The dominant role of CD8$^+$ dendritic cells in cross-presentation is not dictated by antigen capture

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Mouse spleens contain three populations of conventional (CD11c^{high}) dendritic cells (DCs) that play distinct functions. The CD8$^+$ DC are unique in that they can present exogenous antigens on their MHC class I molecules, a process known as cross-presentation. It is unclear whether this special ability is because only the CD8$^+$ DC can capture the antigens used in cross-presentation assays, or because this is the only DC population that possesses specialized machinery for cross-presentation. To solve this important question we examined the splenic DC subsets for their ability to both present via MHC class II molecules and cross-present via MHC class I using four different forms of the model antigen ovalbumin (OVA). These forms include a cell-associated form, a soluble form, OVA expressed in bacteria, or OVA bound to latex beads. With the exception of bacterial antigen, which was poorly cross-presented by all DC, all antigenic forms were cross-presented much more efficiently by the CD8$^+$ DC. This pattern could not be attributed simply to a difference in antigen capture because all DC subsets presented the antigen via MHC class II. Indeed, direct assessments of endocytosis showed that CD8$^+$ and CD8$^-$ DC captured comparable amounts of soluble and bead-associated antigen, yet only the CD8$^+$ DC cross-presented these antigenic forms. Our results indicate that cross-presentation requires specialized machinery that is expressed by CD8$^+$ DC but largely absent from CD8$^-$ DC. This conclusion has important implications for the design of vaccination strategies based on antigen targeting to DC.

antigen presentation | mice | endocytosis | ovalbumin | vaccines

Dendritic cells (DC) possess several mechanisms that make them highly efficient antigen-presenting cells. DC can endocytose a large variety of exogenous antigens for presentation via MHC class II molecules and for cross-presentation via MHC class I (1). The cross-presenting capacity of DC is unusual, because most other cell types are only able to present endogenous antigens (i.e., antigens synthesized by the antigen-presenting cells themselves) on their MHC class I molecules. Thus, DC possess specialized machinery, as yet not fully defined, that allows delivery of exogenous antigens into the MHC class I presentation pathway for cross-presentation (2, 3).

On the other hand, several populations of DC have been described (4, 5), and it is unclear whether all these DC types can cross-present (6). Mouse spleens contain three "conventional" (CD11c^{high}) DC subsets: CD8$^+$ DC, CD4$^+$ DC, and CD4$^-$CD8$^-$ [double negative (DN)] DC. Previous studies have shown that cell-associated antigen was cross-presented by CD8$^+$ but not CD8$^-$ DC (7–10). The unique capacity of the CD8$^+$ DC at cross-presenting this form of antigen was attributed to their ability to capture dead cells (7–9, 11, 12) because antigens in soluble or immunocomplexed form, or associated to bacteria, were reportedly cross-presented by both CD8$^+$ and CD8$^-$ DC (8, 9, 13). These findings led to the hypothesis that all DC can cross-present and that the role of each DC subset in cross-presentation of a given antigen is dictated simply by their ability to capture that antigen. However, our own observations with soluble antigen revealed that both CD8$^+$ and CD8$^-$ DC captured this form of antigen *in vivo*, but only the CD8$^+$ DC cross-presented it efficiently (14). This finding suggested that CD8$^+$ DC possess specialized machinery for cross-presentation that is expressed poorly, if at all, by other DC subsets. We therefore hypothesized that the role of different DC subsets in cross-presentation is determined by their antigen-handling properties.

Understanding whether the capacity to cross-present is dictated by antigen capture or handling is important for the design of vaccination strategies based on antigen targeting *in vivo* (15–19). For instance, if all of the DC subsets can cross-present, a vaccine antigen targeted to a receptor shared by all subsets might be cross-presented by all DC. If, on the other hand, only CD8$^+$ DC are capable of cross-presentation, the antigen carried by such a vaccine might be cross-presented only by this DC subset. Furthermore, in the latter scenario an antigen targeted to any CD8$^-$ DC subset might not be cross-presented at all. Because different DC types may play distinct roles in induction of immunity and tolerance (6, 20–23) the outcome of the vaccination strategy in each scenario might vary (17).

In this study we show that, of the three splenic DC subsets, CD8$^+$ DC are the most efficient at cross-presentation of cellular, soluble, or latex bead-associated antigens. This ability does not appear to be dictated solely by their capacity to capture the antigen, because all three subsets efficiently presented all these forms of antigen by the MHC class II pathway. Our results support the notion that CD8$^+$ DC possess specialized machinery to deliver different forms of antigen to the cross-presentation pathway and that this machinery is largely absent in the other splenic DC subsets. This conclusion should be considered when designing vaccination strategies based on DC targeting.

Results

Mouse spleens contain three conventional DC subsets that can be distinguished by their expression of CD4 and CD8: CD4$^+$ DC, CD8$^+$ DC, and DN DC (Fig. 1*A*) (24). These three DC populations are immature in the steady state, but upon culture *in vitro*

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Abbreviations: CFSE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; OVA, ovalbumin; DN, double negative.

