In Situ Analysis Reveals Physical Interactions Between CD11b+ Dendritic Cells and Antigen-Specific CD4 T Cells After Subcutaneous Injection of Antigen

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In situ staining techniques were used to visualize physical interactions between dendritic cell subsets and naive Ag-specific CD4 T cells in the lymph node. Before injection of Ag, CD8+ dendritic cells and naive OVA-specific CD4 T cells were uniformly distributed throughout the T cell-rich paracortex, whereas CD11b+ dendritic cells were located mainly in the outer edges of the paracortex near the B cell-rich follicles. Many OVA-specific CD4 T cells were in contact with CD8+ dendritic cells in the absence of OVA. Within 24 h after s.c. injection of soluble OVA, the OVA-specific CD4 T cells redistributed to the outer paracortex and interacted with CD11b+, but not CD8+ dendritic cells. This behavior correlated with the uptake of OVA and the presence of peptide-MHC complexes on the surface of CD11b+ dendritic cells, and subsequent IL-2 production by the Ag-specific CD4 T cells. These results are consistent with the possibility that CD11b+ dendritic cells play a central role in the activation of CD4 T cells in response to s.c. Ag. The Journal of Immunology, 2002, 169: 2247–2252.

Materials and Methods

Mice

Six- to 8-wk-old BALB/c and BALB/c SCID mice were obtained from the National Cancer Institute, National Institutes of Health (Frederick, Maryland). DO11.10 BALB/c TCR-transgenic mice expressing an αβ-TCR specific for aa 332–339 from chicken OVA presented by I-A\* (12) were bred in our facilities. DO11.10 BALB/c SCID were produced by backcrossing DO11.10 BALB/c mice to BALB/c SCID mice. DO11.10 BALB/c Rag2–/– mice were the kind gift of Dr. A. Khoruts (University of Minnesota, Minneapolis, MN). All DO11.10 mice were housed in a specific pathogen-free facility. All experiments involving the use of mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Cell transfer

Single cell suspensions of spleen and lymph nodes were prepared from naive DO11.10 or DO11.10 SCID mice. A small sample was stained with biotin-labeled KJ1-26 mAb (an anti-clonotypic mAb that uniquely recognizes the DO11.10 TCR; Ref. 13), streptavidin-FITC (Caltag Laboratories, South San Francisco, CA), and PE-labeled anti-CD4 mAb (BD Pharmingen, San Diego, CA), and analyzed on a FACScan flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software. A portion of the remaining unlabeled cell suspension containing 2.5–5.0 × 10^6 CD4^+ KJ1-26^+ cells was then injected i.v. into each unirradiated BALB/c recipient. In some cases, lymph node cells from naive BALB/c mice were labeled with CFSE (Molecular Probes, Eugene, OR) using a modification (14) of a previously described technique (15), and then injected i.v. into unirradiated BALB/c recipients. Mice were injected s.c. on the back with chicken OVA (Sigma-Aldrich, St. Louis, MO), from which contaminating LPS was removed as described (16), or with FITC- or Texas Red (TR)3-labeled OVA (Molecular Probes, Eugene, OR).

Abbreviations used in this paper: TR, Texas Red; MHC II, MHC class II.
IL-2 production by DO11.10 T cells

Single-cell suspensions were prepared in PBS containing 10 mM EDTA (to ensure the release of activated T cells) from the draining (brachial, axillary, and inguinal) and nondraining (mesenteric) lymph nodes of recipients of DO11.10 T cells at various times after s.c. injection of OVA. The cells were fixed in 2% formaldehyde, permeabilized with 0.5% saponin, and previously described (17), and stained (1-5 10^6/tube) with CyChrome-labeled anti-CD4 mAb RM4-5 (BD PharMingen), FITC-labeled KJ1-26 (Calgt Laboratories), and PE-labeled anti-IL-2 mAb S4B6 (BD PharMingen) or isotype control mAb R35-95 (BD PharMingen). CD4^+ T cells were identified as DEC-205-stained cells. Sorting was performed on a FACS-vantage flow cytometer (BD Biosciences).

