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Initiation of Cellular Organization in Lymph Nodes Is Regulated by Non-B Cell-Derived Signals and Is Not Dependent on CXC Chemokine Ligand 13

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The molecular and cellular events that initiate the formation of T and B cell areas in developing lymph nodes are poorly understood. In this study we show that formation of the lymphoid architecture in murine neonatal lymph nodes evolves through a series of distinct stages. The initial segregation of T and B cells is regulated in a CXCL13-independent manner, characterized by the localization of B cells in a ring-like pattern in the outer cortex on day 4. However, during this CXCL13-independent phase of lymph node modeling, CXCL13 is expressed and regulated in a lymphotoxin-αβ2 (LTαβ2)-dependent manner. Surprisingly, neonatal B cells are unable to respond to this chemokine and also lack surface LTαβ2 expression. At this time, CD45+ CD4+ CD3− cells are the predominant LTαβ2-expressing cells and are also capable of responding to CXCL13. From day 4 on, architectural changes become CXCL13 dependent, and B cells become fully CXCL13 responsive, express LTαβ2, and cluster in anatomically distinct follicles. Because the initial induction of CXCL13 is dependent on LTαβ2, a role for CD45+ CD4+ CD3− cells in inducing chemokine expression in the developing lymph nodes is proposed and, as such, a role in initiation of the shaping of the microenvironment. The Journal of Immunology, 2004, 173: 4889–4896.

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ne of the hallmarks of the adaptive immune system is the presence of secondary lymphoid organs, which provide the appropriate environment for activation and expansion of naïve lymphocytes in response to signals delivered by APCs. Lymph nodes (LNs), Peyer’s patches (PPs), and spleen contain anatomically distinct, B cell-rich microenvironments in which an environment permissive for affinity maturation and the generation of immunologic memory is created. As such, the correct formation of lymphoid follicles is important for the ability to mount adaptive immune responses (1–5). The generation of gene-targeted mice provided insights into the cellular and molecular pathways governing the establishment of B cell follicles, implicating several members of the TNF superfamily as pivotal regulators of this process (6–13). Mice with deficient TNF receptor I (TNF-R1) or lymphotoxin-β receptor (LTβ-R) signaling lack follicle formation in the spleen, whereas impaired LTβ-R signaling in addition leads to a block in the development of LNs and PPs (8, 9, 12, 14–18). The cellular source of LTαβ2 and TNF-α was determined in a series of splenocyte and bone marrow transfer studies (6, 19, 20). B cells have been shown to deliver the signals necessary for both the induction of follicular dendritic cells (FDCs) as well as the maintenance of lymphoid follicles in adult animals. In spleen, engagement of the LTβ-R by B cells results in increased production of the chemokine CXCL13 by stromal cells. Binding of CXCL13 to its receptor, CXCR5, reciprocally induces signaling events, leading to the additional up-regulation of surface LTαβ2 on B cells and subsequent clustering of these cells, effectively establishing a positive feedback loop important for the integrity of B cell follicles. This is illustrated by the fact that in the absence of CXCL13, follicles in both spleen and LNs fail to form (21). The role of B cells in this process was further dissected by preventing B cells from expressing LTαβ2, by selectively ablating the LTβ gene in B cells. In the spleen, this resulted in the absence of FDCs and disruption of the B cell follicles. However, LNs were much less affected, and follicles and FDCs could still be detected (22). Therefore, although B cell-derived signals appear important in the formation and maintenance of lymphoid follicles in the spleen, the requirements for follicle formation in LNs are less clear.

In this study we analyzed the cellular and molecular mechanisms governing the initial segregation of T and B cells in neonatal LNs. The initial migration of B cells to the outer cortex of the LNs occurs in a CXCL13-independent manner, because these processes are indistinguishable in C56BL/6 and CXCL13−/− mice. Non-B cells regulate the induction of CXCL13, which depends on LTαβ2 expression. We propose a role for LTαβ2+ CD45+ CD4+ CD3− cells in inducing CXCL13 and possibly additional chemokines responsible for the earliest B cell migration and, therefore, in the molding of the LN architecture.
Materials and Methods

Animals

C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) and were kept under routine laboratory conditions. LTαβ-/- and SCID mice were bred and kept at the in-house animal facilities of the Vrije Universiteit Medical Center. CXCL13-/- mice were described previously (21) and were bred and kept at the in-house animal facilities of Trudeau Institute.

