A Member of the Dendritic Cell Family That Enters B Cell Follicles and Stimulates Primary Antibody Responses Identified by a Mannose Receptor Fusion Protein

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Summary

Dendritic cells (DCs) are known to activate naive T cells to become effective helper cells. In addition, recent evidence suggests that DCs may influence naive B cells during the initial priming of antibody responses. In this study, using three-color confocal microscopy and three-dimensional immunohistograms, we have observed that in the first few days after a primary immunization, cells with dendritic morphology progressively localize within primary B cell follicles. These cells were identified by their ability to bind a fusion protein consisting of the terminal cysteine-rich portion of the mouse mannose receptor and the Fc portion of human immunoglobulin (Ig)G1 (CR-Fc). In situ, these CR-Fc binding cells express major histocompatibility complex class II, sialoadhesin, and CD11c and are negative for other markers identifying the myeloid DC lineage, such as CD11b, macrophages (F4/80), follicular DCs (FDC-M2), B cells (B220), and T cells (CD4). Using CR-Fc binding capacity and flow cytometry, the cells were purified from the draining lymph nodes of mice 24 h after immunization. When injected into naïve mice, these cells were able to prime T cells as well as induce production of antigen-specific IgM and IgG1. Furthermore, they produced significantly more of the lymphocyte chemoattractant, macrophage inflammatory protein (MIP)-1α, than isolated interdigitating cells. Taken together, these results provide evidence that a subset of DCs enters primary follicles, armed with the capacity to attract and provide antigenic stimulation for T and B lymphocytes.

Key words: dendritic cells • mannose receptor • antibody production • antigen presentation • chemokines

Antigen-specific lymphocytes, to initiate T cell-dependent antibody responses, must extravasate from the circulation into secondary lymphoid tissues, where they encounter signals for activation. Maturing dendritic cells (DCs)1 migrate via blood or lymph into these organs and efficiently prime naïve T cells (1, 2). This process involves the presentation of not only peptides but also costimulatory signals such as CD80 and CD86, the production of cytokines such as IL-12, and the release of chemokines such as IL-8, macrophage inflammatory protein (MIP)-1α, and MIP-1β (3–5).

Although much is known concerning DC–T cell interactions, it is only recently that investigators have found evidence that DCs may directly influence the production of antibody. Highly purified human CD1a+ DCs have been shown to stimulate activated B cells to produce IgM and IgG in vitro (6). Furthermore, Wymes and colleagues have shown that rat DCs can transfer antigen to B cells and initiate a primary antibody response in vivo (7). Hence, it appears that DCs may have a broader role in lymphocyte activation than previously appreciated.

Studies performed by Kelsoe and colleagues originally suggested that early B cell activation occurs in the outer T cell zones of splenic periarteriolar sheaths (8–10). These foci were visualized due to the antigen-binding capacity of the B cells and, with time, contained an increased number of antigen-specific plasma cells. However, a recently published study using an adoptive transfer system of transgenic B and T cells has shown that early cognate interactions lo-
calize to the follicular border in lymph nodes (11). The height of this interaction occurred at day 2–4 after immunization, suggesting that lymph node T and B cell priming events occur in these areas.

Previously in our laboratory, a chimeric protein was generated as a means to determine which cell types express ligands for the cysteine-rich (CR) domain of the murine mannose receptor (12). This chimera consisted of the CR domain of the mannose receptor fused to the Fc region of human IgG1 (CR-Fc). In conjunction with immunohistochemistry, the CR-Fc fusion protein revealed that cells with dendritic morphology accumulated in primary B cell follicles during the initial phases of an immune response while interdigitating cells in the paracortex remained negative (12). This observation was intriguing, leading us to develop methods to characterize this population in situ and ex vivo. The results reported here demonstrate that CR-Fc binding provides a marker for isolating a novel murine subset of CD11c+ DCs that localize to B cell follicles and initiate humoral responses.

