Organizer-Like Reticular Stromal Cell Layer Common to Adult Secondary Lymphoid Organs

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J Immunol 2008; 181:6189-6200; doi: 10.4049/jimmunol.181.9.6189
http://www.jimmunol.org/content/181/9/6189

Supplementary Material
http://www.jimmunol.org/content/suppl/2008/10/17/181.9.6189.DC1

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Mesenchymal stromal cells are crucial components of secondary lymphoid organs (SLOs). Organogenesis of SLOs involves specialized stromal cells, designated lymphoid tissue organizer (LTo) in the embryonic anlagen; in the adult, several distinct stromal lineages construct elaborate tissue architecture and regulate lymphocyte compartmentalization. The relationship between the LTo and adult stromal cells, however, remains unclear, as does the precise number of stromal cell types that constitute mature SLOs are unclear. From mouse lymph nodes, we established a VCAM-1+ICAM-1+MAdCAM-1+ reticular cell line that can produce CXCL13 upon LTβR stimulation and support primary B cell adhesion and migration in vitro. A similar stromal population sharing many characteristics with the LTo, designated marginal reticular cells (MRCs), was found in the outer follicular region immediately underneath the subcapsular sinuses of lymph nodes. Moreover, MRCs were commonly observed at particular sites in various SLOs even in Rag2−/− mice, but were not found in ectopic lymphoid tissues, suggesting that MRCs are a developmentally determined element. These findings lead to a comprehensive view of the stromal composition and architecture of SLOs. The Journal of Immunology, 2008, 181: 6189–6200.
feedback loop by further attracting LTi cells. Mice lacking the above signaling components exhibit various degrees of SLO deficiency and malformation (19). Generation of LTi cells from fetal liver progenitor involves Id2 and RORγt; mice deficient in these gene products show a complete loss of LTi cells and lack all LNs and PPs (20–22). TNF-related activation-induced cytokine (TRANCE), a key factor in osteoclastogenesis, participates in the proliferation and differentiation of LTi cells, particularly in the LN anlagen (23); hence, all LNs but not all PPs are absent in mice deficient in TRANCE or its receptor TRANCE-R (24, 25). Conversely, IL-7Rα expressed on LTi cells and its downstream signaling pathway are essential for the development of PPs but not LNs (16, 26). Formation of splenic white pulp does not require LTi cells, but the maturation of the tissue structure depends on LTα1β2 produced by lymphocytes and LTβR signaling (14). Therefore, despite some similarities, the developmental program and molecular requirements of each SLO are clearly different.

Even after the maturation of SLOs, continuous interplay between lymphocytes and stromal cells is likely to be required for the maintenance of tissue architecture and characteristics of adult stromal cells. Despite their importance in the spatiotemporal regulation of immune cell behavior, however, only limited information about the cytological nature of adult stromal cells has been obtained so far. It remains unknown how many different mesenchymal stromal cell types exist in particular SLOs. The relationship between embryonic LTo cells and such different types of stromal cells in adult SLOs, i.e., the postnatal fate of LTo cells, is also unclear. It is possible that LTo-like cells might still exist in the adult and play some role in the maintenance of SLOs. Since variations in stromal cells might account for the differences in both embryonic LTo cells and such different types of stromal cells, but the maturation of the tissue structure depends on LTα1β2 produced by lymphocytes and LTβR signaling (14). Therefore, despite some similarities, the developmental program and molecular requirements of each SLO are clearly different.

In this study, we report the detailed characterization of a reticular stromal cell line derived from adult mouse LN, which can produce CXCL13 upon LTβR signaling. We also found a layer of unique reticular cells underneath the subcapsular sinus lining of the LNs. These specialized mesenchymal cells share many characteristics with LTo cells and are commonly observed at certain places in various types of adult SLOs. Taken together, our observations provide new insights into the development and tissue organization of SLOs.

Materials and Methods

Mice

Mice were maintained at the animal facility in the Center for Genomic Medicine (Kyoto University). ab/a and a/b mice were purchased from CLEA Japan. Experimental procedures involving animals were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University and conducted according to the guidelines for animal treatment of the Institute for Laboratory Animals (Kyoto University).

Cells

BLS4 and BLS12 cells were established from peripheral LNs of BALB/c mouse as described previously (4). Cells were maintained in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. For ECM network formation, confluent BLS12 cells grown on 8-well chamber slides (Nalgen Nunc International) were cocultured with 2 × 10^6 lymphocytes and 5 × 10^5 BLS12 cells. Twenty-four hours before the assay, the confluent BLS12 monolayer was stimulated with agonistic anti-LTβR Ab (0.5 μg/ml). Primary lymphocytes were labeled with 1 μg/ml 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (Molecular Probes) at 37°C for 20 min. Labeled lymphocytes were applied to the BLS12 monolayer at 5 × 10^5 cells/well and incubated at 37°C for 30 min with or without 20 μg/ml blocking Abs. Alternatively, lymphocytes were pretreated with 0.2 μg/ml pertussis toxin (PTx) or B oligomer (Calbiochem) at 37°C for 2 h. Nonadherent cells were removed by five consecutive washes. Input and bound cells were measured using a fluorescence multimode plate reader (Cytofluor4000; Applied Biosystems).

