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B Cells Control the Migration of a Subset of Dendritic Cells into B Cell Follicles Via CXC Chemokine Ligand 13 in a Lymphotoxin-Dependent Fashion

Ping Yu,* Yang Wang,* Robert K. Chin,* Luisa Martinez-Pomares,† Siamon Gordon,* Marie H. Kosco-Vibois,§ Jason Cyster,§ and Yang-Xin Fu2,*

Certain classes of dendritic cells (DCs) meet rare cognate Ag-specific T and B cells inside primary B cell follicles for the development of germinal centers. However, the mechanisms underlying this coordination are still undefined. Cysteine-rich (CR) domain of the mannose receptor (CR-Fc)+ DCs are a newly discovered subset of DCs that migrate rapidly into the primary lymphoid follicles from marginal zone after immunization. In this work, we uncover the key role of B cells in the establishment of a microenvironment that allows these DCs to be in the B cell area in a lymphotoxin (LT)-dependent fashion. CR-Fc+ DCs are absent from the spleens of both LTβR- and LTα-deficient mice, suggesting that signaling by membrane LT is required for the presence of CR-Fc+ DCs in the spleen. Interestingly, analysis of mutant mice that lack T, B, or NK cells demonstrates that B cell-derived membrane LT is essential for the unique localization of CR-Fc+ DCs in the spleen. Using bone marrow transfer and ligand-blocking approaches, we provide evidence that B cell-derived LT acts indirectly on CR-Fc+ DCs through LTβR+ stromal cells. In analogous fashion to certain Ag-activated T and B cells, CR-Fc+ DCs, expressing CXCR5, localize to primary lymphoid follicles in response to CXC ligand 13 (B lymphocyte chemotactrant). Together, we propose that B cells play a central role in establishing the chemotactic gradient that attracts not only Ag-activated T and B cells but also Ag-carrying CR-Fc+ DCs. In turn, CR-Fc+ DCs and T cells home to B cell follicles to interact with B cells in the developing germinal center. The Journal of Immunology, 2002, 168: 5117–5123.

T-dependent Ab responses require dendritic cells (DCs) for the activation of naive T cells, whose differentiation into effective helper cells promotes B cell activation and Ab production (1–5). Recent evidence from human and rat studies has shown that DCs can also directly regulate naive and memory B cells (6–8). Similar to the T/DC interaction, B cells can also contact APCs to form a B synapse, leading to B cell activation (9). However, the subset of DCs that migrates into the B cell follicle and promotes IgG response in vivo has not been well characterized. Martinez-Pomares et al. (10) have identified a subset of murine cells displaying distinctive dendritic morphology present in the spleen marginal zone and B cell follicles. These cells stain positive with a chimeric protein containing the cysteine-rich (CR) domain of the mannose receptor fused to the Fe region of human IgG1 (CR-Fc). In contrast to the T cell zone localization of the majority of DCs, CR-Fc+ cells migrate toward B cell follicles and accumulate in developing germinal centers (GC) after immunization (10). Later studies performed by Berney et al. (11) confirmed the identity of these CR-Fc+ cells as a unique subset of CD11c+ DCs. In contrast to CR-Fc− DCs, adoptive transfer of purified CR-Fc+ DCs from immunized mice can induce the production of Ag-specific IgM and IgG1 in naive mice. These results validate CR-Fc binding as the marker of a novel subset of murine DCs capable of migration into B cell follicles during humoral immune responses (11).

Humoral immune responses require the physical contact of APCs and rare Ag-specific B and T lymphocytes in secondary lymphoid organs (4, 5). Recent data strongly implicate the intimate involvement of chemokines and their receptors in both immune cell trafficking and the compartmentalization of lymphoid organs (4). DCs are known to migrate to T cell zones to interact with T cells (12, 13). Extensive studies have focused on the mutual regulation of T cells and DCs and the mechanisms controlling the migration of maturing DCs to the T cell zone (5). The coordinated action of several chemokines expressed in the T cell zones of secondary lymphoid organs, including EBV-induced gene 1 ligand chemokine/CCL19 and secondary lymphoid tissue chemokine/ CCL21, attracts mature DCs and naive T lymphocytes (14–16). They are likely to play a key role in facilitating the requisite encounter between Ag-bearing DCs and specific T cells. In contrast to the T/DC interaction, the interaction between B cells and DCs in vivo is significantly less well defined, as is the mechanism underlying the migration of DCs into B cell follicles.