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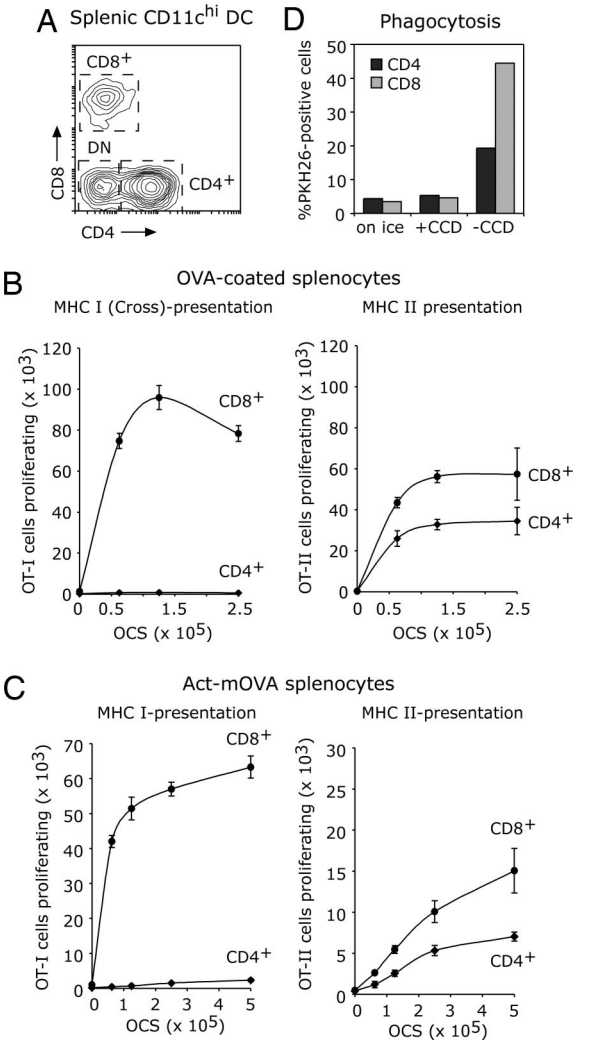

Fig. 1. *In vitro* cross-presentation of cell-associated antigens by splenic DC subsets. (*A*) FACS analysis of a splenic DC preparation. The plot shows the expression of CD4 and CD8 in CD11c$^+$ cells (≈70–80% of the total). Three populations can be distinguished: CD8$^+$CD4$^-$ DC (CD8$^+$ DC), CD8$^-$CD4$^+$ DC (CD4$^+$ DC), and CD8$^-$CD4$^-$ DC (DN DC). B220$^+$ plasmacytoid DC were excluded from the DC preparation (44). (*B*) Purified spleen DC were cultured with irradiated OVA-coated bm1 splenocytes and naïve CFSE-labeled OT-I T cells (*Left*) or with OVA-coated MHC class II-deficient splenocytes and naïve CFSE-labeled OT-II T cells (*Right*). The number of proliferating (propidium iodide$^-$ CFSE^{low}) T cells was determined by FACS analysis at 60–65 h. All of the data point determinations were performed in duplicate. The error bars represent the range of the values obtained. The results shown are representative of multiple experiments. (*C*) Same as in *B*, but the source of OVA was splenocytes from act-mOVA mice backcrossed to bm1 mutant mice (presentation to OT-I cells; *Left*) or backcrossed to MHC class II-deficient mice (OT-II; *Right*). (*D*) Uptake of PKH26-labeled splenocytes by DC subsets. Sorted CD4$^+$ (dark bars) or sorted CD8$^+$ (light bars) DC were cultured for 3 h with γ-irradiated PKH26-labeled splenocytes on ice or at 37°C in the presence or absence of 10 μM cytochalasin D (CCD). The percentage of PKH26$^+$ CD11c$^+$ DC was then determined by flow cytometric analysis (Fig. 4). Data represent one experiment performed with sorted DC, but similar results were obtained in two experiments using unsorted DC.

they become spontaneously activated and acquire a mature phenotype (25, 26). Thus, by purifying these DC and exposing them to antigen and to naïve T cells *in vitro*, it is possible to mimic the process of antigen encounter and maturation *in vivo* (27). We used this approach to compare the ability of the splenic DC subsets to cross-present different forms of antigen.

Presentation of Cell-Associated Antigen by Splenic DC Populations. We purified the CD4$^+$ and CD8$^+$ DC populations and incubated them with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OT-I cells together with 0.6–2.5 $\times$ 10^5 irradiated ovalbumin (OVA)-coated splenocytes, corresponding to 0.5–2 ng of OVA protein (28). To prevent the presentation of OVA by the OVA-coated splenocytes, these cells were derived from H-2^{bm1} mutant (bm1) mice, which express MHC class I molecules that cannot present OVA to OT-I cells (29). In this assay, the OT-I cells could proliferate only if cell-associated OVA was cross-presented by the DC. Proliferation was quantitated 60 h later by flow cytometry by determining the number of CFSE^{low} OT-I cells. As shown in Fig. 1*B Left*, only the CD8$^+$ DC could cross-present OVA-coated spleen cells to OT-I cells. Similar results were obtained when the splenocytes were purified from bm1 transgenic mice expressing OVA under the control of the actin promoter (30) (Fig. 1*C Left*). These results indicated that, whether the antigen was coated on cells or expressed as a membrane protein, cross-presentation of cell-associated antigen was primarily mediated by CD8$^+$ DC.