Immunohistochemistry

Tissues isolated from animals were embedded in OTC compound ( Sakura Finetechical) and then frozen in liquid nitrogen. Frozen sections (10-μm thick) were fixed with cold acetone. BLS12 cells plated on chamber slides (Nalgen Nunc International) with or without coculturing or factor treatment were fixed with 3% paraformaldehyde-PBS and then permeabilized with 0.2% Triton X-100. After blocking with 1% BSA/0.05% Tween 20-PBS, sections or cells were stained with Abs. Nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Sections or cells were examined using a confocal laser scanning microscope (TSC-SP2; Leica). Digital images obtained were prepared using Adobe Photoshop software (Adobe Systems).

Flow cytometry

BLS12 cells were harvested from culture dishes using 0.02% EDTA-PBS. After blocking with PBS containing 1% BSA, the cells were stained with Abs by direct or indirect methods, counted using a FACSCalibur flow cytometer (BD Biosciences), and analyzed using CellQuest software (BD Biosciences).

ELISA

Confluent BLS12 cells in 24-well culture plates were stimulated with mouse TNF-α (10 ng/ml; PeproTech), human TNF-α (LTα3, 10 ng/ml; PeproTech), and/or polyclonal goat anti-mouse LTβR Ab (0.5 μg/ml; R&D Systems). Production of CXCL13 and CCL19 in culture supernatants was detected by sandwich ELISA using DuoSet (R&D Systems) according to the manufacturer’s recommendations.

RT-PCR analysis

RT-PCR analysis was performed as described previously (30). Specific primer pairs used in this study were as follows: GAPDH, 5'-CCATCA CCACTTTCCAGGAG-3' and 5'-CCTGCTTTACACCTCTCTT-3'; CXCL13, 5'-TGGACTCCTACCCATGCG-3' and 5'-CTTACCCGTGTTTGTTTGGT-3'; CCL19, 5'-GCCACAGTCTCTCA GGC-3' and 5'-CCCTCTCTGGTTTGGT-3'; IL-7, 5'-GGATGAGTCTG-3' and 5'-TCTGTTTCCTCTGGTCCCTG-3'.

Lymphocyte adhesion to BLS12

The in vitro adhesion assay was performed as described previously, with slight modifications (31). BLS12 cells were plated on fibronectin-coated (20 μg/ml) 96-well plates and cultured for 2–3 days to form monolayers. Twenty-four hours before the assay, the confluent BLS12 monolayer was stimulated with agonistic anti-LTβR Ab (0.5 μg/ml). Primary lymphocytes were labeled with 1 μg/ml 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (Molecular Probes) at 37°C for 20 min. Labeled lymphocytes were applied to the BLS12 monolayer at 5 × 10^5 cells/well and incubated at 37°C for 30 min with or without 20 μg/ml blocking Abs. Alternatively, lymphocytes were pretreated with 0.2 μg/ml pertussis toxin (PTx) or B oligomer (Calbiochem) at 37°C for 2 h. Nonadherent cells were removed by five consecutive washes. Input and bound cells were measured using a fluorescence multimode plate reader (Cytofluor4000; Applied Biosystems).
BLS12 cells were seeded on fibronectin-coated (20 µg/ml) 6-well plates (Biorad) and cultured for at least 5 days to construct a monolayer. The confluent BLS12 monolayer was stimulated with agonistic anti-LTβR Ab (0.5 µg/ml) for 24 h. Primary B cells (5 × 10⁶) were loaded onto activated BLS12 monolayers in RPMI 1640 medium supplemented with 8% FCS and 10 mM HEPES. After 3 h of incubation, phase-contrast images were obtained every 30 s for 30 min at 37°C on a LSM510 confocal laser microscope (Zeiss) equipped with a heating stage system for obtaining every 30 s for 30 min at 37°C on a LSM510 confocal laser microscope. Bar, 100 µm. C, Cell surface markers expressed on BLS12 cells. EDTA-harvested BLS12 cells were stained for the indicated surface markers and analyzed by flow cytometry. Histograms show overlays of stained (filled histograms) and control (open histograms). D, No significant expression of CR1 or FcγRII/III is induced in BLS12 by dual signaling through TNFR and LTβR. Cells were stimulated with or without TNF-α (10 ng/ml) and agonistic anti-LTβR Ab (0.5 µg/ml) for 2 days. Harvested cells were stained for CR1 and FcγRII/III and analyzed by flow cytometry. E and F, BLS12 constitutively expresses TRANCE mRNA, but TRANCE protein is undetectable at the cell surface. The transcript for TRANCE was detected by RT-PCR (E). The amounts of PCR products amplified from 5-fold serial dilutions of BLS12 cDNAs were standardized relative to GAPDH. Another reticular cell line, BLS4, was used as a control and showed little expression of TRANCE mRNA. Cell surface TRANCE protein was analyzed by flow cytometry (F). G, BLS12 displays carbohydrates recognized by PNA. Cells were stained with FITC-PNA in the presence or absence of 0.2 M galactose and analyzed by flow cytometry. Addition of galactose markedly diminishes the PNA binding to BLS12 cells, indicating that most of the binding is mediated by the lectin activity of PNA.