Lymphotoxin (LT)α is required for the establishment of a microenvironment necessary for the localization of immune cells in
the spleen (4, 17–19). LTα may exist in a secreted form as a homotrimeric protein (LTα3), binding to the two defined TNFRs, TNFRI and TNFRII (20, 21). LTα also exists in a heteromeric form with membrane LTβ, as LTα1β2 (22, 23). Membrane LT is expressed on activated T, B, and NK cells and signals through a high-affinity interaction with LTβR, found predominantly on non-lymphoid cells (21–25). This signal is critical for lymph node and Peyer’s patch development and formation of microenvironments in the secondary lymphoid tissue (18, 26–32). The necessity of membrane LT and its receptor LTβR on normal lymphoid organ development and architecture can be attributed, at least in part, to its ability to induce the expression of various chemokines in lymphoid organs (19). The lack of chemokines may contribute to disorganized T cells, B cells, and DCs in LTα−/− mice (17). Membrane LT has been shown to be required for stromal cell expression of CXC ligand (CXCL)13 (B lymphocyte chemottractant (BLC)) in the B cell area of the spleen (19). CXCL13 is strongly expressed in the follicles of Peyer’s patches, the spleen, and lymph nodes (33). The interaction between CXCL13 and its receptor CXCR5 is essential for the migration of B cells to the B cell follicles to support IgG responses (34).

We now demonstrate that CR-Fc+ DCs, expressing CXCR5, localize to primary lymphoid follicles in response to CXCL13. We demonstrate that B cell-derived, but not T or NK cell-derived, membrane LT is essential for the regulation of CR-Fc+ DCs by controlling CXCL13 expression on the follicular stromal cells. This study reveals a novel mechanism by which a subset of DCs regulates secondary lymphoid tissue chemokine expression, and these findings may have implications for effective IgG responses.

Materials and Methods

Mice

C57BL/6 (B6), B6 μMT (BCR−/−), B6 TCRβ−/−δ−/− (TCR−/−), and B6 recombinase-activating gene (RAG)-1−/− mice as well as CBA-transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). LTα−/− mice (backcrossed to B6 mice for seven generations) and their wild-type littermates on a B6 background were bred under specific pathogen-free conditions. CXCL13−/− mice were described previously (35). LTβR−/− mice were kindly provided by Dr. K. Pfeffer (Technical University of Munich, Munich, Germany) (28). Animal care and use were in accordance with institutional guidelines.

Reagents and in vivo blockage of membrane LT or LIGHT activity

Production and preparation of anti-LTβ Ab (25) LTβR-Ig fusion protein (17) used in this study have been previously described. Chinese hamster ovary cells transfected with pMMV vector expression containing DNA coding sequence of herpesvirus entry mediator (HVEM) extracellular domain fused to that of Fe portion of murine IgG2a were kindly gifts of Dr. L. Chen (Mayo Clinic, Rochester, MN). The HVEM-Ig chimeric protein was purified from supernatants of transfected cells by protein A chromatography. To block membrane LT or LIGHT (homologous to LT, inducible expression, compete with herpesvirus glycoprotein D for HVEM on T cells or TNF superfamily 14) activity in mice, the LTβR-Ig and anti-LTβ Ab or HEVM-Ig (100 μg/injection) were given i.p., and the presence of CR-Fc+ DCs was determined 7–14 days later by immunohistology. The CR-Fc fusion protein was generated as described before (10). In brief, the CR domain of the mannose receptor was amplified by RT-PCR and cloned into the pGEX expression vector. The resulting plasmid was transfected into COS-7 cells. The chimeric protein consisting of CR fused to the Fc portion of human IgG1 was purified from supernatants of transfected cells by protein A column and stored at −20°C.