The dominant cross-presenting capacity of the CD8$^+$ DC could be attributed to two possibilities: (*i*) CD8$^+$ DC might be the only DC that can endocytose cell-associated antigen *in vitro* (antigen uptake), or (*ii*) CD8$^+$ DC might possess specialized machinery to direct endocytosed antigens into the MHC class I presentation pathway (antigen handling). To distinguish between these two possibilities, we determined the capacity of the DC subsets to present cell-associated OVA by the MHC class II pathway to CD4$^+$ OT-II cells. For these experiments, the splenocytes used as an antigen source were deficient in I-A^b to ensure that only the DC would be capable of presenting antigen via the MHC class II pathway. As shown in Fig. 1 *B* and *C Right*, both the CD4$^+$ and CD8$^+$ DC presented these forms of cell-associated antigen to OT-II cells, albeit the CD8$^+$ DC were more efficient than their CD4$^+$ counterparts. Obviously, because antigen uptake is required for MHC class II presentation, this result demonstrated that the CD4$^+$ DC were able to capture cell-associated antigen *in vitro*, although as demonstrated in previous studies they appeared less efficient than the CD8$^+$ DC (8, 9). To directly compare the ability of the two DC subsets to capture cellular antigens *in vitro*, we incubated them with splenocytes labeled with the membrane dye PKH26. After a 3-h incubation period, ≈45% of the CD8$^+$ DC and nearly 20% of the CD4$^+$ DC were positive for PKH26, indicating capture of splenocyte-derived material (Fig. 1*D* and Fig. 4, which is published as supporting information on the PNAS web site). Capture by either subset was prevented if the incubation was carried out at 4°C or if cytochalasin D, an inhibitor of phagocytosis, was included during the 3-h incubation at 37°C. These controls indicated that the acquisition of PKH26 by the DC was due to active phagocytosis of labeled cells, or cell fragments, rather than to adherence of the labeled cells on the DC surface. The difference in capture of cellular material between the CD8$^+$ and CD4$^+$ DC correlated well with the ≈2-fold difference in their efficiency of presentation of cell-associated OVA via MHC class II. Nevertheless, such difference in antigen capture could not explain the inefficiency of cross-presentation by the CD4$^+$ DC, suggesting that these two DC subsets also differed at the level of antigen handling. Separate comparisons of the antigen-presenting capacities of DN and CD8$^+$ DC revealed differences similar to those described between CD4$^+$ and CD8$^+$ DC (data not shown). We stress that these results confirm the observations of other groups, namely that CD8$^+$ DC are more efficient than other DC types at capturing dying cells; however, they also demonstrate that antigen capture alone is not sufficient for cross-presentation because, even in situations where CD8$^-$ DC capture cell-associated antigens and present them via MHC class II, they lack the ability to cross-present those same antigens.

To discard the possibility that the $CD8^+$ DC possess a special feature, e.g., a differentially expressed costimulatory molecule that enables them to preferentially stimulate naïve $CD8^+$ T cells, we incubated the three DC subsets with the antigenic peptides recognized by the OT-I and the OT-II T cells. As expected, all DC populations were equally capable of T cell activation (Fig. 5, which is published as supporting information on the PNAS web site). Furthermore, all three DC populations purified from act-mOVA transgenic mice, which express OVA endogenously (30), induced OT-I and OT-II proliferation similarly (data not shown). Finally, we have previously shown that $CD8^+$ and $CD8^-$ DC present endogenous viral proteins via the "classical" MHC class I presentation pathway and stimulate naïve T cells specific for such antigens, with comparable efficiency (31). These experiments confirm that all splenic DC subsets have a comparable capacity to activate naïve T cells.

Cross-Presentation of Soluble Antigen. Next we measured uptake and presentation of soluble OVA *in vitro*. The $CD8^+$ and $CD8^-$ DC subsets (the latter comprising the $CD4^+$ and DN DC populations) captured comparable amounts of OVA conjugated to the pH-insensitive fluorochrome Alexa Fluor 488 (Fig. 2*A*) or to the fluorochrome FITC (data not shown). Similar results were obtained in measurements of uptake of BSA conjugated to FITC (data not shown). We then measured presentation of OVA antigen. The $CD8^+$ DC were far more efficient than the other two populations at cross-presenting soluble OVA (Fig. 2*B Left*), but all three splenic DC subsets presented this antigen via MHC class II with comparable efficiency (Fig. 2*B Right*). Furthermore, cross-presentation of OVA to OT-I cells by $CD8^+$ DC was clearly and consistently observed after incubations with doses as low as 15 μg/ml antigen for only 45 min. This amount of OVA was also the minimum required to detect presentation via MHC class II to OT-II cells. This finding contrasts with previous reports that used soluble antigen to assess the cross-presenting capacity of mouse bone marrow-derived or human monocyte-derived DC, which concluded that the concentration of antigen that was required for cross-presentation was high and probably nonphysiological (32–37). Our results thus demonstrate that cross-presentation is a highly efficient process in the $CD8^+$ DC population.

Cross-Presentation of Bacterial Antigen. Another form of antigen that has been reported to be cross-presented *in vitro* with similar efficiency by both $CD8^+$ and $CD8^-$ DC is OVA contained in paraformaldehyde-fixed bacteria (8). However, that report did not assess in parallel MHC class II antigen presentation as an indirect measurement of antigen capture or processing, which might have been affected by the fixation of the bacteria. Therefore, we incubated $CD8^+$ and $CD8^-$ DC with fixed OVA^+ *Escherichia coli* and CFSE-labeled OT-I or OT-II cells. Both DC subsets presented this form of OVA via MHC class II and, as previously reported, cross-presented it via MHC class I (Fig. 2*C*). However, this result appeared to reflect an inefficient capacity of the $CD8^+$ DC to cross-present this particular form of OVA rather than enhanced cross-presentation by the $CD8^-$ DC. This conclusion is based on the observation that, for a given amount of soluble or cell-associated OVA, $CD8^+$ DC always induced a stronger proliferative response by the OT-I cells relative to the OT-II cells (Figs. 1 *B* and *C* and 2*B*), but in the case of OVA associated with fixed bacteria, the $CD8^+$ DC induced a poorer OT-I response. We suggest that the fixing process, or the way OVA is expressed by *E. coli* (probably as a misfolded protein), hampers the delivery of the antigen to the cross-presentation pathway so that the cross-presenting capacity of the $CD8^+$ and the $CD8^-$ DC appears similar but poor.

