$ cells were present before depletion, and 8% after depletion, data not shown). The remaining cells consisted of autofluorescent and CD11c$^-$CD11b$^-$ cells and showed almost no OVA presentation. These results demonstrate that cross-presentation of cell-associated OVA is mediated entirely by CD11c$^+$ DCs. Depletion of F4/80$^+$ cells resulted in no loss of activity (data not shown). Therefore, CD11c$^-$ macrophages do not seem to play an important in vivo role in cross-presenting cell-associated antigens.

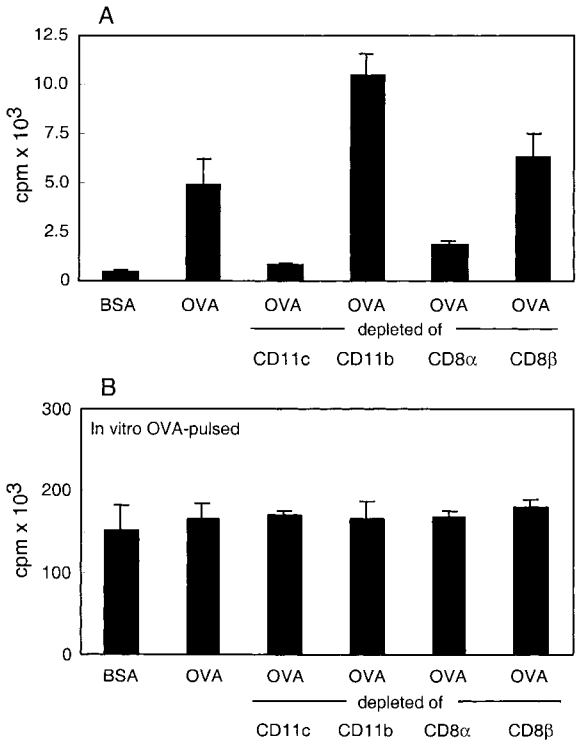

Figure 4. OVA cross-presentation by low density cells depleted of different cell subsets. Low density spleen cells were isolated from B6 mice 14 h after priming with BSA/β2m$^{-/-}$ or OVA/β2m$^{-/-}$ cells. The low density cells from OVA/β2m$^{-/-}$-injected mice were depleted of CD11c$^+$, CD11b^{high}, CD8α^{high}, and CD8β$^+$ cells by magnetic beads and the depleted populations were tested (A) directly for their presenting activity in an in vitro OT-I proliferation assay (2×10^5 stimulator cells/well) or (B) after pulsing with OVA peptide in vitro (2×10^4 cells/well). Error bars indicate SEM of triplicate wells.

Depletion of CD11b$^+$ cells specifically removed myeloid DC (38% predepletion to 9% postdepletion) and concomitantly increased the percentage of lymphoid DCs (26% predepletion to 48% postdepletion). This resulted in a higher stimulatory activity of the DC preparation (Fig. 4). To specifically deplete for lymphoid DCs, we used anti-CD8α antibodies. However, this depletion of the lymphoid DC subset was only ~50% complete (22% predepletion to 13% postdepletion) with the preferential loss of the CD8α^{high}-expressing lymphoid DCs (data not shown). The specific depletion of CD8α^{high} lymphoid DCs resulted in significant loss of stimulatory activity (Fig. 4). Finally, depletion for CD8β$^+$ cells did not lead to a significant change in numbers of the different subsets or in OVA cross-presenting activity. These results suggest that the lymphoid DC was the main cross-presenting APC.

To confirm our findings, we isolated low density cells from mice injected previously with OVA-loaded β2m$^{-/-}$ cells and sorted myeloid and lymphoid DCs on the basis of their CD11b and CD11c expression. Sorting for myeloid and lymphoid DC populations resulted in 87 and 84% pure populations, respectively (Fig. 5 A). The sorted DC subsets were used as APCs for OT-I in an in vitro proliferation assay. Whereas sorted myeloid DCs lacked stimulatory capacity, sorted lymphoid DCs had stimulatory activity that was stronger than that of the starting DC preparation (Fig. 5 B). This result clearly demonstrates that only lymphoid DCs have the ability to cross-present antigen acquired in vivo from injected β2m$^{-/-}$ cells.

