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Influence of Lymphocytes on the Presence and Organization of Dendritic Cell Subsets in the Spleen

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Studies were undertaken to clarify the roles of individual leukocyte populations in maintaining the presence and organization of splenic dendritic cells (DCs). Using Abs specific for DC subsets, we found that the distinct types of DC maintained appropriate compartmentalization within the white pulp of lymphocyte-deficient mice despite an unusual overall distribution of DCs. Even in mice lacking both B and T lymphocytes, the central arteriole remained the structure around which T area DCs were organized. Marginal zone area DCs remained in a peripheral sheath excluded from the T area DCs. Additionally, we revealed an important role for splenic B cells in the presence and organization of marginal zone cells. B-deficient or B- and T-deficient mice lacked sialoadhesin\(^{+}\) marginal zone macrophages and lacked MAdCAM-1 expression in marginal zone reticular endothelial cells. Adop-
tive transfer of B lymphocytes induced MAdCAM-1 expression but failed to recruit marginal zone macrophages. Taken together, our results demonstrate that the arrival, localization, and persistence of DCs in spleen are events not solely dependent upon signals from the mature B and T cells or marginal zone macrophages. We suggest that specific stromal elements in the vicinity of the central arteriole are primarily responsible for providing directional cues to the DC.

knockouts that affect multiple lineages. Using mice that lacked mature B cells (IgM<sup>−/−</sup>), mature αβ T cells (TCR-α<sup>−/−</sup>), mature B, and mature T cells (RAG-1<sup>−/−</sup>), we examined the microanatomical location of CD11c<sup>+</sup> splenic DCs as well as the presence of phenotypically distinct subsets of splenic DCs. The implications of these findings are discussed.

### Materials and Methods

#### Animals
TCR-α<sup>−/−</sup> mice and RAG-1<sup>−/−</sup> mice were purchased from The Jackson Laboratory, Bar Harbor, ME. IgM<sup>−/−</sup> mice were generously provided by Dr. Norman Klinman, Scripps Research Institute. All mice were maintained in the Scripps Research Institute animal colony under specific pathogen-free conditions in accordance with National Institutes of Health and institutional guidelines.

#### Antibodies

The following reagents were used to label for either FACS or immunohistochemistry: anti-CD8<sup>e</sup>, anti-CD8α, anti-MAdCAM-1, anti-B220, anti-TCR-β, streptavidin-PE, PharMingen, San Diego, CA; 3D1 (anti-DC), N418 (anti-CD11c), American Type Culture Collection, Manassas, VA; anti-follicular DC (FDC) clone 4C11, gift from Marie Kosco-Vilbois; NLDC145 (anti-DEC-205), Accurate Chemicals, Westbury, NY; SER-4 (anti-metallphilic macrophage), MOMA-2 (anti-metallphilic macrophage), ERTR9 (anti-marginal zone macrophage), Bachem, Torrance, CA; biotinylated mouse anti-rat, biotinylated goat anti-Armenian hamster, HRP-goat anti-rat, Jackson ImmunoResearch Laboratories, West Grove, PA; FITC-goat anti-rat, Boehringer Mannheim, Mannheim, Germany; avidin-biotin-glucose oxidase conjugate system, glucose oxidase substrate system, Vector Laboratories, Burlingame, CA.

#### Immunohistochemistry

Cryostat sections (10 μm thick) were aceton fixed and air dried before rehydration. Sections were incubated for 30 min with PBS containing 1% BSA before incubation with the following: anti-mouse Abs, biotinylated secondary anti-Ig, streptavidin peroxidase. Peroxidase activity was visualized with 3-amin-9-ethylcarbazole substrate (Sigma, St. Louis, MO) according to the protocol provided by the manufacturer. For double labeling, peroxidase-labeled sections were again incubated for 30 min with PBS containing 1% BSA followed by labeling with anti-mouse Abs, appropriate biotinylated anti-Ig, and avidin-biotin-glucose oxidase conjugate. Bound glucose oxidase was visualized with reagents and protocols provided by the manufacturer (Vector Laboratories).