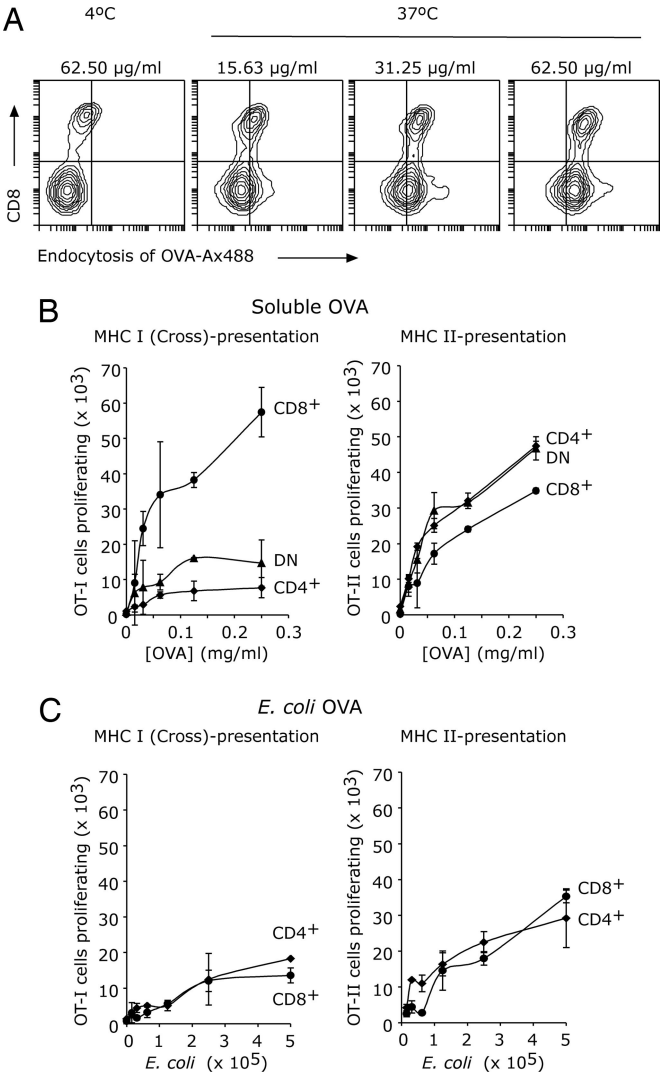

Fig. 2. Uptake and *in vitro* cross-presentation of soluble and bacterial antigens by splenic DC subsets. (*A*) Purified splenic DC were incubated with the indicated concentrations of OVA–Alexa Fluor 488 (OVA-Ax488) for 45 min at 4°C or 37°C. The cells were washed, labeled with antibodies for CD11c and CD8, and analyzed by flow cytometry. The plots show uptake of antigen by the $CD8^+$ and $CD8^-$ DC subset. Endocytosis was measured as an increase in mean linear fluorescence in the FL1 channel. The results shown are representative of multiple experiments. (*B*) Sorted spleen DC subsets were pulsed with the indicated concentration of soluble OVA for 45 min, washed, and cocultured for 60–65 h with naïve OT-I cells (*Left*) or OT-II cells (*Right*). The number of proliferating T cells was then determined by FACS analysis. All determinations were obtained in duplicate; the error bars show the range of the values obtained. The results shown are representative of multiple experiments. (*C*) Sorted spleen DC subsets were cultured with the indicated number of paraformaldehyde-fixed OVA-expressing *E. coli* plus OT-I cells (*Left*) or OT-II cells (*Right*). The number of proliferating T cells was then determined by FACS analysis. All determinations were obtained in duplicate; the error bars show the range of the values obtained. The results shown are representative of four experiments.

Cross-Presentation of Antigen Associated to Fluorescent Latex Beads. The experiments carried out *in vitro* strongly supported the hypothesis that cross-presentation by DC requires not only antigen capture but also the expression of specialized machinery, which is expressed only, or predominantly, by the $CD8^+$ DC. To confirm this finding, we opted to use fluorescent beads associated with OVA. Unlike dead cells, latex beads are captured with comparable efficiency by $CD8^+$ and $CD8^-$ DC *in vivo* (Fig. 3*A*)