OVA uptake in vivo

Single-cell suspensions were prepared by treating the draining lymph nodes of OVA-injected mice with 400 U/ml of collagenase D (Boehringer Mannheim, Indianapolis, IN) and 10 mM EDTA to release dendritic cells from the tissue. In some experiments, dendritic cells were enriched using biotin-labeled anti-CD11c mAb and streptavadin-coated microbeads or anti-CD11c mAb-coated microbeads according to the manufacturer’s protocol (MACS; Miltenyi Biotec, Auburn, CA). Cells were incubated on ice with anti-FcR mAb (2.4G2; American Type Culture Collection, Rockville, MD) to block Fc receptor binding sites. In experiments where unlabeled OVA was injected, cells were stained with PE-labeled anti-CD11c mAb, FITC-labeled anti-CD8α mAb, allophycocyanin-labeled anti-CD11b mAb (in some, but not all experiments), CyChrome-labeled anti-B220 mAb, and CyChrome-labeled anti-CD3 mAb (all from BD PharMingen). In experiments where FITC-labeled OVA was injected, cells were stained with PE-labeled anti-CD11c mAb, allophycocyanin-labeled anti-CD11b mAb, CyChrome-labeled anti-B220 mAb, and CyChrome-labeled anti-CD3 mAb. In other experiments designed to assess CD4 expression on the CD8^+ dendritic cells that took up FITC-labeled OVA, cells were stained with allophycocyanin-labeled anti-CD11c mAb, PE-labeled anti-CD4 mAb or an isotype control mAb, and CyChrome-labeled anti-CD69 mAb. CD11b^+ dendritic cells were identified as cells that expressed CD11c and CD11b, or cells that expressed CD11c and lacked CD8. CD8^+ dendritic cells were identified as cells that expressed CD11c and CD8. Sorting was performed on a FACS-Vantage flow cytometer (BD Biosciences).

Proliferation assay

Naive DO11.10 BALB/c Rag2^−/− T cells (3 10^6) were placed in triplicate in 96-well U-bottom plates. Sorted CD11c^+CD8^− or CD11c^+CD8^+ dendritic cells were added to each well with or without exogenous OVA and with or without anti-MHC class II (MHC II) mAb (M5/114, Forty-eight hours later, tritiated thymidine (1 μCi/well; NEN, Boston, MA) was added to each well. Cells were harvested 18–24 h later with a Wallac cell harvester (Turku, Finland). Tritiated thymidine incorporation into DNA was measured on a Wallac beta counter.

Immunofluorescent microscopy

Draining lymph nodes were harvested from mice sacrificed at various times after OVA injection. The lymph nodes were frozen in precooled isopentane and sectioned using a cryostat microtome to a thickness of 10 μm. Sections were dehydrated in acetone, hydrated in PBS, incubated at room temperature with anti-FcR mAb (2.4G2; American Type Culture Collection), avidin and biotin solutions (Vector Laboratories, Burlingame, CA) and stained sequentially with biotin-labeled anti-CD11c mAb (N418; American Type Culture Collection) or biotin-labeled anti-DEC-205 mAb (NLD1C145; American Type Culture Collection), or biotin-labeled anti-CD11b mAb, digoxigenin-labeled KJ1-26 mAb, streptavadin-labeled HRP (NEN), sheep anti-digoxigenin IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), tyramide-labeled tetramethylrhodamine (NEN), and Cy3-labeled donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories). Dendritic cells of the CD11b^+ subset were identified as anti-CD11b-stained cells present in the paracortex, whereas dendritic cells of the CD8^+ subset were identified as DEC-205-stained cells.

Confocal microscopy and image analysis were performed as previously described (18). Briefly, lymph node sections were analyzed using a Bio-Rad MRC-1000 confocal microscope equipped with a krypton/argon laser (Hercules, CA). Separate images were collected for each mAb in each area scanned and overlaid to produce a composite image containing all colors. The entire surface of each lymph node section was scanned to account for differences in staining intensity throughout the lymph node. Final image processing was performed using the Confocal Assistant program (University of Minnesota) and Adobe Photoshop (Mountain View, CA) as previously described (18). Intersections between CD11b^+ dendritic cells, CD-205^+ dendritic cells, or TR-OVA-containing cells (colored red) and KJ1-26^+ cells or CFSE-labeled polyconal BALB/c cells (colored green) were quantified by measuring the overlap (yellow) between red and green. To account for differences in staining intensity on different sections, the number of yellow pixels was divided by the product of green and red pixels.

Results

Identification of cells that acquire OVA in the draining lymph nodes after s.c. injection

Before searching for physical evidence of Ag uptake, it was necessary to establish the relevant time frame and location of T cell activation. Naive DO11.10 T cells were transferred into BALB/c recipients that were then injected s.c. with OVA. IL-2 production was first detected in the DO11.10 T cells 6 h after OVA injection, peaked at 12 h, and was primarily restricted to the draining lymph nodes (Fig. 1). These results demonstrate that the initial presentation of the OVA occurs in the draining lymph nodes within 24 h after s.c. OVA injection.