Lymphoid DCs Rapidly Acquire Antigen In Vivo. It has been suggested that antigens are transferred from migratory DCs to DCs in the T cell areas (41, 48, 49). Other studies clearly indicate a migration of DCs from the marginal zone where the myeloid DCs are localized into the central T cell areas of the PALS where lymphoid DCs can be found (50–52). This could indicate that marginal zone DCs can transfer antigens to the lymphoid DCs or that marginal zone DCs may mature into lymphoid DCs. Both alternatives would predict that early after injection of OVA/β2m$^{-/-}$ cells, the OVA stimulatory capacity would not be restricted to the lymphoid DC subset. We therefore isolated myeloid and lymphoid DCs soon after injection of OVA/β2m$^{-/-}$ cells and evaluated their OT-I stimulatory capacity (Fig. 6). As early as 2 h after injection, all the OVA stimulatory activity was found in the lymphoid DC subset. Unless transfer of antigens between DCs or the maturation of myeloid DCs into lymphoid DCs is an extremely rapid process, this result suggests that the lymphoid DCs themselves take up and present the cell-associated antigens in vivo.

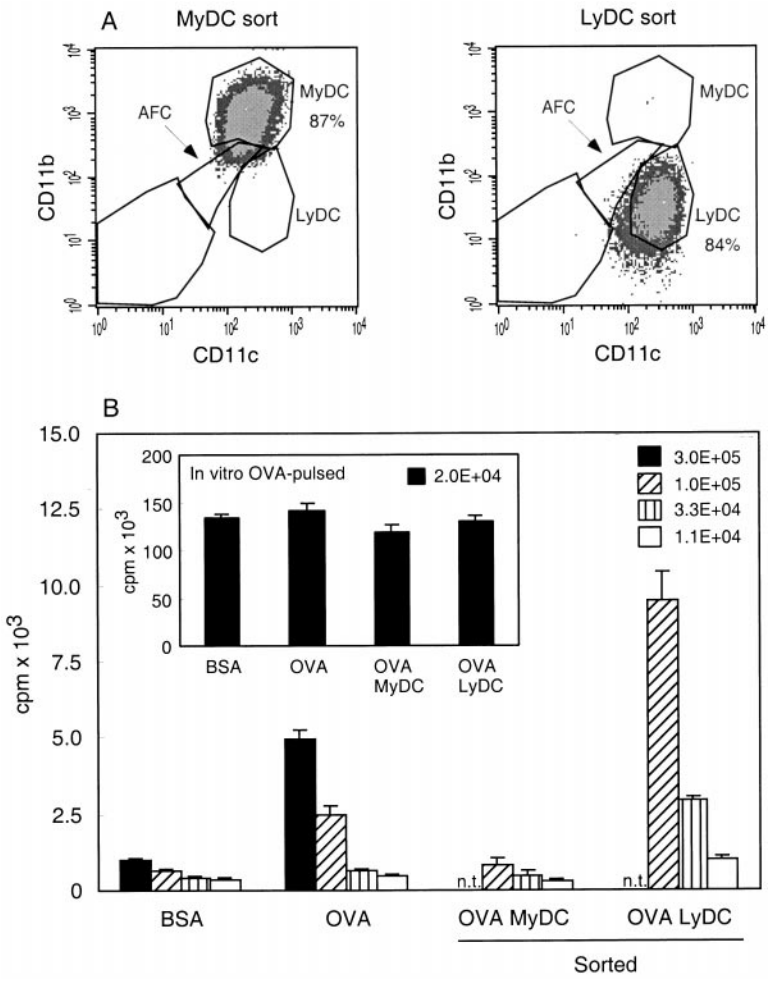

Figure 5. Lymphoid DCs, but not myeloid DCs, cross-present OVA antigen. Low density cells were isolated from mice primed previously with OVA- or BSA-loaded $\beta 2m^{-/-}$ cells. Myeloid and lymphoid DCs from OVA/$\beta 2m^{-/-}$-injected mice were (A) FACS® sorted based on their differential CD11b/CD11c expression and (B) tested for their ability to stimulate naive OT-I cells in vitro. The different bars represent titered numbers of DCs in the well. The proliferation of OT-I induced by peptide-pulsed cells is depicted in the inset. One of three experiments with similar results is shown. Error bars indicate SEM of triplicate wells. MyDC, myeloid DCs; LyDC, lymphoid DCs; AFC, autofluorescent cells, mostly macrophages; n.t., not tested.