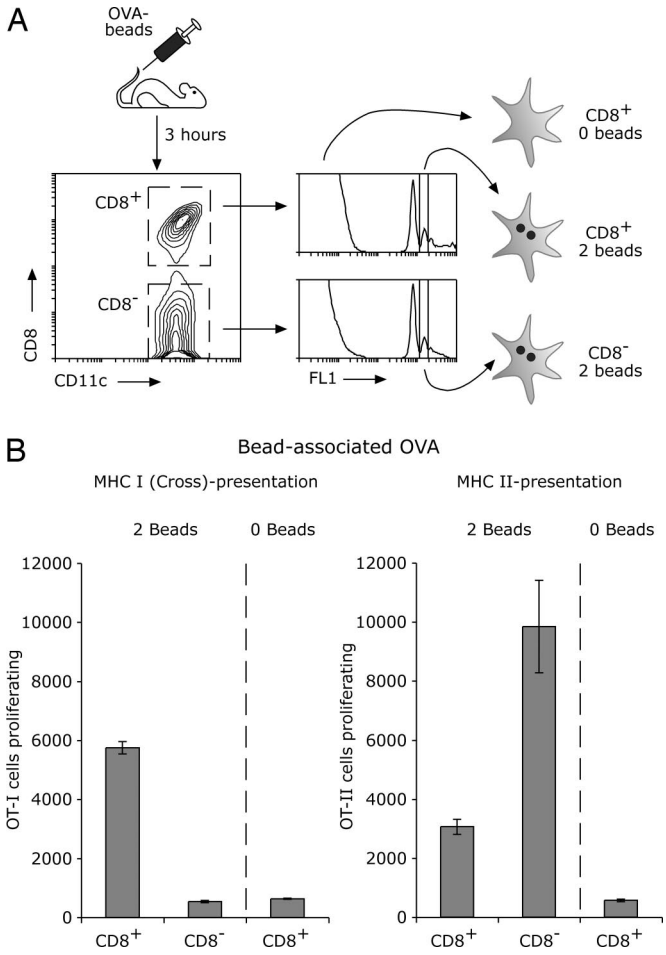

Fig. 3. Cross-presentation of bead-associated antigen by splenic DC subsets. (*A*) Mice were injected i.v. with 200 μl of streptavidin-conjugated fluorescent latex beads that had been conjugated with biotinylated OVA (OVA-beads). Three hours later, spleens were removed and the DC were purified. Cells were stained with antibodies for CD11c and CD8. Preparative flow cytometry was then used to purify $CD8^+$ and $CD8^-$ DC that had captured two beads and $CD8^+$ DC that had not endocytosed any beads. (*B*) Splenic DC subsets were isolated as outlined in *A* and incubated with CFSE-labeled OT-I (*Left*) or OT-II (*Right*) T cells. The number of proliferating $CFSE^{low}$ T cells was determined by flow cytometry 60–65 h later. All determinations were performed in duplicate. The error bars indicate the range of the values obtained. Results shown are representative of three independent experiments.

(9). This property allowed us to compare the relative antigen-presenting capacity of the two subsets after capturing equal amounts of antigen *in vivo*.

We coated avidin-linked fluorescent latex beads with biotinylated OVA and injected them i.v. into mice. Three hours later, $CD8^+$ and $CD8^-$ DC that had captured exactly two beads were purified by FACS, along with $CD8^+$ DC that had failed to capture any beads as a negative control (Fig. 3*A*). The three DC groups were then incubated with OT-I or OT-II cells. Again, the $CD8^+$ DC that captured the OVA-coated beads were the only DC that cross-presented the antigen via MHC class I, whereas the $CD8^-$ DC were more efficient at presenting it via MHC class II (Fig. 3*B*). Because the amount of OVA internalized by the two subsets was the same, this result unequivocally confirms that $CD8^+$ DC are endowed with a unique capacity to deliver exogenous antigens to the cross-presentation pathway.

Discussion

One of the most striking specializations of DC is their capacity to cross-present exogenous antigens on MHC class I molecules. This property may enable DC to present tissue-specific antigens for the induction of tolerance in the steady state and viral antigens for the induction of immunity in the course of viral infections (6, 31). Moreover, cross-presentation might be exploited in vaccination strategies to induce tolerogenic or immunogenic reactions *in vivo*. It has been shown that targeting exogenous antigens to immature DC can induce tolerance, whereas if the antigen is inoculated together with an activatory stimulus the targeted DC induce immunity (15, 16). These observations encourage a thorough investigation of efficient methods for delivery of antigens for cross-presentation *in vivo* (17–19). However, until now it has been unclear whether all DC are equipped with the capacity to cross-present and whether the form of antigen can influence this ability (6). In this report we have compared the relative cross-presenting capacity of the three conventional DC subpopulations found in mouse spleens.

Our results show that the capacity to cross-present is not dictated by antigen capture but by the ability to deliver the antigen to the cross-presentation pathway, a capacity found primarily in the $CD8^+$ DC subset. The strongest experimental evidence that supports this conclusion was obtained by assessing the presentation of OVA bound to fluorescent latex beads administered *in vivo* (Fig. 3). In conditions in which $CD8^+$ and $CD8^-$ DC had captured exactly the same amount of antigen (that associated with two beads) only the $CD8^+$ DC cross-presented OVA, even though the $CD8^-$ DC were more efficient than the $CD8^+$ DC at presenting this form of antigen via the MHC class II pathway. Analyses of presentation of cell-associated, soluble, and bacterial antigens further supported our conclusions.

Several reports have shown that dead cells are endocytosed *in vivo* only by $CD8^+$ DC (9, 11, 12). As a consequence, this is the only DC type that presents cellular antigens *in vivo*, not only on MHC class I molecules (via cross-presentation), but also on MHC class II molecules. We have confirmed that $CD8^+$ DC are the only subset that cross-presents cell-associated OVA *in vivo* (data not shown) and *in vitro* (Fig. 1). However, our analysis shows that the $CD8^-$ DC types can capture this form of antigen and present it via MHC class II *in vitro*, although not as efficiently as the $CD8^+$ DC. These results may appear inconsistent with earlier findings showing that only $CD8^+$ DC captured cellular material *in vitro* (8, 9, 11). This discrepancy might be due to differences in the source of cellular antigen examined by each group, which in our case was irradiated OVA-coated or OVA-expressing spleen cells that were not specifically apoptotic. Nevertheless, our assessment of cross-presentation of cell-associated OVA to OT-I cells, performed in parallel with the assessment of the capacity to present to OT-II cells, demonstrates that only $CD8^+$ DC deliver this form of antigen to the cross-presentation pathway.