**Fc chimeric proteins**

LTβR-Fc and II-6 TCRVα-Fc chimeric proteins were produced as described previously (26, 30). Specifically, X63.653 myeloma cells were stably transfected with each vector construct, and chimeric proteins were purified from culture supernatants or ascites fluid using a protein G-Sepharose column (Amersham Biosciences). Mouse were i.v. injected weekly with 100–200 µg of chimeric proteins and were sacrificed 2–4 wk later to obtain SLOs.

**Results**

**Stromal cell line BLS12 is reticular fibroblast with the ability to produce CXCL13 upon LTβR-NIK signaling**

We previously established a series of stromal cell lines from adult mouse LNs (4). One of these, BLS12, showed typical fibroblastic morphology (Fig. 1A). When cocultured with lymphocytes, BLS12 showed the ability to produce ECM meshwork that contains laminin, fibronectin, and ER-TR7-Ag (Fig. 1B and data not shown), indicating that this cell line preserves FRC features. It is worth noting that BLS12 constitutively expressed MadCAM-1 and BP-3 (CD157), in addition to FRC markers such as VCAM-1, ICAM-1, and gp38 (podoplanin) on the surface (Fig. 1C). BLS12 also expressed LTβR, CD44, and a mesenchymal marker, PDGFRβ (Fig. 1C). In contrast, FDC markers CR1 (CD35) and FcγRII/III (CD16/32) were undetectable, and were virtually undetectable even when the cells were simultaneously stimulated with TNF-α and agonistic anti-LTβR Ab (Fig. 1D) or cocultured with lymphocytes (data not shown). Although surface expression of TRANCE protein was undetectable in BLS12, the mRNA was readily detected, in contrast to another FRC line, BLS4, in which TRANCE mRNA was almost undetectable (Fig. 1, E and F). In addition, BLS12 cells displayed cell surface carbohydrates recognized by PNA (Fig. 1G).

Of prime importance, BLS12 cells exhibited the ability to express CXCL13 upon LTβR ligation and a substantial amount of CXCL13 protein was detected in the culture supernatant (Fig. 2, A and B). Although TNFR ligands, TNF-α or LTα3, did not induce CXCL13 on their own, both of these cytokines markedly augmented the LTβR-induced CXCL13 expression. The stable overexpression of NIK in BLS12 cells resulted in spontaneous CXCL13 production (Fig. 2, C and D), suggesting that excessive NIK is sufficient for inducing CXCL13 in this cell context. We
also detected CCL19 mRNA only when BLS12 cells were simultaneously stimulated with LTα3 or TNF-α and agonistic anti-LTβR Ab. However, the secreted protein level was nearly undetectable (data not shown). In contrast, no CCL21 expression could be detected irrespective of the presence or absence of any stimuli tested (data not shown). BLS12 cells also expressed factors required for lymphoid homeostasis, such as IL-7, BAFF, and CXCL12 (Fig. 3).

**BLS12 supports the motility of primary B cells**

To investigate the interaction between lymphocytes and BLS12, we first examined the adhesion of B cells to BLS12 cells. Approximately 20% of freshly isolated B cells adhered to an unstimulated BLS12 monolayer after several hours of incubation; this adhesion was much more effective than to BLS4 monolayer, which bind only below 5% of primary B cells (Fig. 4A). Prestimulation of BLS12 cells with agonistic anti-LTβR Ab slightly augmented the adhesion. The adhesion of B cells to BLS12 cells was markedly inhibited by anti-α5 integrin Ab and weakly inhibited by anti-α4 integrin Ab (Fig. 4B). The mixture of the two Abs blocked almost all of the adhesion. The pretreatment of B cells with PTx also dramatically inhibited the adhesion, while B oligomer, the noncatalytic subunit of PTx, showed virtually no effect (Fig. 4C). Taken together, these data indicate that Gai-dependent signaling and integrins mediate B cell adhesion to BLS12 in this experimental setting.

We next addressed whether primary B cells are motile on the surface of BLS12. For this purpose, B cells were loaded onto a monolayer of LTβR-stimulated BLS12 and incubated for several hours in a heating chamber system. Under such conditions, time-lapse image analysis revealed that the B cells actively migrated on BLS12, showing significant displacement from the starting point with an average velocity of 5–6 μm/min (Fig. 4, D–F, and video 4). Addition of Abs against integrins (Fig. 4, D–F, and videos 2 and 3) or pretreatment of B cells with PTx (Fig. 4, G–I, and videos 4 and 5) significantly reduced both velocity and displacement, suggesting that the motility of B cells on BLS12 is partially mediated by Gai-dependent signaling and integrins, while residual motile activity is driven by unknown cues. Taken together, the data demonstrate that BLS12 has the unique property of supporting the motility of B cells.