Myeloid DCs Take Up Cell-associated Antigens. The lack of presentation by myeloid DCs could be explained if they do not have access to the injected, antigen-bearing cells. This could either be due to the failure of the injected cells to home to the marginal zone areas where myeloid DCs reside, or the myeloid DCs may lack specific surface receptors for the internalization of cell-associated antigen. To determine whether myeloid DCs take up cell-associated antigens, $\beta 2m^{-/-}$ cells were shocked with both OVA and yellow/green fluorescent beads and injected into B6 mice. Splenic low density cells were isolated 14 h after injection, stained for CD11c and CD11b, and analyzed by flow cytometry (Fig. 7 A). Cells containing one or more fluorescent beads were detected in the FITC channel. The CD11c and CD11b FACS® profile of bead-positive cells clearly shows that in addition to lymphoid DCs, both myeloid DCs and autofluorescent macrophages have taken up beads derived from $\beta 2m^{-/-}$ cells (Fig. 7 B). This result indicates that the restricted presentation by lymphoid DCs is not due to restricted uptake of cell-associated antigens.

Only a Small Fraction of Lymphoid DCs Present OVA. We made two estimates of the fraction of cells within the isolated low density preparation that could present OVA to OT-1 after in vivo uptake of antigen. At 14 h after injection of cells loaded with OVA and fluorescent beads, 0.8% of the total low density cell preparation was bead-positive, as determined by FITC fluorescence (Table I). Within the lymphoid DC subset, which made up 27% of the cells, 1% were bead-positive. Therefore, only 0.27% of the total low density cell preparation was estimated to contain and present OVA to OT-1 cells.

Second, the amount of OT-1 proliferation stimulated by in vivo–derived low density cells was compared with that

Table I. *In Vivo Uptake of Cell-associated Beads*

	Percentage of total	Percentage of bead$^+$ cells in each subset
Low density cell preparation	100	0.8
Myeloid DCs	41	0.6
Lymphoid DCs	27	1.0
Autofluorescent cells	13	1.5

The percentage of different subsets in low density cell preparation and the percentage of each that contain beads.

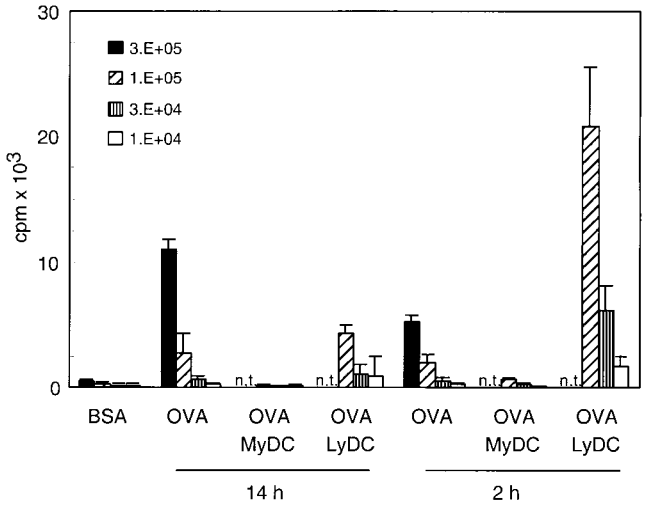

Figure 6. MHC class I–restricted cross-presentation is restricted to lymphoid DCs as early as 2 h after priming. Low density cells were isolated from mice 2 or 14 h after priming with OVA-loaded $\beta 2m^{-/-}$ cells. Myeloid and lymphoid DCs were FACS® sorted based on their differential CD11b/CD11c expression and tested for their ability to stimulate naive OT-I cells in vitro. The different bars represent titered numbers of DCs in the well. Error bars indicate SEM of triplicate wells. MyDC, myeloid DCs; LyDC, lymphoid DCs; n.t., not tested.

stimulated by a titration of in vitro peptide-pulsed low density cells. This comparison suggested that 0.32% of lymphoid DCs, or 0.06% of low density cells, presented OVA (average of three experiments, data not shown). This is presumably an underestimate of the number of OVA-presenting cells, as the amount of OVA acquired in vivo is probably lower than the amount of OVA used for exogenous peptide pulsing. Together, these experiments suggest that only ~1% of the lymphoid DC subset present OVA in vitro.