Analysis of uptake and presentation of soluble OVA enabled us to evaluate more directly the relationship between antigen capture and cross-presentation. All three DC subsets endocytosed comparable amounts of soluble OVA and presented it via the MHC class II pathway, but the $CD8^+$ DC were far more efficient at cross-presenting this antigen. These experiments have also allowed us to gain a better understanding of the efficiency of the cross-presentation pathway. The reported inefficiency of cross-presentation of soluble antigen by several types of DC grown *in vitro* (32–37), and the high doses of antigen required to be injected *in vivo* to observe cross-presentation (7, 13, 14), have called into question the importance of this pathway under physiological conditions (38). However, as we have shown here, the minimum concentration of soluble OVA that the $CD8^+$ DC required for cross-presentation was similar to the minimum required for MHC class II presentation (in the 10–20 μg/ml range; Fig. 2). Moreover, in our *in vitro* assays the DC were incubated with soluble OVA for only 45 min and then were washed before exposure to the T cells. Therefore, our results

demonstrate that cross-presentation by $CD8^+$ DC is a highly efficient process that does not require overloading with antigen. The reason cross-presentation of soluble OVA injected i.v. requires high doses of the antigen is probably that only a small amount of antigen reaches the DC in the spleen. Indeed, detecting the presentation of soluble antigen by DC via the MHC class II pathway *in vivo* also requires high doses of antigen (14), and no one would argue that DC are inefficient at MHC class II presentation.

Finally, we also tested the ability of $CD8^+$ and $CD8^-$ DC to cross-present bacteria-associated antigen, which has been previously shown to be cross-presented comparably by the two DC groups (8). This form of antigen appeared to access poorly the cross-presentation pathway in all DC. This conclusion was suggested because, for any given amount of soluble, cell-associated, or bead-associated antigen captured, the $CD8^+$ DC consistently induced stronger proliferative responses of OT-I cells than of OT-II cells. However, the OT-I responses induced by either the $CD8^+$ or $CD8^-$ DC exposed to fixed OVA^+ *E. coli* were lower than the OT-II responses. Our interpretation of this result is that the way OVA is expressed in *E. coli* (most likely as a misfolded aggregated protein), or the fixation process required to conduct the experiment with this form of antigen, prevents the correct delivery of OVA to the cross-presentation pathway. Because the specific mechanisms used by DC to cross-present antigens are still poorly understood (2, 3), we did not attempt to identify the exact cause for the poor presentation of this particular form of antigen.

We conclude that $CD8^+$ DC are constitutively equipped with machinery that efficiently delivers exogenous antigens into the cross-presentation pathway. These conclusions contribute to a better understanding of the unique roles played by different DC populations and for the design of antigen-targeting strategies for the induction of tolerogenic or immunogenic reactions *in vivo*. Our results predict that antigens taken by $CD8^-$ DC will not be efficiently cross-presented, so vaccination strategies based on antibody-mediated antigen targeting to induce cross-priming (15–19) should be designed to target specifically the $CD8^+$ DC subset. Naturally, our results do not discard that other DC populations not analyzed in this study, such as the "tissue-derived DC" (5) found in lymph nodes, may also be endowed with cross-presentation machinery. Indeed, a recent report by Stoitzner *et al*. (39) showed that Langerhans cells, a DC type derived from the epidermis, can cross-present soluble antigen, albeit less efficiently than $CD8^+$ DC.

Experimental Procedures

Mice. We used 6- to 12-week-old C57BL/6, B6.CH-2^{bm-1} (bm1), OT-I (40), OT-II (41), MHC class II-deficient (42), and act-mOVA transgenic mice (30) backcrossed to bm1 or MHC class II-deficient mice. All mice were bred and maintained under specific pathogen-free conditions at The Walter and Eliza Hall Institute animal breeding facility according to institute guidelines. Where indicated, mice were injected i.v. in the tail vein with 1.0-μm streptavidin-coated Fluoresbrite YG microspheres (Polysciences, Warrington, PA) coupled to biotinylated OVA (Sigma) as described below.

DC Isolation, Flow Cytometric Analysis, and Sorting. DC were isolated from mouse spleens as described elsewhere (24, 26, 43). This protocol excluded $B220^+$"plasmacytoid DC" from the DC preparation (44). To prevent DC maturation during the isolation protocol, the procedure was carried out on ice, except for an initial 20-min digestion with collagenase/DNase, which was performed at room temperature. Analytical and preparative FACS was carried out as described (26) by employing a FACStar Plus, FACScan, FACSDiva, LSR (Becton Dickinson), or Mo-Flo (Cytomation, Fort Collins, CO) instrument. Culture of DC *in vitro* was carried out in RPMI medium 1640 supplemented with 10% FCS/50 μM 2-mercaptoethanol/2 mM L-glutamine/100 units/ml penicillin/100 μg/ml streptomycin/200 units/ml GM-CSF (PeproTech, Rocky Hill, NJ) (complete medium).

Phagocytosis of Splenocytes. Splenocytes were labeled with a 2 μM concentration of the lipophilic fluorescent dye PKH26 (Sigma) for 4 min at room temperature, washed, and γ-irradiated (1,500 rads). Purified DC were prestained with anti-CD11c, anti-CD4, and anti-CD8 and subsequently cultured at 2.5×10^5 cells per well in 24-well plates in 500 μl of complete medium together with 10^6 PKH26-labeled splenocytes. The cocultures were incubated on ice or at 37°C in the presence or absence of 10 μM cytochalasin D, harvested 3 h later, and analyzed by flow cytometry.

Endocytosis of Soluble OVA–Alexa Fluor 488. Purified DC were incubated in complete medium with 15.6–62.5 μg/ml OVA conjugated to Alexa Fluor 488 for 45 min at 37°C or on ice. Cells were washed, stained with anti-CD11c and anti-CD8, and analyzed by flow cytometry.