Therefore, we focused our attention on the draining lymph nodes during the first 24 h after s.c. injection of fluorochrome-labeled OVA to determine which cell types could potentially act as APC in vivo. Flow cytometric analysis revealed that a subset of B lymphocytes (B220^+ cells) and CD8^+ dendritic cells contained low levels of OVA, whereas all CD11b^+ dendritic cells and macrophages (CD11c^−/−CD11b^+ CD11c^−/CD11b^+ ) contained larger amounts of OVA (Fig. 2), 4 h after Ag injection. The only fluorochrome-containing cells that could be detected by immunofluorescent microscopic examination of tissue sections at this time were present in the subcapsular sinus (Fig. 3). These cells were probably macrophages because macrophages are the major occupant of the subcapsular sinus (19) and because macrophages contained the largest amount of labeled OVA as revealed by flow cytometry (Fig. 2). No fluorochrome-labeled OVA was detected by microscopy at 4 h in the follicles or in the paracortex of the lymph node where naive T cells reside (Fig. 3B). Thus, the level of fluorochrome detected by flow cytometry in a subset of B cells, which should reside in the follicles, and in all CD11b^+ dendritic cells and a subset of CD8^+ dendritic cells, both of which reside in the paracortex, was below the limit of detection by microscopy at this early time after OVA injection.

Eighth hours after s.c. injection of fluorochrome-labeled OVA, the amount of fluorochrome associated with B cells, macrophages, CD8^+ dendritic cells, and some CD11b^+ dendritic cells fell compared with the 4 h levels (Fig. 2). However, a subset of
CD11b⁺ dendritic cells bearing very large amounts of fluorochrome-labeled OVA appeared at this time (Fig. 2). Further characterization revealed that these cells do not express CD4 (data not shown) and were located within the paracortex, usually near the B cell-rich follicles (Fig. 3, C and D).

**CD11b⁺ dendritic cells produce OVA peptide-MHC complexes**

The capacity of dendritic cells purified from draining lymph nodes to produce OVA peptide-MHC complexes in vivo was tested by assessing their ability to directly stimulate DO11.10 T cells in vitro without the addition of exogenous OVA. As shown in Fig. 4A, neither subset of dendritic cells from the lymph nodes of mice injected with OVA 4 h earlier stimulated DO11.10 T cells in vitro.

Similar results were obtained at 2 h (data not shown). In contrast, 18 h after OVA injection, CD11b⁺ but not CD8⁺ dendritic cells stimulated the DO11.10 T cells, and the CD11b⁺ dendritic cells that took up the largest amounts of OVA in vivo were the most effective stimulators (Fig. 4A). Similar results were obtained at 12 h, the peak of IL-2 production (data not shown). Addition of an anti-MHC II mAb completely blocked the Ag-driven proliferation of the DO11.10 T cells (17,937 ± 1,280 cpm for cultures without mAb, 111 ± 39 for cultures with anti-MHC II mAb).

The failure of CD8⁺ dendritic cells to produce detectable OVA peptide-MHC complexes in vivo could have been due to an inability to process Ag. This possibility was tested by culturing sorted dendritic cells and DO11.10 T cells with intact OVA, which must be processed to produce OVA peptide-I-Ad complexes (20). As shown in Fig. 4B, CD8⁺ dendritic cells stimulated DO11.10 T cells at least as efficiently as the CD11b⁺ dendritic cells proving that they are capable of producing OVA peptide-MHC complexes from intact OVA. Thus, the failure of CD8⁺ dendritic cells to...
produce peptide-MHC complexes from OVA in vivo could not be explained by an inherent defect in Ag processing. It is formally possible that Ag is sequestered and not substantially processed by dendritic cells in vivo. To address this, we studied dendritic cells before in vitro culture. This did not change the finding that the CD8<sup>+</sup> and not the CD8<sup>-</sup> dendritic cells stimulated DO11.10 T cells (25,437 ± 709 cpm for cultures with CD8<sup>+</sup> dendritic cells, 679 ± 234 for cultures with CD8<sup>-</sup> dendritic cells).