Discussion

Cross-presentation of exogenous antigens in association with MHC class I molecules has been studied extensively in vitro. Both macrophages and DCs have been shown to present peptides derived from soluble and particulate antigens in the context of MHC class I molecules (for a review, see reference 7). In addition, antigens from apoptotic or necrotic cells can be processed and presented in MHC class I by both cell types in vitro (15, 19, 53, 54). In contrast, there is little information concerning the identity of cross-presenting cells in vivo. DCs have been shown to present soluble protein-derived and cell-associated antigens in MHC class II molecules (48, 51, 55), but the cell that cross-presents antigens in association with MHC class I molecules in vivo has remained elusive. In our study, we have isolated low density spleen cells from in vivo–primed mice and shown that they present exogenously derived antigens in association with MHC class I in a TAP-dependent manner. Further analysis of the cross-presenting cells identified them as $CD11c^+$ DCs. This is the first isolation and characterization of DCs in the central lymphoid organs that have taken up cell-associated antigens to present them to $CD8^+$ T cells in vivo.

Further experiments showed that only the lymphoid DC subset cross-presents cell-associated antigens in MHC class I molecules in the spleen. The number of actual OVA-presenting cells is very low. We estimated that only 1% of lymphoid DCs present OVA to T cells. The lymphoid DC subset–restricted presentation was evident as early as 2 h after immunization, which strongly suggests that lymphoid DCs themselves take up the exogenous cell-associated antigen. Transfer of antigen between different DC subsets, as had been suggested, is therefore unlikely (41, 48, 49). In support of the notion of direct uptake of antigen by lymphoid DCs, rat DCs containing apoptotic cell remnants have been found to migrate to the T cell areas of lymph nodes. These DCs were $CD4^-$ and may be the rat equivalent of the mouse lymphoid DCs (56). Our finding that only lymphoid DCs cross-present cell-associated antigens in the spleen does not preclude the presence of other types of APCs with cross-priming function in other tissues. APCs have been isolated from tumors and islets of Langerhans that could stimulate $CD8^+$ T cells specific for tumor and islet antigens, respectively (57–59). One of these studies defined the phenotype of the APCs as macrophage like (57).

Why do only lymphoid and not myeloid DCs cross-present cell-associated antigens to $CD8^+$ T cells? In principle, it could be the result of differences in the uptake of cell debris, processing ability, or their T cell stimulatory capacity. We show that, in addition to lymphoid DCs, both au-

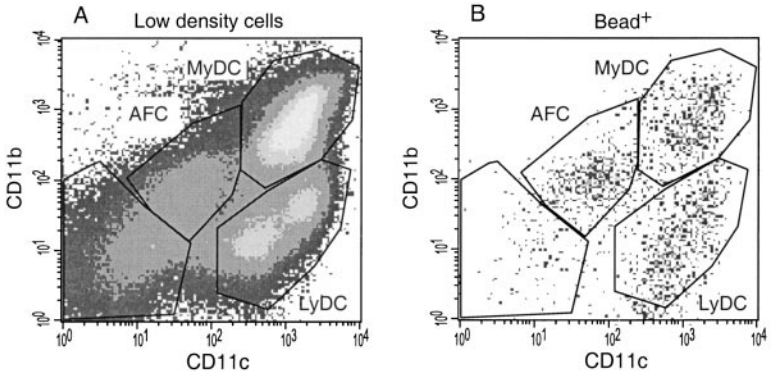

Figure 7. Uptake of cell-associated beads by both DC subsets and autofluorescent macrophages. Low density cells were isolated from mice 14 h after priming with OVA/bead-loaded $\beta 2m^{-/-}$ cells. (A) The cells were stained for CD11c and CD11b and analyzed by FACS®. MyDC, myeloid DCs; LyDC, lymphoid DCs; AFC, autofluorescent cells, mostly macrophages. (B) CD11c and CD11b profile of the gated low density cells containing beads.