Preparation of T Cells. For OT-I (OVA-specific $CD8^+$ T cells), single-cell suspensions were made from pooled s.c. and mesenteric lymph nodes of OT-I transgenic mice and depleted with mAbs against M1/70 (macrophage and DCs), F4/80 (macrophages), Ter 119 (RBC), RB6 (granulocytes), M5/114 (MHC class II^+ cells), and GK 1.5 ($CD4^+$ cells). The antibody-coated cells were incubated with anti-rat IgG-coupled magnetic beads (Dynal) following the manufacturer's protocols. For OT-II (OVA-specific $CD4^+$ T cells), single-cell suspensions from the lymph nodes of OT-II transgenic mice were treated with mAbs against M1/70, F4/80, Ter 119, RB6, M5/114, and 53-6.72 ($CD8^+$ T cells) and were depleted as for OT-I T cells. All T cell preparations were between 85% and 95% pure as determined by flow cytometry using the antibodies for anti-CD8, anti-CD4, and anti-Vα2.

OVA-Coated Spleen Cells. To prepare cell-associated OVA (45), bm1 and MHC class II-deficient spleen cells were γ-irradiated (1,500 rads), washed, incubated with 10 mg/ml OVA in RPMI medium 1640 for 10 min at 37°C, and washed three times with RPMI medium 1640 supplemented with 3% FCS. The amount of OVA associated to the cells was quantitated to be 8 ng of OVA per 10^6 cells (28).

Analysis of Antigen Presentation to Naïve T Cells *in Vitro*. For the experiments using bacterial and soluble OVA or synthetic peptides, individual DC populations were plated in 96-well plates (Costar-Corning) at 5×10^3 cells per well with the indicated number of fixed OVA-expressing *E. coli* (a kind gift from C. Reis e Sousa, London Research Institute, London), soluble OVA (Sigma), or $OVA_{257–264}$ (OT-I) or $OVA_{323–339}$ (OT-II) synthetic peptide (Auspep, Melbourne, Australia) for 45 min at 37°C in complete medium. The DC were washed three times and resuspended in 200 μl of complete medium containing 5×10^4 CFSE-labeled OT-I or OT-II cells. Proliferation was analyzed after 60–65 h of culture as described elsewhere (26). Each determination was performed in duplicate. For the experiments using cell-associated OVA, 2.5×10^4 purified DC were plated in 96-well plates with the indicated amounts of bm1 or MHC class II-deficient splenocytes coated with OVA or expressing OVA endogenously and 5×10^4 CFSE-labeled OT-I or OT-II cells and incubated for 60–65 h. T cell proliferation was determined by flow cytometry as described elsewhere (26).

Presentation of OVA Conjugated to Fluoresbrite YG Microspheres. Streptavidin-coated 1.0-μm Fluoresbrite YG microspheres were conjugated with biotinylated OVA (Sigma) at 250 μg of OVA protein per 100 μl of beads, washed, and injected i.v. Three hours

later, the spleens were harvested, and DC populations were purified as described above. DC (5×10^3 per well) were plated in 200 μl of complete medium containing 5×10^4 CFSE-labeled OT-I or OT-II cells. T cell proliferation was determined by flow cytometry at 60–65 h as described elsewhere (26).