#### Reconstitution assays

Normal B220<sup>+</sup> lymphocytes from spleen or lymph nodes were sorted and injected retroorbitally into B-deficient or normal hosts. Control animals received B220<sup>−</sup>, CD4<sup>+</sup>, and B220<sup>−</sup>CD8<sup>+</sup> cells sorted from lymph node. In some experiments, B lymphocytes were isolated by depletion of CD11b<sup>CD62<sup>e</sup></sup>CD4<sup>+</sup>CD8<sup>+</sup>F480<sup>−</sup>CD3<sup>−</sup> cells by Ab labeling followed by removal with anti-Ig magnetic beads. In each case, 10–20 million cells were transferred. Seven days after transfer, animals were sacrificed, and tissues were removed for histological examination or FACS analysis of isolated splenic DCs.

#### FACS analysis

DCs were recovered from spleens by enzymatic digestion followed by isolation of low density cells (17). Enriched populations of cultured DCs were obtained by adherence of low density cells followed by overnight deadherence of mature DCs. For FACS phenotyping, cells were labeled with various anti-mouse Abs followed by FITC-anti-Ig. Identification of DCs was performed by counterstaining with PE-conjugated CD86.

#### Results

**Histological findings in normal mice**

We used a panel of mAbs to investigate the correlation between splenic marginal zone organization and the localization of DCs. In the normal mouse spleen, the majority of CD11c<sup>+</sup> DCs are found concentrated in two distinct areas within the white pulp: in macrophage-free regions at the marginal zone border and in the T areas as interdigitating cells (10) (Fig. 1a). Sialoadhesin<sup>+</sup> marginal zone macrophages (MZM) were often present as a sheath of single cells around the white pulp with regular breaks occurring at areas enriched in T cells and DCs (Fig. 1e). The MZM colocalized with another population of marginal zone cells that expressed the lymphocyte homing receptor MAdCAM-1 (14–16). Two-color immunohistochemistry demonstrated that the MAdCAM-1<sup>+</sup> reticular cells were distinct from the sialoadhesin<sup>+</sup> MZM (Fig. 1b).

**In TCR<sup>−</sup> mice, the lack of mature T lymphocytes did not appear to affect the overall organization of splenic white and red pulp or localization of macrophages and DC**

As in normal spleens, CD11c<sup>+</sup> DC in TCR<sup>−</sup> mice found concentrated near the marginal zone at breaks in the macrophage sheaths and in the T area of the white pulp (Fig. 1c). Marginal zone macrophages and MAdCAM-1<sup>+</sup> reticular cells were present at the outer border of the white pulp in the same highly organized structures observed in normal spleens (Fig. 1d). The DCs in both the T area and marginal zone area took on an appearance that was more compact than seen in normal spleens (Fig. 1e). The unusual plaque-like appearance of T area DCs was likely due to the inability to visualize individual DC bodies and dendritic processes that were normally apparent when the DCs were interdigitated between T lymphocytes. Interestingly, the marginal zone appeared more heavily populated with MAdCAM-1-expressing cells in T-deficient spleens than in normal spleens (Fig. 1e). Nevertheless, despite these unusual aspects, it was clear that DCs still arrived and resided in the appropriate non-B, perivascular areas of the splenic white pulp and marginal zone quite independent of mature T lymphocytes.

**The spleens of B lymphocyte-deficient animals displayed several abnormalities involving macrophage and DC organization**

The positioning of the marginal zone DCs in B-deficient mice represented a major departure from normal architecture. Marginal zone DCs were no longer sequestered into specific DC areas bordering the T area. Instead, the DC areas encircled the T-containing white pulp (Fig. 1f). Another striking change was the near complete absence of marginal zone macrophages and MAdCAM-1<sup>+</sup> cells (Fig. 1g). The few MZM and reticular cells were sometimes colocalized at the marginal zone borders and appeared to align in a network such as in the normal spleen. The MZM deficiency was confirmed using ERTR9 and MOMA-2 Abs specific for marginal zone macrophages or metalphilic macrophages, respectively (results not shown). Importantly, despite the meager representation of those specific marginal zone cells, there remained good segregation of white and red pulp. T-rich areas were well defined, interdigitating DCs in the T areas were easily identifiable and separate from marginal zone DCs, and F4/80<sup>−</sup> macrophages were restricted to areas outside of the T area (not shown).