Materials and Methods

Animals and Immunizations. Female B6C3F1 mice were purchased from Janvier and kept under pathogen-free conditions. They were used at 8–12 wk of age. Mice were primed subcutaneously in each limb (50 μg OVA precipitated in alum (Sigma Chemical Co.). These mAbs, with the exception of NLD-145, were either obtained from American Type Culture Collection and produced in house or, when available, purchased from Pharmingen. Secondary antibodies and reagents included Texas red-conjugated rat anti-mouse IgM (Southern Biotechnology Associates), Cy5-conjugated mouse anti-human IgG (Jackson Immunoresearch Laboratories), streptavidin-Cy5 (Caltag Laboratories), FITC-conjugated mouse anti-rat IgG (Southern Biotechnology Associates), FITC-conjugated mouse anti-rat IgG, FITC-conjugated mouse anti-human IgG (both from Jackson Immunoresearch Laboratories), and FITC-conjugated goat anti-hamster IgG (Southern Biotechnology Associates). Human IgG (Jackson Immunoresearch Laboratories) was used as a control for nonspecific binding of CR-Fc.

Three-dimension Immunohistograms. The photomicrographs were computer analyzed using a program developed in house. The program divides the image into 4,096 squares (64 × 64). The mean fluorescence intensity (MFI) is calculated for each of the squares and stored in a matrix. The MFI is then represented as a three-dimension (3D) histogram (x, y = position of the square, z = MFI). The 3D immunohistograms can represent single parameters as well as an overlay of two or more colors.

Isolation of CR-Fc binding cells. Mice were injected with OVA precipitated in alum (50 μg sc per limb and 100 μg sc behind the neck). 24 h later, the draining lymph nodes were collected and the cells recovered using an enzyme cocktail containing collagenase and DNase (14). The low buoyant density cells (6 × 10^5–1.065 g/ml) were collected from Percoll gradients, and B cells were depleted with anti-B220–coated Dynabeads (Dynal). The remaining cells were sequentially incubated with CR-Fc (10 μg/ml) and N418 (10 μg/ml), biotinylated mouse anti-human IgG (1:100) and goat anti-hamster FITC (1:100; Southern Biotechnology Associates), and Streptavidin-PE (1:100; Serotec) before separation by flow cytometry. All washes and dilutions of reagents for FACScan® used a solution consisting of PBS containing 10% FCS.

T Lymphocyte Priming In Vivo. For assessing in vivo T cell priming, the N418+ /CR-Fc binding cells were injected subcutaneously into the right side of naive mice. 7 d later, the draining (right side) and nondraining (left side) lymph nodes were collected separately, and the cells were recovered using the enzyme cocktail. The cells (obtained from the nondraining versus draining lymph nodes) were then fractionated using discontinuous Percoll gradients (15). The T cell band (6 × 10^8–1.081–1.085 g/ml) was collected and incubated with a cocktail of anti-CD8 (54.6.7), anti-MHC class II (14.4.4), and anti-B220 (14.8) coated Dynabeads according to the manufacturer’s specifications. This method produced >98% CD4+ T cells The T cells (2 × 10^6 cells/well) were then incubated for 48 h in medium (IMDM, 5% FCS, l-glutamine, penicillin/streptomycin, and 2-ME) with spleen cells (irradiated with 30 Gy; 2 × 10^6 cells/well) in the presence or absence of 10 μg/ml OVA. For the final 24 h of culture, [3H]thymidine was added and incorporation measured using a β-counter (Wallac).

T Lymphocyte Priming In Vitro. N418+ /CR-Fc binding cells were isolated as specified above and cultured overnight with T cells isolated from DO11.10 mice (i.e., transgenic for an OVA-specific T cell receptor [16]). At 24 h, [3H]thymidine was added, and incorporation was measured 24 h later.