BM transplantation

Bone marrow (BM) was harvested and recipients were prepared as described previously (32, 36). Recipient mice were lethally irradiated with 1050 rad and reconstituted with 5 × 107 donor BM cells. Six weeks after transplantation, recipients were sacrificed and the presence of CR-Fc+ DCs in the spleen was determined by immunohistology.

Transfer of lymphocytes

Whole spleen cell suspensions were prepared from a single mouse donor by mincing with scissors and teasing between two frosted microscope slides. B cells were enriched using a nylon wool column as described (37) and further purified by using a column-based purification technique (27). Cell Technologies, Paisley, U.K.) according to the manufacturer’s instructions. In brief, B cell-enriched spleen cell suspensions were incubated with a mixture containing anti-CD3, CD11b, TER119, and Gr-1 Abs. After the incubation at 4°C, magnetic colloids were added to cell-Ab complexes to deplete cells stained with the above Abs by using a column and a magnet. The purity of cells after column depletion was determined by flow cytometric analysis of purified cells. These preparations contained 95–97% B cells, defined as IgM+ B220+ by flow cytometry. There were no detectable contaminating T cells. A total of 107 purified B cells was injected i.v. into each recipient. Three, 4, or 5 wk after transfer, spleens from these mice were analyzed by immunohistology.

Immunohistology

Spleens were harvested, embedded in OCT compound (Miles-Yeda, Rehovot, Israel) and frozen at −70°C. Frozen sections (6–10 μm thick) were fixed in cold acetone. For immunohistochemical staining, endogenous peroxidase was quenched with 0.06% H2O2 in methanol. The sections were preblocked with 2% normal mouse serum, rabbit serum, and goat serum in PBS for half an hour at room temperature in a humidified chamber. Staining for B cells and CR-Fc+ DCs was done by first incubating with FITC-conjugated anti-B220 (BD PharMingen, San Diego, CA) at a 1/100 dilution and CR-Fc at a 17.5 μg/ml concentration (1/200 dilution of the stock preparation) in blocking buffer. HRP-conjugated rabbit anti-FITC (DAKO, Carpinteria, CA) and alkaline phosphatase-conjugated rabbit anti-human IgG (H and L chains) (Southern Biotechnology Associates, Birmingham, AL) were added 1 h later. Staining for metallophilic macrophages was done with MOMA-1 (Serotec, Oxford, U.K.) at a 10 μg/ml concentration followed by a secondary incubation with HRP-conjugated goat anti-rat IgG (Southern Biotechnology Associates) (38). Staining of CD11c+ DCs was done with biotin-conjugated anti-CD11c (BD PharMingen) at a 1/100 dilution. Alkaline phosphatase-conjugated streptavidin (Vector Laboratories, Burlingame, CA) was used as second-step Ab. Color development for alkaline phosphatase and HRP was performed with an alkaline phosphatase reaction kit (Vector Laboratories) and with 3,3′-diaminobenzidine (Sigma-Aldrich, St. Louis, MO), respectively. For immunofluorescence staining, sections were blocked with 2% normal mouse serum, rabbit serum, and goat serum in PBS for half an hour at room temperature in a humidified chamber. Blocking solution was replaced with 50 μl of primary Abs, PE-conjugated anti-B220 (BD PharMingen), CR-Fc, or biotin-conjugated anti-CD11c (BD PharMingen), diluted 1/100 in blocking solution, and sections were incubated for 1 h at room temperature in a humid chamber. FITC-conjugated donkey anti-human Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) and PE-conjugated streptavidin (Immuno Technologies, Cedx, France) were used as secondary reagents for CR-Fc and biotin-conjugated anti-CD11c, respectively. Specimens were mounted in Mowiol 4-88 (BD Biosciences, La Jolla, CA) containing 10% 1,4-diazobicyclo [2.2.2] octane. Samples were analyzed within 48 h using a Zeiss Axiosplan microscope (Zeiss, Oberkochen, Germany) and a Photometrics PXL CCD camera (Photometrics, Tucson, AZ). No-neighbour deconvolution was performed using Openlab v2.0.6 (Improvision, Lexington, MA).