**Marginal reticular cell (MRC) layer is a unique stromal network in adult LNs**

From the aforementioned results, we noticed that BLS12 cells share some characteristics with FDCs, e.g., the expression of MadCAM-1 and BP-3, LTβR-dependent CXCL13 production, and the capacity to support B cell behavior. However, these cells express neither CR1 nor FcyRII/III, both of which are crucial and functional markers of FDCs (32). In addition, FDCs are generally weak producers of reticular fibers (3). These facts prevent us from considering BLS12 to be a FDC line.

To obtain a clue about the origin of BLS12, we examined in detail the stromal structure of the LNs. As has been well established, the FDC network was clearly observed at the center of the follicles, which

![FIGURE 2. BLS12 produces CXCL13 upon LTβR-NIK signaling. **A** and **B**, BLS12 but not BLS4 produces CXCL13 in response to LTβR ligation. BLS cells were stimulated with TNF-α, LTα3, anti-LTβR Ab, or combinations of these stimulants for 5 days. CXCL13 protein in the supernatant was measured by ELISA (A). The results are shown as means ± SD. Note that TNF-α and LTα3 used in our experiments exert almost equivalent enhancing effects on LTβR-mediated CXCL13 production. The transcript for CXCL13 was detected by semiquantitative RT-PCR analysis 2 days after the stimulation (**B**). **C** and **D**, Overexpression of NIK induces spontaneous CXCL13 production in BLS12. BLS12 stably transfected with control (vector) or NIK was stimulated with TNF-α, LTα3, anti-LTβR Ab, or combinations thereof for 2 days. CXCL13 expression was analyzed by ELISA (**C**) and RT-PCR (**D**).](http://www.jimmunol.org/)

![FIGURE 3. BLS12 cells express some lymphoid homeostatic factors. BLS4 and BLS12 cells were stimulated with LTα3, anti-LTβR Ab, or combinations thereof for 2 days. Transcripts for the indicated factors were detected by RT-PCR. The amounts of PCR products amplified from 5-fold serial dilutions of cDNAs were standardized relative to GAPDH.](http://www.jimmunol.org/)
highly expressed CR1, MAdCAM-1, BP-3, and CXCL13, as well as VCAM-1, ICAM-1, and gp38 (Fig. 5A). The nuclear accumulation of RelB in this region was also evident (Fig. 5Ad). All of the markers overlapped well in the follicular center. During the course of careful examinations, we found that there was a notable stromal cell layer at the outer margin of the cortex, i.e., the lining of the subcapsular sinus (SCS). This cell layer was brightly positive for MAdCAM-1, CXCL13, BP-3, VCAM-1, ICAM-1, and gp38, but not present in paracortical and medullary sinuses (Fig. 5A, red arrows). At most, a faint expression of CR1 was detected in this region. Stromal cells in this restricted area strongly expressed TRANCE, in contrast with the FDC network, which was barely stained for TRANCE (Fig. 5Af). Higher magnification views revealed that the layer is composed of a kind of reticular cell network (Figs. 5B), which extends several 10s of micrometers from the abluminal side of the SCS immediately underneath the layer of LYVE-1+ lymphatic endothelial cells and the basement membrane-like ECM “floor,” indicated by laminin and ER-TR7 (Fig. 5C). CXCL13 was detected in a filamentous pattern concurrent with the network (Fig. 5Ba). Nuclear RelB accumulation and PNA binding were also evident in this stromal layer (Fig. 5Ad and data not shown). Taking these observations together, we concluded that this specialized type of reticular cells represents a distinct population from stromal cells in the other regions, including FDCs and T zone FRCs, and hence designated these cells the MRCs (Fig. 5D).

**MRC layer is a stromal structure common to different types of SLOs**

It is well known that MAdCAM-1+ FRCs, termed the marginal sinus-lining cells, encircle the inner lymphoid sheath of the splenic white pulp (Fig. 6A). This stromal layer expressed almost the same marker set with MRC in LNs, including CXCL13, TRANCE, BP-3, gp38, RelB, PNA-binding carbohydrates, laminin, and ER-TR7 (Fig. 6A and data not shown), suggesting that reticular cells aligned in this region are equivalent to LN MRCs. The layer was more obvious in the outer margin of the follicles (Fig. 6A, arrows) than in the interfollicular channel region (Fig. 6A, asterisks). MRC-like stromal networks were also observed in mucosal SLOs such as PPs, NALTs, ILFs (Fig. 6, B–D), and cecal lymph patches (data not shown). In all cases, MRC-like cells constituted reticular
layers restricted to the subepithelial dome region immediately beneath the follicle-associated epithelium (FAE; Fig. 6E). In contrast, a MRC-like population was not found in ectopic lymphoid tissues in the stomach induced in mouse models for gastric autoimmunity (30, 33) (data not shown). These data indicate that the MRC layer is a common stromal structure in SLOs.