B Lymphocyte Priming In Vivo. For assessing in vivo B cell activation, both the N418+ /CR-Fc binding fraction and the
Figure 1. Localization of CR-Fc binding cells in lymph nodes with time after a primary immunization. Three-color confocal analysis was used in order to determine when and where CR-Fc binding cells appeared in the tissue. Cryostat sections prepared from murine lymph nodes obtained at days 0–4 (A–E) of a primary response were processed for IgM expression (red), N418 labeling (green), and CR-Fc binding (blue). The images were then digitally transformed using a computer program in order to map the MFI (in arbitrary units) for either single (CR-Fc⁺; N418⁺) or double (CR-Fc⁺/ N418⁺) positive cells. The resulting 3D immunohistograms revealed that CR-Fc binding cells accumulated with time in the outer part of the B cell follicles. Many of these cells were also N418⁺ (see the CR-Fc⁺/ N418⁺ column). The scale 0–250 represents the MFI, with the colors yellow, blue, green, and red representing values in the 100–150, 150–200, 200–250, and >250 range, respectively.
Table I. Profile of CR-Fc Binding Cells in B Cell Follicles on Days 0, 1, 2, 3, and 4 of a Primary Immune Response

<table>
<thead>
<tr>
<th>Marker (mAb)</th>
<th>CR-Fc Binding</th>
<th>CR-Fc Nonbinding</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c (N 418)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>MHC class II (14.4.4)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Sialoadhesin (Ser-4/3D6)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>F4/80 (F4/80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11b (M 1/70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8α (53-6.7)</td>
<td>−/+</td>
<td></td>
</tr>
<tr>
<td>Dec 205 (N LDC-145)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FDC-M2 (209)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD90 (T24)</td>
<td>−/+</td>
<td></td>
</tr>
<tr>
<td>CD4 (GK 1.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220 (14.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD138 ( Syndecan-1)</td>
<td>−/+</td>
<td></td>
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* A minor subset of cells was identified as positive on days 3 and 4.

N418+/CR-Fc nonbinding population (representing interdigitating cells) were isolated as specified above. The CR-Fc binding cells were injected subcutaneously into the right forelimb while the CR-Fc nonbinding cells were injected subcutaneously into the left forelimb of naive mice. 7 d later, three sets of lymph nodes were collected and processed separately: (1) the draining lymph nodes receiving CR-Fc+ cells (i.e., right axillary and brachial), (2) the draining (i.e., left axillary and brachial) lymph nodes receiving CR-Fc nonbinding cells, and (3) the lymph nodes receiving no cells (inguinal and paraaortic). Cells were recovered by removing adherent cells. The nonadherent cells were then incubated with enzyme cocktail and fractionated using discontinuous Percoll gradients. The lymphocytes were cultured for 1 h at 37°C to remove adherent cells. The nonadherent cells were then incubated (3 × 10^5 cells/ml) in 96-well plates containing medium. After 7 d, the supernatants were collected and OVA-specific antibody titers were assessed using standard ELISA procedures. After 7 d, the supernatants were collected, and OVA-specific antibody titers were assessed using standard ELISA procedures.

Chemokine Production. N418+/CR-Fc binding and N418+/CR-Fc nonbinding cells were purified using flow cytometry according to the protocol mentioned above. In addition, DCs were derived from mouse bone marrow according to the protocol by Metlay et al. In brief, the bone marrow was flushed from femurs, pipetted into a single cell suspension, and then incubated at 37°C, 5% CO₂ for 2 d in the presence of gentle pipetting, discarded, and new medium containing GM-CSF was added to the remaining adherent cells. On day 6, the nonadherent cells were harvested and shown to contain ≥99% N418+ cells by flow cytometry. Cells were incubated at 37°C in 96-well plates (2 × 10^5 cells/well) in 200 μl IMDM plus 5% FCS (GIBCO BRL). Supernatants were harvested at 24 h, and chemokine levels were determined by ELISA using mouse RANTES (kit MM R00; R&D Systems) and mouse MIP-1α (kit MAA00; R&D Systems) kits according to the manufacturer's specifications.