Enrichment of DCs

B6 mice were injected s.c. in each limb (50 μg per limb) and at the end of the tail (100 μg) with chicken egg OVA (Sigma-Aldrich) emulsified in IFA. Forty-eight hours later, the draining lymph nodes were collected and digested with 2 mg/ml collagenase and 100 μg/ml DNase for 30 min at 37°C and then gently pipetted in the presence of 0.01 M EDTA for 1 min. CD11c+ DCs were positively enriched by magnetic cell sorting (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. In brief, $^{1}$H-labeled cells were sequentially incubated with biotinylated mouse anti-CD11c (BD PharMingen) at 10 μg/ml and MACS streptavidin MicroBeads (Miltenyi Biotec) before proceeding to magnetic separation. Positive selection column type MS RS7 was used. CXCR5 expression on
CR-Fc<sup>+</sup> cells was analyzed on FACScan (BD Biosciences) by staining positively enriched CD11c<sup>+</sup> DCs.

**FACS analysis**

The hybridomas 2A4G2 (anti-CD32) were obtained from American Type Culture Collection (Manassas, VA) and used to block FcR binding. A polyclonal rabbit antiserum prepared by immunization with CXCR5 peptides has been described previously (39). The enriched CD11c<sup>+</sup> DCs were stained and analyzed by two-color flow cytometry on a FACScan (BD Biosciences). In brief, the enriched CD11c<sup>+</sup> DCs were first blocked with anti-CD32 before being incubated with anti-CXCR5 Ab at a 1/50 dilution and CR-Fc at a 10 μg/ml concentration. The secondary reagents were goat anti-rabbit IgG (H and L chains) conjugated to PE (Southern Biotechnology Associates) and donkey anti-human IgG (H and L chains) conjugated to FITC (Jackson ImmunoResearch Laboratories).

**Results**

**LPS drives the migration of marginal zone CR-Fc<sup>+</sup> DCs into the B cell but not T cell zone**

In the spleens of wild-type mice, CR-Fc<sup>+</sup> DCs are found predominantly in the marginal zones, with a small proportion inside the B cell follicles (Fig. 1A). Pathogenic products such as LPS trigger the migration of the majority of CD11<sup>high</sup> DCs into the T cell areas of lymphoid organs (40). To study the migration pattern of CR-Fc<sup>+</sup> DCs in response to LPS, we analyzed serial splenic sections of wild-type mice 24 h after LPS injection. We found dramatically decreased numbers of CR-Fc<sup>+</sup> DCs in the marginal zone and increased LPS-activated CR-Fc<sup>+</sup> DCs in the B cell area (Fig. 1A). Staining of spleen sections with anti-CD11c verified the migration of the majority of CD11c<sup>+</sup> DCs into the T cell zone, in the center of the white pulp (Fig. 1B). Double staining of these sections with CR-Fc and anti-CD11c confirmed the unique response of this CD11c<sup>+</sup>-CR-Fc<sup>+</sup> DC subset in migrating to the B rather than T cell zones (Fig. 1B). These results suggest that activated CR-Fc<sup>+</sup> DCs in marginal zone may respond to trafficking cues differently from the majority of conventional DCs.