**The white pulp of RAG-1<sup>−/−</sup> spleens consisted almost entirely of densely packed CD11c<sup>+</sup> DCs**

It was obvious that the DCs in RAG-1 knockout mice still received their positional cues because they organized themselves around central arterioles throughout the spleen. Similar to the situation in B-less mice, marginal zone components were disrupted (Fig. 1i). The DC areas were often associated with the few MAdCAM-1<sup>+</sup> cells also present in these spleens (Fig. 1h). Sialoadhesin<sup>+</sup> macrophages were occasionally present but not aligned in any way representative of a marginal zone. Evidently, CD11c<sup>+</sup> DCs leave venous sinuses, traverse to the vicinity of a central arteriole, and generate white pulp areas in response to signals not derived from mature lymphocytes. In the absence of lymphocytes, it was not obvious whether there remained any true segregation of the two
subsets as was observed in normal spleens. This issue was ad-
dressed in a later section.

B cell areas exert a shaping force on DC distribution

Our findings in B-deficient mice indicated that mature B lympho-
cytes had a principal role in bringing about specific aspects of splenic architecture. To confirm this, we adoptively transferred normal B lymphocytes into B lymphocyte-deficient animals. Seven days after transfer, B lymphocytes were found in several B areas of the splenic white pulp. DCs took on a more polar distribution next to the T area (Fig. 2a) instead of the circumferential distribution originally observed in B-deficient animals (Fig. 2b). Strong MAdCAM-1 expression occurred only in those tracts abutting the areas containing transferred B cells (Fig. 2a). MAdCAM-1 induction was not apparent in B-less animals that received T cells only (Fig. 2b). In TCR−/− mice, the intensity of MAdCAM-1 staining is greater than in normal spleen (left) (f). In contrast, marginal zone MAdCAM-1 expression and MZMs are sparse in BCR−/− (g), and RAG-1−/− (i) mice. Magnification of the original images a, c, d, f, g, and h was ×100, b was ×200, j was ×50, and e was ×25.

DC subsets segregate properly in the absence of mature lymphocytes.

DCs released from the mouse spleen may be divided into several subsets by their distinct surface Ag phenotypes. The two major CD11c+ subpopulations identified are also distinct by virtue of their microanatomical locations (10). Myeloid DCs expressing macrophage markers such as CD11b, CD16/CD32, and the DC-specific marker 33D1 correspond to the marginal zone DCs described earlier. They are found primarily in the area bordering the marginal zone and the white pulp (Fig. 3a). Interdigitating DCs, alternatively called lymphoid DCs, bear the DEC-205 surface Ag and CD8 but not 33D1 or any macrophage markers, and are located in the deep cortex of the T area (Fig. 3a). CD11c+ DCs in the white pulp of RAG−/− spleens were so densely packed that the distinction between the marginal zone subset and the normally interdigitating subset was not apparent. We investigated this issue using the anti-CD11c Ab, which reacts with both DC subsets in the spleen, in combination with DEC-205, which reacts with the lymphoid DC subset found in the T area. In this manner, we revealed
that the densely packed DCs in lymphocyte-deficient mice still segregated according to subset. Reminiscent of the tight knot seen in the T-deficient spleens (Fig. 3b), CD11c+DEC-205+ lymphoid DCs were tightly arranged around the arteriole in the center of the white pulp of RAG-1−/− spleens (Fig. 3c). A similarly compact sheath of DEC-205+CD11c+ myeloid DCs encircled the T area DCs. Immunostaining of serial sections revealed that the rare MAdCAM-1+ reticular cells were positioned between the two DC subsets (not shown). Thus, in the absence of signals derived from mature T and B cells, DC subsets may enter and localize appropriately within the splenic white pulp.