Results

Determining the Phenotype In Situ of CR-Fc Binding Cells. During an immune response, it has been previously shown that within lymph nodes, cells with dendritic morphology bind the CR-Fc fusion protein (12). To further investigate their nature, we developed a system involving immunohistochemistry and three-color microscopy. As shown in Figs. 1 and 2, the B cell follicles were visualized using an anti-IgM antibody conjugated to Texas red. CR-Fc binding cells were labeled with Cy5-conjugated reagents. A third marker associated with FITC was then used to determine either the phenotype of the CR-Fc binding cells (double positive = cyan) or the proximity of these cells to other cell types. 3D histograms were then created in order to accurately assess the number and location of single or double positive cells.

The coexpression of the murine DC-specific marker, CD11c (using the N 418 mab [17]), was assessed on lymph node sections obtained before (day 0) and 1, 2, 3, and 4 d after a primary immunization. In general, as shown in the histograms of Fig. 1, CR-Fc binding cells represented a subset of N418+ cells. Before antigen stimulation (Fig. 1, Day 0), no CR-Fc binding cells were observed below the subcapsular sinus. Within 24 h of antigen injection (Fig. 1, Day 1), CR-Fc single and CR-Fc/N418 double positive cells appeared in the follicle. By day 2, the cells were scattered throughout the follicle, becoming more organized towards the edge of the follicle during days 3 and 4. At day 5, very few CR-Fc/N418 double positive cells were observed except in the subcapsular sinus (data not shown).

As a control to address nonspecific Fc-mediated binding, the sections (days 0–3) were incubated with human IgG instead of the fusion protein CR-Fc. No binding was observed on the tissue sections (data not shown). Finally, very few, if any, N418+ interdigitating cells in the paracortex demonstrated the ability to bind CR-Fc (Fig. 1).

Using this procedure, the expression of several proteins was analyzed (Table I). The CR-Fc binding cells in the follicles were observed to also express MHC class II and sialoadhesin, the latter being one of the ligands for CR-Fc (Martinez-Pomares, L., and S. Gordon, manuscript in preparation). A subset of cells expressed CD90 (Thy 1), especially the CR-Fc binding cells present at the border of the B cell follicle and paracortex. At days 3 and 4, a minor subset of cells also expressed CD8α and CD138 (Syndecan-1). However, CD11b, Dec205, CD4, FDC-M2, F4/80, and B220 were all absent. Taken together, these results re-
veal a novel pattern of protein expression for the CR-Fc binding cells that localize in B cell follicles.

CR-Fc Binding Cells Associate with B and T Lymphocytes in the Outer B Cell Follicles. Interestingly, many CR-Fc binding cells tended to localize at the border of the B cell follicle (Fig. 1). As it had recently been reported that adoptively transferred antigen-specific transgenic T and B cells colocalize in this region (11), we asked whether T cells could be observed in the vicinity of CR-Fc binding cells. As shown in Fig. 2 (see areas within boxes), indeed T cells (CD4+) were found to be mingled with B cells (IgM+) and CR-Fc binding cells at the periphery of the follicle between days 3 and 4 after antigen injection.

CR-Fc Binding Cells Prime T Cells In Vivo. The kinetics of their appearance in the particular anatomical location and their expression of the murine DC-specific protein, CD11c, suggested that CR-Fc binding cells play a role in lymphocyte stimulation. To address this hypothesis, CR-Fc

Figure 2. B cells, T cells, and CR-Fc binding cells colocalize in the outer B cell follicles. Cryostat sections prepared from murine lymph nodes obtained at day 3 (A, C, and E) and day 4 (B, D, and F) of a primary response were processed for IgM expression (red), CD4 labeling (green), and CR-Fc binding (blue). Especially at the higher magnification (E and F) of the areas outlined in C and D, CR-Fc binding cells (blue) and T cells (green) can be observed within the B cell follicles (red).
binding cells were isolated from draining lymph nodes 24 h after subcutaneous injection of OVA and purified by flow cytometry. As shown in Fig. 3, the CR-Fc binding cells had a high side scatter value and were all CD11c+ sorting the CR-Fc binding cells using the R1 gate (Fig. 3) produced a purity of >99%.