**LT is required for the presence of CR-Fc<sup>+</sup> DCs in the marginal zones and B cell follicles of the spleen**

We have previously reported that LTα<sup>−/−</sup> mice present with disorganized DC distribution and disrupted T/B cell segregation, and fail to generate both primary and secondary IgG response to foreign Ags (17, 32, 41). Therefore, LT is a candidate cytokine for regulating the positioning of CR-Fc<sup>+</sup> DCs. To explore this possibility, spleens from LTα<sup>−/−</sup> mice were stained for CR-Fc<sup>+</sup> DCs. Interestingly, no CR-Fc<sup>+</sup> cells were detectable in the spleen (Fig. 2A), suggesting an essential role for LT in regulating CR-Fc<sup>+</sup> DC presence in the spleen. As LTα<sup>−/−</sup> mice lack both soluble LTα3 and membrane LTαβ2, it was important to isolate the contributions of each. To study whether LTα3 is required for the presence of CR-Fc<sup>+</sup> DCs, spleens from TNFRI<sup>−/−</sup> mice were analyzed. Staining with CR-Fc showed a pattern of distribution of CR-Fc<sup>+</sup> cells indistinguishable from wild-type (data not shown), pointing to the dispensability of LTα3 signaling through TNFRI. To isolate the involvement of LTβR, spleens from LTβR-deficient mice were stained for CR-Fc<sup>+</sup> DCs. These mice showed CR-Fc<sup>+</sup> DC staining in neither the marginal zone nor the B cell follicle (Fig. 2A). These experiments suggest that both LT and LTβR are essential for the presence of CR-Fc<sup>+</sup> DCs in the spleen.
Membrane LT is required for the maintenance of CR-Fc⁺ cells in the spleen

We have previously reported that LT participates in the organization of lymph nodes and Peyer’s patches early in development and actively maintains lymphoid microenvironments throughout life. To determine whether membrane LT is required for the maintenance of CR-Fc⁺ DCs in the spleen, we treated adult B6 mice with 100 μg LTβR-Ig fusion protein. Within a few days, CR-Fc⁺ DCs had completely disappeared from these spleens (Fig. 2B), suggesting that LTβR ligands are required. However, soluble LTβR-Ig can bind both membrane LT and LIGHT (TNF superfamily 14), a newly defined TNF superfamily member (42, 43). To confirm the role of membrane LT in the maintenance of CR-Fc⁺ DCs in the spleen, mice were treated with anti-LTβ Ab, which specifically blocks membrane LT but not LIGHT. CR-Fc⁺ DCs were undetectable in both the marginal zone and B cell area (Fig. 2B). To exclude a possible contribution by LIGHT, wild-type mice were treated with soluble HVEM-Ig. HVEM-Ig blocks LIGHT function without any interference with membrane LTα1β2 (42, 43). Immunohistochemical analysis of HVEM-Ig-treated mice showed unperturbed CR-Fc⁺ DC distribution (Fig. 2B). These results substantiate our finding that membrane LT, but not LIGHT, is essential for the maintenance of CR-Fc⁺ DCs in the spleen.

Control of CR-Fc⁺ DCs in the spleen by B cells but not T cells or NK cells

LTα1β2 is expressed predominantly on T, B, and NK cells. To test whether T and B cells are required for the regulation of CR-Fc⁺ DCs in the spleen, RAG-1⁻/⁻ mice, which lack both T and B cells, were used. RAG-1⁻/⁻ mice showed barely detectable CR-Fc⁺ cells in spleen (Fig. 3A), suggesting that either T or B cells, or both, may regulate splenic CR-Fc⁺ DCs in the adult mice (Table 1). To investigate whether T cells were required for the homeostasis of CR-Fc⁺ DCs, spleens from TCR⁻/⁻ mice were stained with CR-Fc. Spleens from TCR⁻/⁻ mice manifest a strong staining of CR-Fc, similar to that of wild-type mice (Fig. 3A). Normal staining of CR-Fc⁺ DCs in the CD3e-transgenic mice, which are defective in NK and T cells, further verifies that neither NK nor T cells are involved in the regulation of CR-Fc⁺ DCs (Table 1). Interestingly, BCR⁻/⁻ mice show undetectable staining of CR-Fc⁺ DCs in the spleens (Fig. 3A). These data indicate that B cells, but not T or NK cells, are essential for the regulation of CR-Fc⁺ DCs.