tofluorescent macrophages and myeloid DCs contain cell-associated beads. This indicates that uptake of cell-associated antigen is not restricted to lymphoid DCs. This contrasts with the study by Fossum and Rolstad, in which allogeneic lymphocyte cell debris was found mainly in lymphoid DCs (60). However, differences in the in vivo model systems used may explain this discrepancy. Second, myeloid and lymphoid DCs do not seem to differ in their capacity to stimulate T cells, as both DC subsets have been shown to stimulate $CD8^+$ T cells efficiently in vitro and in vivo (39). This leaves differences in antigen processing pathways between myeloid and lymphoid DCs as the most likely explanation. A selective transport of internalized antigens to the cytosol has been shown to occur in DCs and to be absent in macrophages (20). Both bone marrow–derived DCs and a splenic-derived DC line exhibited this ability, but ex vivo DCs were not evaluated. Our results suggest that this endosome to cytosol transport will be limited to lymphoid DCs.

Cross-presentation of cell-associated antigens to $CD8^+$ T cells has been shown to result in either T cell activation or tolerance (1, 2, 8–10, 61, 62). In both cases, bone marrow–derived cells are necessary (8–10). Lymphoid DCs have been speculated to mediate peripheral tolerance, whereas myeloid DCs have been suggested to be essential for T cell stimulation (49, 63). Our studies do not specifically address the outcome of the DC–T cell interaction, as T cell proliferation is involved in both processes. However, our method of intravenous immunization with OVA-loaded cells has repeatedly been shown to lead to T cell activation and memory (10, 61, 64). Therefore, we are convinced that we have isolated the APC required for $CD8^+$ T cell cross-priming.

An attractive alternative explanation for the different outcomes of DC–T cell interaction is offered by several studies indicating that the activation state of the DCs is an important factor. DCs involved in cross-priming are activated via CD40–CD40L interaction provided by $CD4^+$ T cells (64–66). $CD8^+$ T cell cross-priming does not occur in the absence of $CD4^+$ T cells, illustrating the crucial role of $CD4^+$ T cells in this process (10). Likewise, $CD4^+$ T cells play an important role in preventing the induction of $CD8^+$ T cell tolerance to self-antigens, as the addition of antigen-specific $CD4^+$ T cells converts $CD8^+$ T cell tolerance induction into $CD8^+$ T cell activation (67). Furthermore, in several other tolerance induction systems, in vivo CD40 activation resulted in abrogation of tolerance and in T cell activation (68–71). Taken together, these studies strongly suggest that the activation state of the DCs is an important factor in determining the outcome of the DC–T cell interaction and that lymphoid DCs may be implicated in both activation and tolerization of antigen-specific $CD8^+$ T cells.

Recent studies suggest different functions for lymphoid and myeloid DCs in the activation of $CD4^+$ T cells, preferentially inducing Th1 and Th2 responses, respectively (40–42). The capacity of lymphoid DCs to drive Th1 responses is explained by their production of IL-12 and IFN-γ (27, 52, 72). Furthermore, the localization of the different DC and Th subsets appears to be similar. Th1 cells primarily reside in proximity to the lymphoid DCs in the central T cell zone of the PALS, whereas Th2 cells are found in the outer PALS near the B cell follicles and the marginal zone where myeloid DCs are located (73). Differential chemokine receptor expression patterns in both Th and DC subsets are essential in establishing this homing pattern (73–76). Our data add a new role for the lymphoid DCs as the main stimulator of $CD8^+$ T cell responses specific for cell-associated antigens. This points to an interaction between lymphoid DCs, $CD8^+$ T cells, and Th1 cells. It remains to be investigated whether $CD8^+$ T cell cross-priming is specifically associated with and dependent on Th1 responses.

In summary, our data demonstrate that lymphoid DCs play a key role in cross-presentation of cell-associated antigens to $CD8^+$ T cells in vivo. Because cross-presentation enables the activation of naive T cells specific for antigens that are not expressed by the APCs themselves, we envisage that lymphoid DCs will be essential for the induction of a large spectrum of $CD8^+$ T cell responses.

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