**LTo cells in the marginal area of LN anlagen expand to form the MRC layer during postnatal development**

Given that MRCs and LTo stromal cells share many markers, we speculated that there is some relationship between the two mesenchymal lineages. To address this issue, we examined the transitional process of stromal architecture from anlagen to postnatally developing SLOs. A structural examination of fetal LN anlagen has already been reported (34) and the authors showed that ICAM-1highVCAM-1high (IVMhigh) LTo cells expressing chemokines and TRANCE are concentrated in the outer region of the anlagen surrounded by LYVE-1− lymphatic vasculature, while IVMlow cells are localized in deeper regions. We confirmed similar histology of LNs on the day of birth, at which time the LNs still retain the characteristics of anlagen, as few lymphocytes have yet migrated (Fig. 7A). CD4+CD3+ LTo cells accumulated in the outer region adjacent to presumptive SCS, where stromal cells highly expressing VCAM-1 and ICAM-1 formed a dense layer (Fig. 7A, a and b). In addition to blood vessels, these LTo also expressed MAdCAM-1 (Fig. 7Ac). Although TRANCE staining illuminated the whole anlage, a group of stromal cells with higher TRANCE expression clearly delineated the boundary of the lymphatic sinus (Fig. 7Ad). A faint signal for CXCL13 was detected in the same cells (Fig. 7Ac). Overall, the LN anlagen seem to be segregated into roughly outer and inner parts during ontogeny, and the stromal cells in the former exhibit a phenotype typical of LTo.

At day 6, the size of LNs markedly increased as the influx of lymphocytes and their compartmentalization in the cortical area...
began (Fig. 7). The expression of MAdCAM-1 and TRANCE was markedly reduced in the developing paracortex and medulla, while the outermost part still retained high expression of the two molecules in conjunction with the colonization of CD4<sup>+</sup>CD3<sup>+</sup> cells. These observations are consistent with the idea that, with the

**FIGURE 6.** MRC layers are present in particular regions of various SLOs. A, Reticular stromal cells in the marginal sinus lining of splenic white pulp show characteristics of MRCs. Serial sections of spleen were stained for various markers and examined by confocal microscopy. A composite image of a section stained for laminin, CD3, and B220 shows tissue architecture and lymphocyte localization (a). Well-known MAdCAM-1<sup>+</sup> marginal sinus-lining cells highly express the set of MRC markers (arrows). The MRC-like layer is obscure in the interfollicular channel region (asterisks). B–D, Reticular stromal cells in the subepithelial dome of PP (b), NALT (c), and ILF (D) show characteristics of MRCs (arrows). Arrowheads indicate FAE. Luminal surface of the epithelia is often nonspecifically stained by anti-CXCL13 Ab. Higher expression of MAdCAM-1 is observed in FDCs and high epithelial venules. Bars, 200 μm. E, Higher magnification view of the subepithelial dome region in the PP. Section was stained with Abs against laminin, CXCL13, and MAdCAM-1 and counterstained with DAPI for the visualization of the nucleus. MRC network is localized immediately underneath the FAE layer (laminin<sup>−</sup> CXCL13<sup>−</sup> MAdCAM-1<sup>−</sup>). Bar, 100 μm.

**FIGURE 7.** Continuity between LTo stroma and MRC layer during postnatal development of LNs. A, Stromal architecture of day 0 mesenteric LNs (MLN). Serial sections of mesentery, including mesenteric LNs from day 0 mouse, were stained for the indicated markers. CD4<sup>+</sup>CD3<sup>+</sup> LTi cells (a) are accumulated in the marginal region of the anlagen, where LTo stromal cells with higher expression of ICAM-1, VCAM-1 (b), MAdCAM-1 (c), and TRANCE (d) are condensed underneath presumptive SCS (arrows). Bar, 200 μm. B, Expansion of the MRC layer in postnatally growing LNs. Composite images of day 6 mesenteric LNs and peripheral LNs (PLN) are shown. Presumptive MRC layers highly expressing MAdCAM-1 and TRANCE are observed at the outermost regions (arrows in left and middle panels). In the right panels, CD4<sup>+</sup>CD3<sup>+</sup> mature T cells accumulate in the inner cortex (asterisks), while CD4<sup>+</sup>CD3<sup>+</sup> cells are still present in the outer cortex (arrows). Note that MAdCAM-1 is still expressed by blood vessels even in peripheral LNs at this time. Bar, 200 μm.
expansion of the organ, LTo stromal cells subsequently form a thin MRC layer in the tissue periphery.

An analogous process occurred in the area surrounding the central artery in the developing spleen, in which a stromal layer similar to LTo/MRCs expanded outward as lymphocytes accumulated in presumptive white pulp; eventually, this layer constituted the lining of the marginal sinus (Fig. 8). Interestingly, MAdCAM-1+ mesenchymal cells showed relatively diffuse distribution in the day 0 spleen, but thereafter became concentrated around the artery at day 6 and then expanded to form the MRC layer. Accordingly, there is a dynamic redistribution of MRC lineage in the developing spleen.