Antibodies

For flow cytometry and immunohistology, the following Abs were used: 6B2 (anti-B220), GK-1.5 (anti-CD4), 2.4G2 (anti-CD16/CD32; all were affinity-purified from hybridoma cell culture supernatants with protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden) and labeled in our laboratory if needed), FDC-M2 (anti-FDC; AMS Biotechnology, Oxfordshire, U.K.), anti-IgM F(ab)2 (Cappel, West Chester, PA), anti-IgD-PE (Dako-Cytomation, Glostrup, Denmark), H139.52.1 (anti-κ-L chain-FTTC; Beckman Coulter, Fullerton, CA), 1D3 (anti-CD19; BD Pharmingen), anti-CXCL13 and anti-CXCR5; BD Pharmingen). Biotin-labeled anti-CXCL13 and anti-CCL21 were purchased from R&D Systems Europe (Oxon, U.K.). For the paracortical region. B cells appear as a ring-like structure in which no follicles can be distinguished.

Chemotaxis

Chemotactic activity was determined using a Transwell system with 5-μm pores (Costar, Corning, NY). Recombinant murine CXCL13 (1 μg/ml; R&D Systems Europe) was used in the lower compartment, whereas 2.5 × 10^6 cells were placed in the upper compartment. Cells were allowed to migrate for 3 h at 37°C, followed by a 30-min incubation with a LTβR-human IgG fusion protein for 60 min (15). Anti-human-PE (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as second-step conjugate. Splenocytes from adult LTαβ-/- mice were used as negative controls.

Flow cytometry and immunohistology, the following Abs were used: 6B2 (anti-B220), GK-1.5 (anti-CD4), 2.4G2 (anti-CD16/CD32) supplemented with 5% normal mouse serum for 30 min and subsequently incubated with an LTβR-human IgG fusion protein for 60 min (15). Anti-human-PE (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as second-step conjugate. Splenocytes from adult LTαβ-/- mice were used as negative controls.

Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences). 7-Aminoactinomycin D ( Molecular Probes, Eugene, OR) was used to exclude dead cells.

Immunofluorescence microscopy

Six-micron cryosections were fixed in dehydrated acetone for 5 min and air-dried for an additional 10 min. Sections were incubated with primary Ab for 1 h at room temperature, followed by a 30-min incubation with a Fluor-Alexa-labeled conjugate (Molecular Probes) when needed. Sections were embedded in Fluorstab (ICN Biomedicals, Aurora, OH) and analyzed on an Eclipse E800 microscope (Nikon Europe, Haarlem, The Netherlands). For visualization of chemokines, sections were incubated overnight at 4°C with chemokine-specific Abs. The Alexa-Fluor 594 tyramide amplification system was subsequently used to enhance the signal according to the manufacturer’s protocol (Molecular Probes).

CXCL13 mRNA expression

RNA was extracted on the day of birth from whole mesenteric lymph nodes (C57BL/6) or from mesenteric lymph node rudiments (LTαβ-/-) using TRIzol (Invitrogen Life Technologies, Gaithersburg, MD) and reverse transcribed with oligo(dT)12-18 (Invitrogen Life Technologies) and random hexamer primers (Invitrogen Life Technologies) using standard protocols. cDNA was analyzed for the expression of CXCL13 and LTβR by TaqMan PCR assay using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).

In situ hybridization was performed on 6-μm cryostat sections using a digoxigenin-labeled CXCL13 probe as described previously (23).

Results

B cell follicles develop during the first week of life

To gain insight into the cellular and molecular processes underlying the initial shaping of the LN microdomains, immunohistochemical analysis of inguinal, axillary, brachial, and mesenteric LNs was performed on the day of birth and 4 and 7 days after birth.

Three distinct phases could be distinguished in the segregation of T and B lymphocytes and the formation of B cell follicles. On the day of birth (day 0) the developing LNs showed no apparent organization, with B cells scattered throughout the LN, and T cells being virtually absent (stage I; Fig. 1A). Starting on approximately day 2, the first wave of αβTCR+ thymic emigrants colonized the LNs, and B cells started to localize to the outer cortex (24, 25). On day 4, the first phase of B cell migration was completed, and B cells were organized in a ring-like structure in the outer cortex of the LNs, the paracortex being occupied by numerous T lymphocytes. However, no follicles were detectable at this time (stage II; Fig. 1B). Between days 4 and 7, B cells localized in cortical clusters, and the first clearly distinguishable B cell follicles became evident on day 7 (stage III, Fig. 1, C and D). The generation of these follicles was accompanied by the emergence of FDCs, as detected by the FDC-M2 Ab (Fig. 1D) as well as positive staining for complement receptor 1 (data not shown). Because FDCs are dependent on TNF-α and LTαβ2 signaling, as described for the adult spleen, CXCL3 expression was compared between C57BL/6 MLNs isolated at birth and rudimentary MLNs (rMLNs) from LTαβ-/- mice (Fig. 2A). To control for possible differences in stromal cell populations between these two mouse strains,
CXCL13 levels were normalized to the expression of the LTβ-R, expressed on stromal cells. Quantitative analysis of LTα−/− and C57BL/6 mice revealed a 5-fold decrease in CXCL13 message in the absence of LTα,β2 signaling in total LNs as well as in sorted CD45− stromal cells. CXCL13 message was normalized to LTβ-R message using TaqMan analysis. In the absence of a LTα,β2 signal, an average 5-fold reduction in CXCL13 message was observed, both in total LN as well as in sorted CD45− stromal cells. C. Popliteal LNs (PLNs) were dissected on the day of birth and on days 2, 4, and 7 after birth, and CXCL13 mRNA was visualized by in situ hybridization. At birth, no CXCL13 message could be detected; the first signal became apparent on day 2. CXCL13 was expressed in the outer cortex, although not in a follicular pattern. On day 4, CXCL13 is organized in a follicle-like expression pattern, which was even more obvious on day 10.