So far, we have found that membrane LTα1β2 and B cells are required for the regulation of CR-Fc⁺ DCs. To further substantiate the role of membrane LTα1β2 on the B cells collected in the control of CR-Fc⁺ DCs, TCR⁻/⁻ mice were administered with 100 μg LTβR-Ig fusion protein and the spleens were collected 7 days later. No CR-Fc⁺ cells could be detected in either the splenic sinus or the follicular DC (FDC) area in TCR⁻/⁻ mice treated with LTβR-Ig fusion protein (Fig. 3A), confirming that constant signalizing from LT-expressing B cells is required for regulation of CR-Fc⁺ DCs. To exclude the possibility that the absence of CR-Fc⁺ DCs is due to the absence of the marginal zone, we stained metallophilic macrophages normally present in the marginal zones with MOMA-1 Ab. MOMA-1⁺ cells were clearly present (Fig. 3A), suggesting that there are intact marginal zones in the spleens of both TCR⁻/⁻ and BCR⁻/⁻ mice. Therefore, the lack of CR-Fc⁺ DCs in the BCR⁻/⁻ mice may not be attributed by the absence of marginal zone. It also suggests that MOMA-1⁺ cells are not the same cells as CR-Fc⁺ DCs. In fact, many MOMA-1⁺ cells did not colocalize with CR-Fc⁺ DCs when double staining was performed (data not shown). Whether the two types of cells are derived from the same lineage remains to be determined.

BM from TCR⁻/⁻ mice may contain other type of cells different from those of BCR⁻/⁻ mice. To investigate whether mature B cells are sufficient for the regulation of CR-Fc⁺ DCs, purified B cells from the splenocytes of TCR⁻/⁻ mice were transferred into RAG-1⁻/⁻ mice. Three weeks after transfer, lymphoid tissues from these mice were analyzed. CR-Fc expression was restored in the reconstituted mice (Fig. 3B), suggesting that mature B cells are sufficient to support the presence of CR-Fc⁺ DCs in the absence of T and NK cells. To further prove that it is LT derived from B cells that restored the CR-Fc⁺ DCs in the RAG-1⁻/⁻ mice, purified B
cells from the spleens of LT-deficient mice were transferred into RAG-1$^{-/-}$ mice. CR-Fc staining in the spleens of the recipients was analyzed by histology 3, 4, or 5 wk after transfer. We found that B cells were present in the spleens in numbers similar to RAG-1$^{-/-}$ recipients of wild-type B cells. However, CR-Fc$^+$ DCs clearly did not recover in mice that received LT-deficient B cells (Fig. 3B). These experiments clearly demonstrate that LT derived from B cells is sufficient to support the presence of CR-Fc$^+$ DCs in the spleen.

**LTβR on stromal cells is required for the presence of CR-Fc$^+$ DC in the spleen**

Membrane LT signaling through LTβR is necessary for the presence of CR-Fc$^+$ DCs in the spleen. To determine whether BM-derived cells or radiation-resistant stromal cells are the membrane LTα1β2 responding cells, reciprocal BM transplantation was performed. In lethally irradiated wild-type mice reconstituted with BM from wild-type mice, CR-Fc staining was maintained in the FDC area and marginal sinus (Fig. 4). While no CR-Fc$^+$ cells were detected in the spleens of LTβR$^{-/-}$ mice reconstituted with BM from wild-type mice, CR-Fc staining was normal in wild-type mice that received BM from LTβR$^{-/-}$ mice (Fig. 4). These data indicate that LTβR on BM-derived cells is not required for the presence of CR-Fc$^+$ DCs in the spleen. In contrast, LTβR expressed on stromal cells in the spleen is important for the presence of CR-Fc$^+$ DCs in the spleen. It is likely that LT does not act directly on CR-Fc$^+$ DCs, a BM-derived population, but rather on LTβR$^+$-positive stromal cells.

**CR-Fc$^+$ DCs express CXCR5, while the chemokine CXCL13 is required for the normal positioning of CR-Fc$^+$ DCs in the spleen**