Maintenance of MRC property requires LTβR signaling

To address the role of LTβR signaling in the maintenance of the MRC layer in mature SLOs, LTβR-Fc chimeric protein was injected into adult mice, and SLOs were examined after 2–4 wk of weekly administration of the chimeric proteins. No discernable alterations in the architecture of SLOs were observed in control experiments in which mice were injected with IL-6 TCRVβ-Fc chimeric protein (30) or PBS compared with untreated animals (Fig. 9 and data not shown). Consistent with previous reports, the structure of splenic white pulp (in particular, follicular assembly) was disorganized as a result of LTβR-Fc treatment (35, 36) (Fig. 9A). FDC networks also disappeared (data not shown). LTβR-Fc treatment abolished MAdCAM-1 expression and the typical boundary structure of the marginal sinus, which also caused the complete disappearance of the MRC layer highlighted by CXCL13, TRANCE, and BP-3 staining. Similar views were obtained in the spleen in aly/aly mice, which bear a point mutation in NIK (37) (Fig. 9A), and the observations are in good accordance with a previous report showing the absence of sinus-lining FRCs and MAdCAM-1 expression in the aly/aly spleen (38). These data indicate that the maintenance of the MRC layer in the spleen strongly depends on LTβR-NIK signaling.

Although LTβR-Fc treatment dramatically diminished the expression of CXCL13 and MAdCAM-1 in the MRC layer of the LNs, TRANCE expression was comparable to or slightly reduced compared with that in control LNs; furthermore, we found no remarkable alteration in the structure of the SCS (Fig. 9B). As FDC networks indicated by CR1 and FDC-M2, which are highly depending on LTβR signaling, disappeared (data not shown), circulating LTβR-Fc protein was suggested to reach a high enough level to block the pathway. This suggests that TRANCE expression in MRCs is independent of LTβR-signaling or another TRANCE-expressing cell types still exist in the case of LNs, although some MRC properties still depend on this pathway. It is also clear that the LTβR dependence in MRC layers differs among SLOs.

FIGURE 8. Development of the white pulp in spleen. Spleen sections from days 0, 6, and 14 and adult mice were stained for the indicated markers and examined by confocal microscopy. MRC layer/marginal sinus-lining structure expands and becomes prominent as lymphocytes are accumulated. In day 0 spleen, CD4+CD3+ LTi-like cells are concentrated at presumptive white pulp surrounding large blood vessels (arrows in B). Bars, 200 μm.
Lymphocytes are dispensable for the formation of MRC layer

To address whether lymphocytes are required for the MRCs in adult SLOs, we next examined Rag2^-/-^ mice. Rag2^-/-^ mice have rudimentary LNs due to the lack of lymphocytes; nonetheless, we could clearly observe the MRC layer in the subcapsular region of each LN, in which weak but significant expression of CXCL13 was present (Fig. 10A). Thus, at least two compartments, i.e., the MRC layer and inner stroma, are unambiguously formed even in...
the LN s from lymphocyte-deficient animals. Likewise, we observed reduced but significant expression of MRC markers at the sheath-like stromal structure surrounding the artery in the Rag2−/− mice spleen (Fig. 10B). These findings indicate that lymphocytes are not essential for the differentiation and maintenance of MRCs.

Discussion

In this study, the characterization of the lymphoid stromal cell line BLS12 prompted us to notice a unique lymphoid stromal population, MRC. MRC could be classified into a novel category of mesenchymal lineage common to adult SLOs, defined as the population of specialized reticular fibroblasts localized at a particular area of the tissues. These cells exhibit high expression of VCAM-1, ICAM-1, MadCAM-1, CXCL13, TRANCE, BP-3, and gp38, as well as various reticular matrix components. Although FDCs show a similar marker set, they exhibit high expression of CR1 but low or no TRANCE, whereas MRCs express TRANCE but little or no CR1. Furthermore, FDCs do not generally produce typical reticular structure such as the one that surrounds the ECM fiber to form the conduit. Since the phenotypic characteristic of BLS12 shows good agreement with the criteria for MRC, we consider this cell line to be of MRC origin. However, there are some discrepancies between the nature of BLS12 and MRCs. For instance, BLS12 cells in culture constitutively display VCAM-1, ICAM-1, and MadCAM-1, whereas in vivo the expression of these molecules is regulated by LTβR signaling. In addition, TRANCE protein is undetectable in BLS12, although TRANCE mRNA is readily detected. Immortalization and expansion in vitro presumably caused these alterations in BLS12.