**Splenic DC subsets in lymphocyte-deficient mice**

All the factors that determine the DC localization are not known; hence, it is unclear whether there exists a relationship between the specific location of a subset and the survival of that subset in the tissue. We investigated whether the absence of specific leukocyte subsets and disruption of the DC-lymphocyte interaction and microanatomical location resulted in alteration of the relative numbers of specific subsets.

Of the fresh splenocytes released by collagenase digestion, CD11c+ cells represent 1–2% of the cells or 1.5–2 million DCs (not shown). Of those, ~0.8–1.0 million were recovered after enrichment by adherence and 10–15 h culture (Table I). In the cultured DC population, ~8–10% of CD86high DCs were identified as DEC-205+ lymphoid DCs (Fig. 4). The remainder were 33D1+DEC-205− myeloid DCs (not shown). Surprisingly, in the absence of T cells, the number of lymphoid DCs appeared unaffected, whereas there were slightly fewer marginal zone MAdCAM-1+ cells and no FDCs, and DCs remained in an abnormal circular distribution (b, d). Adoptively transferred B cells did not restore all marginal zone components. Despite MAdCAM-1 induction, SER-4+ macrophages were not observed to colocalize with MAdCAM-1-expressing cells in the marginal zone as was observed in normal animals. Magnification of the original images was ×100.

**FIGURE 2.** Adoptive transfer of B lymphocytes reestablished some components of splenic architecture. a, c, e. Serial sections of spleen from a B-deficient mouse that received sorted T lymphocytes only. a, c, e. Serial sections of spleen from a B-deficient mouse that received sorted B lymphocytes. MAdCAM-1 expression (a, b, e, red) and FDC formation (c, d, red) were induced where adoptively transferred B cells had established follicles (a, c). CD11c+ DCs (a, b, d, blue) adopted a more polar distribution when B follicles were formed (a). Animals that received T cells only had little marginal zone MAdCAM-1 expression (b) and no FDCs (d), and DCs remained in an abnormal circular distribution (b, d). Adoptively transferred B cells did not restore all marginal zone components. Despite MAdCAM-1 induction, SER-4+ macrophages (e, blue) were not observed to colocalize with MAdCAM-1-expressing cells (e, red) in the marginal zone as was observed in normal animals. Magnification of the original images was ×100.
25–30% of the cultured DCs were DEC-205+ (Fig. 4d). In each case, the DC deficiency was due to a loss in 33D1+ myeloid DCs, not DEC-205+ lymphoid DCs (Table I and unpublished FACS results). Apparently, the DEC-205+ DCs in T areas were not greatly affected by the loss of mature T and B lymphocytes or marginal zone macrophages. However, the extensive loss of certain marginal zone cells or total cellularity of the spleen did correlate with a loss of marginal zone DCs.

**Discussion**

DC subsets enter and occupy restricted regions in the white pulp of the murine spleen. The widely accepted explanation for existing in vitro and in vivo data is that receptors for specific chemokines or other cellular products are expressed by the DC at appropriate times during its maturation process. This selective expression directs the DC to the site in a tissue where it functions appropriately according to the situation. Thus, the decision to migrate to a particular area depends on the type of DC and its maturation stage. The maintenance of specific trafficking routes is responsible for the consistent appearance of DCs and other leukocytes in specific splenic compartments. Consequently, disrupting the delivery of certain signals to the DC would result in failure to enter the organ, disorganization within the organ, untimely death, or inappropriate exodus from the organ. The experiments described here examined the DCs in mice lacking defined lymphocyte populations. Using this approach, we were able to distinguish the contributions of specific leukocyte populations to the presence and overall organization of splenic DCs.