It has been reported that membrane LT is required for the CXCL13 (BLC) production by follicular stromal cells (19). CXCL13 is thought to pair exclusively with CXCR5 to attract activated Ag-specific T and B cells into B cell follicles (4). We wondered whether CXCL13 is also required for the normal presence of CR-Fc$^+$ DCs in the spleen. To test that hypothesis, spleens from CXCL13 (BLC)-deficient (CXCL13$^{-/-}$) mice were stained for CR-Fc$^+$ cells. Interestingly, few CR-Fc$^+$ were detected in the spleens of CXCL13$^{-/-}$ mice (Fig. 5A). This result confirms the requirement for CXCL13 for the presence of CR-Fc$^+$ DCs in both the marginal zones and B cell follicles in the spleen. It was interesting to test whether CR-Fc$^+$ DCs express CXCR5 (Burkitt’s lymphoma receptor-1) so that they can directly respond to CXCL13 gradient. To test this hypothesis, we enriched for CR-Fc$^+$ DCs from the lymph nodes after immunization with OVA and stained the CR-Fc$^+$ DCs with anti-CXCR5 Ab. Most of the purified CR-Fc$^+$ DCs were positive for CXCR5 (Fig. 5B). This result supports our hypothesis about the possible mechanism explaining the role of LT in regulating CXCR5$^+$ CR-Fc$^+$ DCs via a CXCL13 gradient established in B cell follicles by B cells.

**Discussion**

This study has revealed the molecular and cellular requirements regulating the localization of CR-Fc$^+$ DCs in the spleen. We have demonstrated that the presence of CR-Fc$^+$ DCs in the spleen is primarily regulated by membrane LT. 1) Neither LTα$^{-/-}$ nor

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**Table 1. B cells, but not T and NK cells, are required for the presence of CR-Fc$^+$ DCs in the spleen**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Absent Cell Lineages</th>
<th>CR-Fc$^+$</th>
<th>MOMA-I$^+$</th>
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<tbody>
<tr>
<td>WT</td>
<td></td>
<td>+</td>
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<td>RAG$^{--}$</td>
<td>T, B</td>
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<td>TCR$^{--}$</td>
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<td>CD3$^+$-transgenic</td>
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<tr>
<td>BCR$^{--}$</td>
<td>B</td>
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<td>+</td>
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* +, Detectable; +/-, weakly detectable; -, undetectable.
LTβR−/− mice have CR-Fc+ DCs in the spleen (Fig. 2A), while TNFR1- or TNFR2-deficient mice have relative normal staining of CR-Fc (P. Yu and Y.-X. Fu, unpublished data). 2) Transfer of LT-expressing lymphocytes into Rag-1−/− mice restores the presence of CR-Fc+ DCs in the spleen while LT-deficient lymphocytes fail to do so (Fig. 3B). 3) Preexisting CR-Fc+ DCs in wild-type mice disappeared completely within 7 days after blockade of LTβR signaling, by either LTβR-Ig or anti-LTβR Ab (Fig. 2B). In contrast, total CD11c+ cells were only slightly reduced after 7 days and reduced up to 60% in the second week of treatment.

We have also determined that B cells, but not T or NK cells, are essential for the regulation of CR-Fc+ DCs. 1) BCR−/− but not TCR−/− mice show undetectable CR-Fc staining (Fig. 3A). 2) CR-Fc staining disappears completely after the blockade of LT signaling in TCR−/− mice (Fig. 3A). 3) CR-Fc staining in Rag-1−/− mice recovers completely after LT-expressing B cells are transferred (Fig. 3B). It is possible that T cells, mostly located in T cell zones, have little opportunity to physically contact stromal cells in B cell zone via membrane LT. This is consistent with the result that B cell-derived membrane LT, rather than soluble LT, plays an important role in establishing a microenvironment inside B cell follicle conducive for the formation of GCs.

We have further demonstrated that B cell-derived LT does not act directly on CR-Fc+ DCs to regulate its localization, but rather acts indirectly through LTβR-expressing stromal cells. It has been shown that membrane LT is required for stromal cell expression of CXCL13 in the B cell area of the spleen (19). We show in this study that CR-Fc+ DCs express CXCR5, and that the chemokine CXCL13 is required for the normal presence of CR-Fc+ DCs. These lines of evidence strongly support the proposal that B cell-derived LT acts on follicular stromal cells to form a CXCL13 gradient, which brings Ag-carrying CR-Fc+ DCs and Ag-specific T and B cells together. In addition to providing Ag, CR-Fc+ DCs also produce high levels of lymphocyte chemoattractant, macrophage inflammatory protein-1α, and other factors to attract and activate T cells (11). The dynamic direct contact is also required for the interaction between membrane ligands and receptors, such as CD40 and CD40 ligand, for effective GC formation and IgG response (3, 5). A recent report has shown that B cells can contact APC directly to form a B synapse and acquire Ags from APCs (9). Therefore, B cells can play a central role in bringing relevant DCs and Ag-specific T and B cells together via the same chemokine to coordinate them for developing GCs.