Now that MRCs have been added to the list of stromal cells, every SLO turns out to be composed of at least three different types of mesenchymal stromal cells, i.e., FRCs in T zone, FDCs, and MRCs. Among these, the former two, FRCs expressing CCL19/ CCL21 in the T zone and FDCs expressing CXCL13 in the follicular center, have been established as major anatomical backbones for the T and B compartments, respectively (7). It was recently demonstrated that these stromal networks support lymphocyte movement, acting as guidance footholds (39). Although the medulla of LN s and the marginal zone of the spleen are supported by types of FR/S clusters distinct from those in the T zone (3, 14), these populations are not to be included in the common elements because they reside in varying anatomical compartments depending on SLO type. T zone FRCs and follicular FDCs are closely associated with the corresponding lymphocyte subsets. In addition, both of these types of stromal cells are induced in chronic inflammatory diseases, even in ectopic lymphoid tissues (30, 33, 40, 41). Therefore, we would suggest that the existence of mature lymphocytes induces the differentiation and maintenance of these stromal cells. In accordance with this, no obvious subcompartmentalization by these stromal lineages are observed in Rag2−/− mice SLOs, whereas adoptive transfer of lymphocytes restores them (Ref. 1 and our unpublished observation). In contrast, MRCs are probably present in all SLOs, even in Rag−/− mice, but absent in ectopic lymphoid tissues, strongly supporting the notion that MRCs are a developmentally programmed element and tightly fixed to the organ, irrespective of the existence of mature lymphocytes. This notion is consistent with previous observations that organogenesis and even some tissue compartmentalization of SLOs, possibly concomitant with the separation of MRCs from other stromal cells, can occur in SCID mice (42–44).

The fact that MRCs display the set of molecular markers that is also expressed by LTo cells suggests the relevance of them. Indeed, we observed that LTo descendants in the outer margin of the LN anlagen seem to subsequently form the MRC layer. Likewise, periarteriolar LTo-like sheaths in the neonatal spleen gradually expand to form MRC rings, previously known as marginal sinus-lining cells. These observations prompt us to speculate that the organizer-like stromal cells are still present as MRCs in mature SLOs. MRC-like cells are also observed in the apical dome region of mucosal-associated lymphoid organs. These are likely the same cells reported previously as TRANCE+ stromal cells in PPs, ILFs, and CPs (45). In general, CPs contain few lymphocytes but are colonized by LTi-like hematopoietic cells and the stromal cells exhibit characteristics similar to LTo/MRC (13, 46). It was recently suggested that ILFs are inducible structures derived from CP in response to the intestinal bacterial flora (13). Therefore, the maturation process of ILFs recapitulates the organogenesis of SLOs in the adult environment.

Based on the findings in this study and slightly modifying the models presented previously (10, 47), we propose following four sequential stages of SLO organogenesis from anatomical and stromal viewpoints (Fig. 11). In the earliest phase (stage I), a developmentally programmed “address code” determines the location of anlagen by attracting LTi cells or converting the adjacent mesenchyme to LTo conglomerates. Cross-talk between LTi and LTo cells facilitates the maturation of LTo stroma, which further drives a positive feedback loop. As anlagen grow (stage II), the stromal network differentiates into outer (genuine LTo layer?) and inner (presumptive lymphocyte compartment) parts (primary differentiation and compartmentalization of stromal cells). Hashi et al. (43) demonstrated the compartmentalization of the PP anlagen before lymphocyte entry and several other reports also have presented clear pictures showing uneven distributions of LTo and LTi cells within the SLO anlagen (22, 34). After birth (stage III), the influx of lymphocytes begins and the inner part of anlagen is further divided into lymphocyte subcompartments with corresponding adult stromal subsets (secondary differentiation and compartmentalization of stromal cells); meanwhile, the outermost part expands to form the MRC layer. Tissue architecture (stage IV) is maturated in the adult SLO. Continuous LTβR signaling is required for maintenance of the properties of MRCs; however, the dependence on this pathway varies depending on the individual SLO. The administration of LTβR-Fc completely disrupts the marginal sinus structure, with loss of the MRC layer in the splenic white pulp, although this treatment does not lead to the immediate disappearance of the white pulp structure. Likewise, LTβR-Fc partially diminishes markers in LN MRCs, but has little effect on TRANCE expression or overall tissue geometry, suggesting that MRCs are dispensable for the accumulation and compartmentalization of lymphocytes, at least once the construction of SLO architecture has been accomplished.

Although the functional significance of MRCs in adult SLOs remains largely unknown, we consider it important to note that all MRC layers in various SLOs are faced toward the major route of antigenic entry (Fig. 11). DCs capturing Ags in peripheral tissues migrate to the draining LN s via afferent lymph. They first reach the SCS, from which they pass across the MRC layer to enter the paracortex (48). Low-molecular weight soluble Ags can pass the SCS lining, penetrating into the conduit network in the T zone, where they can be picked up by resident DCs (5, 6). Analogously, MRC networks in the subepithelial dome of mucosal SLOs harbor unique DC subsets by which Ags are transported from the FAE to the follicular region (49, 50). In the spleen, blood-borne Ags and immune cells must pass through the marginal stromal layer to enter the inner lymphoid compartment of the white pulp (14). Accordingly, the MRC layer possibly regulates these Ag-transporting pathways. Of note along these lines, follicular B cells in the LN
directly capture lymph-borne Ags, either in a soluble form penetrates from the SCS or in a particulate form from SCS-resident cells (51–54). As the network of MRCs covers the outer part of the follicle, MRCs are likely to be involved in the Ag transport along with a polarity axis directed toward the major route of Ag entry. It is also likely that the MRC network is the foothold for the migration of primary B cells in vitro, which partially depends on Gαi-mediated signaling and integrins. However, recent studies have shown that integrins are not the major adhesion machinery, at least for the interstitial migration of T cells and DCs within the LN (56, 57). Careful examination of the integrin requirement for the migration of B cells in this area will be required in future studies.