**CD11b<sup>+</sup> dendritic cells undergo OVA-dependent interactions with specific CD4 T cells in situ**

We next searched for physical interactions between dendritic cell subsets and Ag-specific CD4 T cells during the time frame when OVA peptide-MHC complexes and IL-2 were produced in the draining lymph nodes. This was assessed by measuring changes in the proximity between dendritic cells and DO11.10 T cells by two-color immunofluorescence before and after OVA injection. Initial experiments were performed to define the anatomic location of the dendritic cell subsets. Lymph node tissue sections were stained with anti-CD11c mAb to detect all dendritic cells and anti-CD11b or anti-DEC-205 mAbs to detect the different dendritic cell subsets. Anti-DEC-205 Ab was used instead of anti-CD8 Ab because of the confounding effect of the many CD8<sup>+</sup> T cells within the paracortex. As expected (2, 18), anti-CD11c mAb stained dendritic cells throughout the paracortex but minimally stained cells in the subcapsular sinus (data not shown). Anti-CD11b mAb intensely stained macrophages in the subcapsular sinus, and dendritic cells in the peripheral region of the paracortex near the B cell-rich follicles (parafollicular region) (Fig. 5, B and D). This is the area where CD11b<sup>+</sup> dendritic cells containing large amounts of OVA appeared by 18 h after injection of fluorochrome-labeled OVA (Fig. 3, C and D). In contrast, CD8<sup>+</sup> dendritic cells (identified by DEC-205 expression) were uniformly distributed throughout the paracortex (Fig. 5, A and C).

Sections were then stained with KJ-1-26 mAb labeled with one fluorochrome (shown green) and anti-CD11b or anti-DEC-205 mAb labeled with a different fluorochrome (shown red) in an attempt to visualize Ag-dependent cell-cell interactions. The sections were analyzed by confocal immunofluorescent microscopy at a magnification where yellow color was produced at the point of contact between red and green cells (Fig. 6A).

The DO11.10 T cells were uniformly distributed throughout the paracortex before OVA injection (Fig. 5, A and B). Some interactions between DO11.10 T cells and dendritic cells were detected in
accompanied by enrichment of the DO11.10 T cells in the dendritic cells increased as early as 6 h after OVA injection, with a B and OVA-containing cells were rarely observed. The degree of interaction between polyclonal BALB/c T cells (shown purple) and KJ1-26+ T cells (green) and not polyclonal BALB/c cells (purple). A. Quantification of the overlap pixels per BALB/c cell or KJ1-26+ cell pixels ± SD. These data are representative of two independent experiments.

Discussion

Using in situ methods, we show in this study that CD11b+, but not CD8+, dendritic cells undergo physical interactions with Ag-specific CD4 T cells after soluble Ag injection when the T cells produce IL-2. In the absence of stimulation, naive T cells and CD8+ dendritic cells were homogeneously distributed throughout the T cell-rich paracortex, whereas CD11b+ dendritic cells were concentrated in the outer portion of the paracortex close to the B cell-rich follicles. The restriction of CD11b+ dendritic cells to a subdomain of the paracortex may explain our observation that naive T cells are more likely to interact with the more widely distributed CD8+ dendritic cells in the absence of Ag.

Four hours after s.c. injection, most CD11b+ dendritic cells and about one-third of the CD8+ dendritic cells contained a low level of fluorochrome-labeled OVA that could be detected by flow cytometry, but not immunofluorescent microscopy, in the T cell area. Thus, although it is formally possible that both dendritic cell types could have presented Ag to naive T cells in vivo, we could not document this because neither dendritic cell subset isolated from the draining lymph nodes at this time stimulated specific T cells ex vivo. In contrast, during the period of IL-2 production (6–18 h after Ag injection), CD11b+ dendritic cells containing large amounts of OVA appeared in the T cell area, and, when isolated from the draining lymph nodes, stimulated specific T cells ex vivo. Furthermore, the DO11.10 T cells redistributed to the CD11b+ dendritic cell-rich parafollicular region and interacted with CD11b+, but not CD8+, dendritic cells in vivo. These results are strong evidence that CD11b+, but not CD8+, dendritic cells present peptide-MHC II complexes to Ag-specific CD4 T cells in the draining lymph nodes after s.c. injection of soluble Ag.

In summary, our results together with those in the literature suggest several explanations for the finding that CD11b+ dendritic cells are the dominant APC for CD4 T cells in situations where a pathogen enters the body through the skin. These dendritic cells are situated in the outer T cell area and thus are well-positioned to take up bacterial debris that leaks through the subcapsular sinus where bacterial Ags would first appear (21, 22). Alternatively, the CD11b+ cells that we observed could be tissue monocytes or perhaps dermal dendritic cells that engulf pathogens in the skin and carry particulate Ag from the s.c. injection site to the lymph node, and in the process differentiate into CD11b+ dendritic cells as suggested by Randolph et al. (23). Finally, CD11b+ dendritic cells may be the most efficient producers of peptide-MHC II complexes from soluble Ag as suggested by Shortman and colleagues (24). In contrast, CD8+ dendritic cells may be more important as APC for cross-priming CD8 T cells because these dendritic cells are uniquely capable of producing peptide-MHC class I complexes from exogenous Ag (10, 24).

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References