Accumulating evidence indicates that B cell function may not be limited to Ab production. B cells may also actively create lymphoid microenvironments that promote their interaction with other cells. A recent study demonstrated that early T cell responses to Listeria monocytogenes in μMT (BCR−/−) mice is impaired despite the transfer of serum from Listeria-infected wild-type mice, even though B cell Ab presentation is not involved in this model (44). Others have recently reported that BCR−/− mice are more susceptible to acute infection, and this cannot be completely restored by the transfer of additional sera from wild-type mice (45). We also found BCR−/− mice were much more susceptible to acute L. monocytogenes infection and harbor higher titers of Listeria in the spleen 2–4 days after infection (P. Yu and Y.-X. Fu, unpublished data). It is possible that B cells are also important for the establishment of appropriate microenvironments in the spleen to clear Listeria infections. For example, FDC development is also dependent on signals provided by B cell-derived membrane LT (18, 46), as is the maintenance of the expression of marginal zone mucosal addressin cell adhesion molecule-1 and other adhesion molecules. The marginal zone area may play a role in host response to infection and Ab response (38). The impaired marginal zone in BCR−/− mice may also increase their susceptibility to infection. B cells actively regulate and organize lymphoid structure and microenvironments to facilitate effective immune responses by providing membrane LT and possibly other signals. LT-deficient B cells have no intrinsic defects in generating Abs (32, 47). Rather, the lack of LT from B cells impairs the formation of a proper microenvironment for effective IgG responses.

LTβR-deficient mice fail to generate both primary and secondary IgG response to foreign Ags (32, 41). Although FDCs play an important role in presenting Ag-Ab complexes to activated B cells in the GC, the lack of FDC in LTβR−/− mice cannot be the major reason for the absence of early primary IgG response. In contrast to LTβR−/− mice, TNFR2−/− mice also lack FDC/GC, yet show minor reductions in IgG responses. Initial Ag may be carried by a subset of DCs that are also able to stimulate T and B cells (T and B synapse). We suspect that a subset of Ag-carrying DCs may migrate into B cell follicles to facilitate a positive feedback loop for developing GCs. Chemokines, such as CXCR13, generated from B cell follicles may be required for such migration. CR-Fc+ DCs that traffic to primary lymphoid follicles are suitable candidates for the early activation of B cells. They were initially identified as Ag-carrying cells. Most of them are strategically localized around the marginal zone, an area critical to the transport of Ags into the B cell follicles, and are capable of moving into B cell zones soon after immunization to promote GC formation. Not coincidentally, they express CXCR5 and only move into B cell follicles, even after the LPS stimulation that drives most other DCs into the T cell zone. We have found marginal changes in BLC production in the B cell follicle or CXCR5 expression in CR-Fc+ DCs after LPS stimulation. LPS may have multiple effects on the activation and maturation of DCs. What LPS triggers to allow the migration of CR-Fc+ DCs into B cell zone remains to be determined.

In summary, B cell-derived LT controls the development and maintenance of CR-Fc+ DCs, leading to the formation of secondary B cell follicles. Therefore, this study presents an example of B cells playing multiple roles in orchestrating efficient IgG responses by constantly interacting with their surrounding cells. More specifically, we propose that B cell-derived membrane LT controls CR-Fc+ DCs by regulating CXCL13 expression on the LTβR-expressing follicular stromal cells. In turn, CXCR5+ “CR-Fc+” DCs respond to the B cell-mediated CXCL13 gradient and home to B cell follicles to regulate and present Ag to B cells for an Ab response to occur. Better understanding of such interactions may help us to design proper approaches to alter unwanted immune responses and promote a more desired effect.

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**References**