In summary, the MRC layer is a common landmark of mature SLOs; these stromal cells are presumably the adult counterpart of LTo. The organogenesis of SLOs proceeds like a layer-forming reaction. Supposing the LTo/MRC layer as the organizing front of developing SLOs, this is quite reasonable, because the anatomical arrangement of SLOs must necessarily be optimized for capturing and detecting external Ags most efficiently. Since there are multiple mesenchymal lineages, each with distinct functions, tightly integrated into tissue microanatomy, tracing stromal components during the organogenesis and remodeling of SLOs is a suitable system for studying the specialization and diversification of mesenchymal cells via close interaction with lymphoid or myeloid cells. BLS12 cells will be a unique and highly valuable tool for exploring the cytological and biochemical nature of lymphoid stromal cells.

Acknowledgments

We thank W. C. Greene for NIK cDNA, A. G. Farr for anti-gp38 Ab, M. D. Cooper for anti-BP-3 Ab, M. H. Kosco-Vilbois for anti-FDC-M2 Ab, and T. Gonda-Ohtlli, K. Araki, and T. Hayashi for technical assistance.

Disclosures

The authors have no financial conflict of interest.

References


FIGURE 11. A common model for SLO organogenesis that progresses through four sequential stages. Stage I: In the earliest step, intimate interaction between organizer mesenchyme and inducer cells forms the SLO anlagen, under the control of an address code specific in embryonic environment. Stage II: As the anlage grows, the stromal compartment is gradually segregated into outer and inner parts, i.e., the presumptive MRC layer and lymphocyte compartments, respectively (primary differentiation and compartmentalization of stromal cells). Stromal cells in the outer part show the organizer phenotype more remarkably. Stage III: After birth, lymphocytes are accumulated in the inner area, in which they are further separated into outer (B cells) and inner (T cells) areas with the differentiation of corresponding stromal lineages (secondary differentiation and compartmentalization of stromal cells); meanwhile, the outermost stromal population proceeds to expand and eventually forms the MRC layer. B cells in the outer cortex are further assembled to form follicles. Stage IV: Mature architecture of adult SLO. In response to antigenic stimuli, a germinal center is occasionally developed in the follicle, with the asymmetry of dark and light zones. Note that common stromal elements in mature SLOs are MRCs, FDCs, and T zone FRCs. Regular tissue architecture is arranged along with a polarity axis directed toward the major route of Ag entry.


Supplemental Data Legends

Video 1. BLS12 supports the motility of primary B cells.
Time-lapse microscopy of primary B cells on BLS12 monolayer. Primary B cells were loaded onto agonistic anti-LTβR antibody-activated BLS12 monolayer. After 3 h of incubation, images were obtained every 30 sec for 30 min. Playback speed is 300x (1 sec movie=5min).

Video 2. Control IgG has no influence on the motility of B cells on BLS12.
Time-lapse microscopy of primary B cells on BLS12 monolayer in the presence of control rat IgG. Control IgG (final concentration; 20 μg/ml) was added 60 min before commencement of image capture. Playback speed is 300x (1 sec movie=5min).

Video 3. Blocking of αL- and α4-integrins significantly reduced the B cell motility on BLS12.
Time-lapse microscopy of primary B cells on BLS12 monolayer in the presence of anti-αL and anti-α4 antibodies. Blocking antibodies (final concentration; 20 μg/ml each) were added 60 min before commencement of image capture. Playback speed is 300x (1 sec movie=5min).

Video 4. Pretreatment of B cells with B-oligomer, non-catalytic subunit of PTx, has no influence on the motility on BLS12.
Time-lapse microscopy of B-oligomer-treated primary B cells on BLS12 monolayer. B cells were pretreated with 0.2 μg/ml B-oligomer at 37°C for 2 h. Then, B cells were further incubated on BLS12 monolayer before commencement of image capture. Playback speed is 300x (1 sec movie=5min).

Video 5. Pretreatment of B cells with PTx significantly reduced the motility on BLS12.
Time-lapse microscopy of PTx-treated primary B cells on BLS12 monolayer. B cells were pretreated with 0.2 μg/ml PTx at 37°C for 2 h. Then, B cells were further incubated on
BLS12 monolayer before commencement of image capture. Playback speed is 300x (1 sec movie=5min).