The cells were then adoptively transferred into naive mice, and 7 d later, the T cells were isolated separately from the draining versus non-draining lymph nodes. As shown in Fig. 4 A, the CR-Fc binding cells were able to prime naive T cells, as no antigen-specific proliferation occurred in T cells isolated from the non-draining side. These results suggested that CR-Fc binding cells were capable of antigen uptake in vivo followed by processing and presentation of peptides to naive T cells.

CR-Fc Binding Cells Present Antigen to T Cells In Vitro. To insure that CR-Fc binding cells could present antigen they obtained in vivo, the cells, isolated in a similar manner as above, were incubated in decreasing concentrations with antigen-specific T cells. As few as 3,000 CR-Fc binding cells (Fig. 4 B) were able to significantly stimulate the DO11.10 transgenic T cells to proliferate in vitro. Therefore, not only can CR-Fc binding cells take up antigen in vivo, they can also act as antigen-presenting cells both in vitro and in vivo.

CR-Fc Binding Cells Induce Antigen-specific Antibody Production by B Cells. As B cells are stimulated to produce the initial wave of antigen-specific Ig in the outer T cell zones, an experiment was designed to ascertain if CR-Fc binding cells played a role in this process. Similar to the protocol mentioned above, CR-Fc binding cells were isolated from draining lymph nodes 24 h after a subcutaneous injection of OVA. In addition, CR-Fc nonbinding cells were also obtained for comparison. The CR-Fc binding cells were then adoptively transferred into the right forelimb of naive mice while the CR-Fc nonbinding cells were injected into the left. 7 d later, the B cells were isolated separately from the right versus left axillary and brachial lymph nodes as well as the inguinal and paraaortic lymph nodes (i.e., the sites receiving no cells). They were then cultured for a further 7 d, at which time the supernatants were harvested. As shown in Fig. 5, only cells isolated from lymph nodes receiving the CR-Fc binding cells produced antigen-specific antibody. Although only a remote possibility due to the 24-h time point used for isolation, contamination by preplasma cells in the CR-Fc preparations was ruled out by putting these cells in culture for 7 d and assaying for antibody production by ELISA (none was detected; data not shown). In addition, the same results as shown in Fig. 5 were obtained when CR-Fc binding preparations were subjected to a round of B220 depletion using magnetic beads before injection into mice (data not shown).

Next, to determine the characteristics of the antigen-specific antibody response in vivo after transfer of CR-Fc
binding cells, serum was collected from mice after a single and second injection of cells. As shown in Fig. 6, a significant increase was observed for OVA-specific IgG1 titers after both the primary and secondary injections. IgM did not increase, although this was clearly observed when B cells were assessed ex vivo (as in Fig. 5). These data suggest that although the B cells that encounter CR-Fc cells can become IgM-secreting cells ex vivo, left in vivo, they will encounter signals that drive IgG1 production and can be induced to produce an increased level of antibody after a secondary application of the stimulus IgG2a and IgG2b antigen-specific serum titers showed a slight but not statistically significant change. This observation suggests that transfer of CR-Fc cells themselves do not promote the development of microenvironments for downstream Ig class switching events.

Cell Surface Molecules Expressed by Isolated CR-Fc Binding DCs. Double immunolabeling in conjunction with flow cytometry revealed that the CR-Fc binding cells were rich in Fc (CD32) and complement (CD21/35) receptors (Fig. 7). In addition, they were positive for intercellular adhesion molecule 1 (ICAM-1, CD54), CD40, and CD86 (B7-2) whereas they expressed little to no CD80 (B7-1). The cells also expressed siaiophilin (Ser-4/3D6), one of the ligands for CR-Fc. Finally, neither CD3 nor CD23 were expressed.

CR-Fc Binding Cells Differ from Interdigitating Cells and Bone Marrow-derived DCs in Their Ability to Produce MIP-1α. N418<sup>+/</sup>CR-Fc nonbinding cells (i.e., interdigitating cells) and N418<sup>+/</sup>CR-Fc binding cells isolated 24 h after antigen injection as well as N418<sup>+</sup> DCs derived from bone marrow (BM-DCs) were assessed for their capacity to produce T cell chemoattractants. As shown in Fig. 8, RANTES (for regulated upon activation, normal T cell expressed and secreted) production followed the hierarchy of BM-DC < CR-Fc<sup>+</sup>/N418<sup>+</sup> ≤ CR-Fc<sup>+</sup>/N418<sup>−</sup> (interdigitating cell). In contrast, only N418<sup>+</sup>/CR-Fc binding cells produced MIP-1α.