If resident and circulating lymphocytes were responsible for delivery of organizational and survival signals by splenic DCs, then lymphocyte-deficient mice should suffer profound defects in splenic DC populations. This was not the case. We found no deficit in numbers of lymphoid DCs, and only in mice that lacked B lymphocytes did there seem to be an impact on numbers of myeloid DCs. The separation of distinct DC subsets in the white pulp was not disrupted. In the absence of mature B and T lymphocyte populations, DCs enter the spleen from the blood, traverse along the length of the white pulp, and accumulate in discrete areas according to subset. Even in the case of RAG-1 knockout mice where all CD11c+ DCs resided in uniform DC areas of white pulp, it was shown that the DC subsets remained segregated. The DEC-205+ lymphoid DCs associated closely with the central arteriole and the myeloid DCs remained outside the lymphoid DC zone. Thus, the arrangement of splenic DC subsets in lymphocyte-deficient mice demonstrated clearly that in general, the homing instructions to DC areas were independent of lymphocytes.

**Table 1. Numbers of DCs released from wild-type (WT) and lymphocyte-deficient animals**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Total DCs B7-2high (×10⁴)</th>
<th>Lymphoid DCs B7-2high/IDC+ (×10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>80</td>
<td>6–10</td>
</tr>
<tr>
<td>TCR−/−</td>
<td>70</td>
<td>6–8</td>
</tr>
<tr>
<td>BCR−/−</td>
<td>60</td>
<td>6–8</td>
</tr>
<tr>
<td>RAG-1−/−</td>
<td>20–30</td>
<td>6–8</td>
</tr>
</tbody>
</table>

* DC quantities in spleens of normal and knockout mice. The numbers of cultured DEC-205+ and DEC-205− DCs per spleen were enumerated from FACS analysis of CD86high cells recovered after culture (Fig. 4). The DC subsets are not equally impacted by the altered architecture and decreased spleen volume given that roughly equivalent numbers of lymphoid, DEC-205− DCs are isolated from each of the mutant animals. Data are from a single experiment representative of five separate examinations of splenic DCs in the various knockout mice.

**FIGURE 3.** Detail of splenic DC subsets in normal and lymphocyte-deficient animals. Sections from normal (a) and T-deficient (b) animals were stained with mAbs N418 (CD11c, red) and NLDC145 (DEC-205, blue). Using this protocol CD11c+, DEC-205+ cells appear blue/black and CD11c+ DEC-205− cells appear red. In the absence of T cells (b), DEC-205+ T area DCs are densely packed around the central arterioles (arrowheads) and surrounded by the expansive B area (*). Compare this with the dispersed arrangement of T area DCs in the normal spleen (a). Brown/red DEC-205− cells are sometimes apparent in T areas of spleen. These cells may indeed be negative for DEC-205 expression or instead may appear due to an artifact of dual Ab staining on DEC-205− cells. Marginal zone DCs are apparent on the periphery of T areas in the normal spleen. The white pulp in RAG-1−/− mice (c) consists of lymphoid DCs (DEC-205−, red) densely packed around a central arteriole (arrow), and surrounded by DEC-205− myeloid zone DCs (CD11c+, blue). This staining protocol is reversed from a and b. Anti-DEC-205 immunostaining was developed first with red substrate followed by anti-CD11c immunostaining visualized with blue substrate. Although T area DCs are CD11c+, over development of the red substrate was performed to inhibit subsequent visualization with blue substrate. In this way, the two subsets could be differentiated. Magnification of the original image in a was 25×, b was 50×, and c was 100×.
Although the recruitment of DCs into the white pulp of the spleen and segregation of subsets was not greatly impacted, some aspects of the spatial arrangement of DC subsets were affected by the loss of lymphocytes. The abnormal distribution may have occurred due to a loss of cell volume. For example, in T lymphocyte-deficient mice, the white pulp consisted primarily of B cells. As a result, the T area DCs were reduced to a compact plaque of cells. Similarly, the marginal zone area DCs experienced greater than usual pressures excluding them from regions adjacent to B areas resulting in a small patch of marginal zone area DCs compacted to a higher cell density. In the converse situation, B lymphocyte-deficient mice lacked the large B follicles that normally drive the DCs into proscribed areas adjacent to the T cells. As a result, when marginal zone DCs migrated to white pulp that lacked B cells, the DCs remained fairly evenly dispersed around the T areas. Another explanation for the abnormal distribution of DCs in these mice may be altered chemotactic signals. Diminished chemokine levels in B-deficient mice and strengthened chemotactic signals in white pulp of T-deficient mice might result in a diffuse arrangement of myeloid DCs in B-deficient mice and the compact arrangement in T-deficient mice. Indeed, B lymphocytes are a source of macrophage inflammatory protein (MIP)-1α and MIP-1β, both of which are chemotactants for blood-derived DCs (19–21). Furthermore, if macrophages in the marginal zone also bear receptors for these factors, then macrophage-free areas would be the sites where factors would be accessible to myeloid DCs.