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1. Wilson, N. S. & Villadangos, J. A. (2005) *Adv. Immunol.* **86,** 241–305.
2. Cresswell, P., Ackerman, A. L., Giodini, A., Peaper, D. R. & Wearsch, P. A. (2005) *Immunol. Rev.* **207,** 145–157.
3. Touret, N., Paroutis, P., Terebiznik, M., Harrison, R. E., Trombetta, S., Pypaert, M., Chow, A., Jiang, A., Shaw, J., Yip, C., *et al*. (2005) *Cell* **123,** 157–170.
4. Shortman, K. & Liu, Y. J. (2002) *Nat. Rev. Immunol.* **2,** 151–161.
5. Villadangos, J. A. & Heath, W. R. (2005) *Semin. Immunol.* **17,** 262–272.
6. Heath, W. R., Belz, G. T., Behrens, G. M., Smith, C. M., Forehan, S. P., Parish, I. A., Davey, G. M., Wilson, N. S., Carbone, F. R. & Villadangos, J. A. (2004) *Immunol. Rev.* **199,** 9–26.
7. den Haan, J. M., Lehar, S. M. & Bevan, M. J. (2000) *J. Exp. Med.* **192,** 1685–1696.
8. Schulz, O. & Reis e Sousa, C. (2002) *Immunology* **107,** 183–189.
9. Iyoda, T., Shimoyama, S., Liu, K., Omatsu, Y., Akiyama, Y., Maeda, Y., Takahara, K., Steinman, R. M. & Inaba, K. (2002) *J. Exp. Med.* **195,** 1289–1302.
10. Naik, S. H., Proietto, A. I., Wilson, N. S., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M. H., O'Keeffe, M., Shao, Q. X., Chen, W. F., *et al*. (2005) *J. Immunol.* **174,** 6592–6597.
11. Valdez, Y., Mah, W., Winslow, M. M., Xu, L., Ling, P. & Townsend, S. E. (2002) *J. Exp. Med.* **195,** 683–694.
12. Kerksiek, K. M., Niedergang, F., Chavrier, P., Busch, D. H. & Brocker, T. (2005) *Blood* **105,** 742–749.
13. den Haan, J. M. & Bevan, M. J. (2002) *J. Exp. Med.* **196,** 817–827.
14. Pooley, J. L., Heath, W. R. & Shortman, K. (2001) *J. Immunol.* **166,** 5327–5330.
15. Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J. V., Steinman, R. M. & Nussenzweig, M. C. (2001) *J. Exp. Med.* **194,** 769–779.
16. Bonifaz, L., Bonnyay, D., Mahnke, K., Rivera, M., Nussenzweig, M. C. & Steinman, R. M. (2002) *J. Exp. Med.* **196,** 1627–1638.
17. Corbett, A. J., Caminschi, I., McKenzie, B. S., Brady, J. L., Wright, M. D., Mottram, P. L., Hogarth, P. M., Hodder, A. N., Zhan, Y., Tarlinton, D. M., *et al*. (2005) *Eur. J. Immunol.* **35,** 2815–2825.
18. Wille-Reece, U., Flynn, B. J., Lore, K., Koup, R. A., Kedl, R. M., Mattapallil, J. J., Weiss, W. R., Roederer, M. & Seder, R. A. (2005) *Proc. Natl. Acad. Sci. USA* **102,** 15190–15194.
19. Wille-Reece, U., Wu, C. Y., Flynn, B. J., Kedl, R. M. & Seder, R. A. (2005) *J. Immunol.* **174,** 7676–7683.
20. Belz, G. T., Heath, W. R. & Carbone, F. R. (2002) *Immunol. Cell Biol.* **80,** 463–468.
21. Belz, G. T., Behrens, G. M., Smith, C. M., Miller, J. F., Jones, C., Lejon, K., Fathman, C. G., Mueller, S. N., Shortman, K., Carbone, F. R. & Heath, W. R. (2002) *J. Exp. Med.* **196,** 1099–1104.
22. Chilton, P. M., Rezzoug, F., Fugier-Vivier, I., Weeter, L. A., Xu, H., Huang, Y., Ray, M. B. & Ildstad, S. T. (2004) *Diabetes* **53,** 1995–2002.
23. O'Keeffe, M., Brodnicki, T. C., Fancke, B., Vremec, D., Morahan, G., Maraskovsky, E., Steptoe, R., Harrison, L. C. & Shortman, K. (2005) *Int. Immunol.* **17,** 307–314.
24. Vremec, D., Pooley, J., Hochrein, H., Wu, L. & Shortman, K. (2000) *J. Immunol.* **164,** 2978–2986.
25. Inaba, K., Witmer-Pack, M., Inaba, M., Hathcock, K. S., Sakuta, H., Azuma, M., Yagita, H., Okumura, K., Linsley, P. S., Ikehara, S., *et al*. (1994) *J. Exp. Med.* **180,** 1849–1860.
26. Wilson, N. S., El-Sukkari, D., Belz, G. T., Smith, C. M., Steptoe, R. J., Heath, W. R., Shortman, K. & Villadangos, J. A. (2003) *Blood* **102,** 2187–2194.
27. Wilson, N. S., El-Sukkari, D. & Villadangos, J. A. (2004) *Blood* **103,** 2187–2195.
28. Li, M., Davey, G. M., Sutherland, R. M., Kurts, C., Lew, A. M., Hirst, C., Carbone, F. R. & Heath, W. R. (2001) *J. Immunol.* **166,** 6099–6103.
29. Nikolic-Zugic, J. & Bevan, M. J. (1990) *Nature* **344,** 65–67.
30. Ehst, B. D., Ingulli, E. & Jenkins, M. K. (2003) *Am. J. Transplant.* **3,** 1355–1362.
31. Wilson, N. S., Behrens, G. M., Lundie, R. J., Smith, C. M., Waithman, J., Young, L., Forehan, S. P., Mount, A., Steptoe, R. J., Shortman, K. D., *et al*. (2006) *Nat. Immunol.* **7,** 165–172.
32. Shen, Z., Reznikoff, G., Dranoff, G. & Rock, K. L. (1997) *J. Immunol.* **158,** 2723–2730.
33. Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P. & Amigorena, S. (1999) *Nat. Cell Biol.* **1,** 362–368.
34. Schuurhuis, D. H., Ioan-Facsinay, A., Nagelkerken, B., van Schip, J. J., Sedlik, C., Melief, C. J., Verbeek, J. S. & Ossendorp, F. (2002) *J. Immunol.* **168,** 2240–2246.
35. Nagata, Y., Ono, S., Matsuo, M., Gnjatic, S., Valmori, D., Ritter, G., Garrett, W., Old, L. J. & Mellman, I. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 10629–10634.
36. Delamarre, L., Holcombe, H. & Mellman, I. (2003) *J. Exp. Med.* **198,** 111–122.
37. Schnurr, M., Chen, Q., Shin, A., Chen, W., Toy, T., Jenderek, C., Green, S., Miloradovic, L., Drane, D., Davis, I. D., *et al*. (2005) *Blood* **105,** 2465–2472.
38. Zinkernagel, R. M. (2002) *Eur. J. Immunol.* **32,** 2385–2392.
39. Stoitzner, P., Tripp, C. H., Eberhart, A., Price, K. M., Jung, J. Y., Bursch, L., Ronchese, F. & Romani, N. (2006) *Proc. Natl. Acad. Sci. USA* **103,** 7783–7788.
40. Hogquist, K. A., Jameson, S. C., Heath, W. R., Howard, J. L., Bevan, M. J. & Carbone, F. R. (1994) *Cell* **76,** 17–27.
41. Barnden, M. J., Allison, J., Heath, W. R. & Carbone, F. R. (1998) *Immunol. Cell Biol.* **76,** 34–40.
42. Cosgrove, D., Gray, D., Dierich, A., Kaufman, J., Lemeur, M., Benoist, C. & Mathis, D. (1991) *Cell* **66,** 1051–1066.
43. Henri, S., Vremec, D., Kamath, A., Waithman, J., Williams, S., Benoist, C., Burnham, K., Saeland, S., Handman, E. & Shortman, K. (2001) *J. Immunol.* **167,** 741–748.
44. O'Keeffe, M., Hochrein, H., Vremec, D., Caminschi, I., Miller, J. L., Anders, E. M., Wu, L., Lahoud, M. H., Henri, S., Scott, B., *et al*. (2002) *J. Exp. Med.* **196,** 1307–1319.
45. Carbone, F. R. & Bevan, M. J. (1990) *J. Exp. Med.* **171,** 377–387.