Figure 6. OVA-specific serum titers of mice receiving CR-Fc binding cells. CR-Fc binding cells were isolated from OVA-immune mice, then adoptively transferred subcutaneously into naive mice (10<sup>5</sup> per mouse). 14 d later, the same mice were injected subcutaneously a second time with a freshly isolated preparation of CR-Fc binding cells from OVA-immune mice (5 × 10<sup>4</sup> per mouse). Serum was obtained before the primary (D0) and secondary (D14) injections. Isotype-specific anti-OVA titers were assessed by ELISA. Each data point represents the maximum dilution at which a titer was still observed for an individual mouse.
Discussion

Using the CR-Fc binding property, we have identified a population of cells that localize within B cell follicles. These cells appear to be members of the DC family, as they express CD11c in situ and ex vivo and are capable of priming naive T cells. Whether the cells belong to the myeloid or lymphoid lineage is unclear, as CD11b and DEC205, proteins normally associated with the myeloid and lymphoid DC lineage, respectively, were not detected. The lack of F4/80, CD4, and FDC-M2 expression, molecules found on macrophages (18), germinal center DCs (19), and FDCs (20), respectively, further define these as a unique DC subset.

As CR-Fc binding DCs appear mainly in the follicles and not the paracortex, these cells may represent a population specifically suited to precipitating the early phases of humoral immunity. These cells provoke antigen-specific antibody responses in vivo after adoptive transfer, and the isotypes (i.e., IgM and IgG1) produced are restricted to that associated with initial antibody responses (8, 9). These data together with the in vitro and in vivo T cell priming results are significant because they suggest that CR-Fc binding cells have a mechanism not only for presenting peptide but also for transporting native antigen to the B cell compartment.

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The functional profile of these murine-derived CR-Fc binding DCs parallels that of the human CD14⁻⁻ derived DCs characterized by Caux and colleagues (22). The CD14⁻⁻ derived DCs have a robust and long-lasting antigen uptake capacity (22) resulting in the induction of efficient T cell proliferation, and demonstrate a unique ability to induce naive B cell differentiation into IgM-secreting plasma cells. In addition, the accessory molecule profiles of the murine and human DC subtypes are similar, as only CD40 and CD86 are necessary for CD14⁻⁻ induced T cell proliferation while CD80 plays no role. As shown in Fig. 7, the murine CR-Fc binding DC expresses CD40 and CD86 but is negative for CD80.

Assessing the chemokine production by various murine DC subsets demonstrated that CR-Fc binding DCs have a unique phenotype compared with immature (day 6) BM-DCs and interdigitating cells (the remaining population of CD11c⁺ lymph node cells isolated 24 h after antigen injec-
tion). Although the significance of a more robust MIP-1α production remains to be shown, this may relate to the CR-Fc binding DC ability to recruit cell types quickly into the tissue (23). MIP-1α is reported to attract both T cells and other DCs (24, 25). Furthermore, MIP-1α may also act as a B cell chemotactrant, as B cells have been shown to express mRNA for CC chemokine receptor CCR1 (26, 27). As other reagents become available to determine the levels of murine chemokine production, we will be able to further define the function of this cell type during immune responses.

Is the CR-Fc binding DC a migratory cell, or are we witnessing upregulation of a receptor on a resident population? Based on the ability of the CR-Fc binding DCS to prime naive T cells in lymph nodes after a subcutaneous adoptive transfer as well as their similarity to human CD14+-derived DCs, we favor the former. The relationship of the murine CR-Fc binding DC to the germinal center DC remains to be further investigated. In the future, the use of imaging systems as described by Robert and colleagues (28) should provide the means to trace the exact fate of CR-Fc binding cells in vivo.

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