B lymphocyte transfer into B-deficient mice partly restored some but not all aspects of normal architecture. In B-deficient mice or in mice that received B cells by adoptive transfer 1–2 wk before examination, marginal zone macrophages were not detected with Abs specific for sialoadhesin (SER-4), marginal zone metallophilic (MOMA-1), or MZM (ERTR-9). FDCs were restored in B areas of B cell recipients. MadCAM-1 expression by reticular cells was reestablished in marginal zones bordering all areas populated with transferred B lymphocytes. It is likely that lymphotxin expression by B lymphocytes is responsible for marginal zone MadCAM-1 expression in the spleen (22). As predicted, the B-containing white pulp had the effect of sequestering the DCs into a more polar distribution. These observations suggest that B follicles have a significant effect on the DC distribution even though other elements are the principal issuers of DC guidance signals. The nature of the contribution of B lymphocytes to the organization of DCs remains unclear.

We report a correlation between marginal zone disorganization and decreased numbers of marginal zone DCs. One possible explanation for the loss of myeloid DCs is that these DCs are sensitive to changes in chemokine levels brought about by the absence of B cells. Indeed, myeloid DCs reportedly undergo apoptosis if they do not receive maturational or survival signals (23). We and others have found decreased levels of SLC, ELC, and B lymphocyte chemoattractant in lymphocyte-deficient mice (Ref. 24 and unpublished results). In this case, it is possible that other factors are also present in diminished concentrations at an increasing distance from the white pulp source. Consequently, the myeloid DCs in the marginal zone border would not receive appropriate levels for survival or maturation. Attractive candidate factors to provide such a signal are B cell-derived chemokines MIP-1α and MIP-1β (19–21).

Our observations support models that depict non-bone marrow-derived endothelial cells as the source of important organizational signals. We provide evidence that factors that direct DC migration to splenic white pulp are present independently of mature lymphocytes. The task ahead lies in identifying the key factors and the specific cell type responsible. Several reports have shown that the relevant source is in the T area in the vicinity of the central arteriole (7, 24). The efferent lymphatic vessels of the spleen girdle the central arteriole in the deep cortex of the white pulp. Factors produced by endothelial cells or macrophages resident in these structures may be responsible in part for directing the DCs to the T area. For example, endothelial cells can produce a number of chemokines including RANTES (25) and MCP-1 (26) for which DCs express receptors. It had also been shown that MIP-3β expression was restricted to T areas and that only mature, activated DCs had a chemotactic response to this chemokine (1, 27). Lympathic endothelial cells in the vicinity of the central arteriole may express this or other factors chemotactic for DCs. Indeed, two recent reports indicate a role for stromal cell-derived SLC and ELC in recruitment of DCs to T zones in peripheral lymphoid organs, although the mechanisms of their actions are not well understood (5–7). The identification of other ligands and receptors that promote DC chemotactic activity in the spleen will shape future models.
Acknowledgments

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References