CXCL13-independent B cell migration in neonatal lymph nodes

The fact that in adult CXCL13−/− mice, B cell follicles were disrupted indicated the necessity of CXCL13 for follicle maintenance and/or development (21). Therefore, we set out to determine during which stage of development follicle formation in CXCL13-deficient mice is halted, and MLNs dissected from neonatal CXCL13−/− mice were analyzed by immunofluorescence. In contrast to the defective LN architecture reported in MLNs of adult mice, neonatal CXCL13−/− MLNs appeared completely normal until 4 days after birth (stage II; Fig. 3A). As can easily be appreciated from Fig. 3A, the initial migration of B cells to the outer cortex seen during stage II (Fig. 1B) was completed in CXCL13−/− mice by day 4. This indicates that the first phase of B cell migration to the outer cortex was regulated in a CXCL13-independent manner. The subsequent progression to stage III, clustering of B cells in anatomically distinct follicles between days 4 and 7, did not occur in CXCL13−/− mice (Fig. 3A) and was therefore dependent on CXCL13.
FIGURE 3. CXCL13-independent initiation of architectural changes in developing lymph nodes. A, MLNs isolated from day 4 and adult CXCL13−/− mice were analyzed by immunofluorescence. B cells were detected with anti-B220 Abs (green), and T cells with anti-CD3ε (red). Development of the lymphoid architecture appeared normal until day 4. B. To determine the CXCL13 responsiveness of neonatal B cells, day 0, day 4, and adult B cells from MLNs were allowed to migrate in a Transwell assay in response to 1 μg/ml CXCL13 for 3 h. The average specific migration of adult B cells was set at 100%, and neonatal values are shown as the relative migration. On the day of birth B cells are virtually unresponsive, whereas on day 4 migratory capacity is comparable to that of adult cells, albeit with a higher degree of variety than seen in adult cells. Shown are averages from three independent experiments. C, B cells from newborn and adult MLNs were stained for CXCR5. Newborn B cells express lower levels of CXCR5 than adult B cells.

CXCL13 unresponsiveness of neonatal B cells

The findings that B cells only start organizing in a CXCL13-dependent manner after day 4 prompted us to determine the capacity of neonatal B cells to migrate in response to CXCL13. B cells were isolated from MLNs on the day of birth, on day 4, and from adult mice and tested for their CXCL13 responsiveness in an in vitro Transwell assay (Costar, Corning, NY) (Fig. 3B). Shown are specific migrations relative to migration of B cells from adult MLNs. Surprisingly, on the day of birth, B cells were virtually CXCL13-unresponsive (Fig. 3B). The ability to respond to CXCL13 was acquired during the first days of life, reaching levels comparable to those in adults on day 4. However, at this time the B cell pool was still highly heterogeneous with regard to CXCL13-induced migration. To find an explanation for this unexpected unresponsiveness of newborn B cells, neonatal and adult MLNs were disected, and expression levels of CXCR5, the receptor for CXCL13 (26), were compared between newborn and adult B cells. In agreement with their unresponsiveness to CXCL13 in Transwell assays, neonatal MLN-derived B cells showed lower expression of CXCR5 compared with adult B cells, explaining the inability of these cells to respond to CXCL13. The acquisition of normal levels of expression and full CXCL13 responsiveness represents an additional, previously unappreciated, developmental step in B cell maturation.

Neonatal B cells are LTα2β2 negative

Because neonatal LN-derived B cells still have to acquire a distinct feature of mature B cells (CXCL13 responsiveness), an additional phenotypic analysis was performed to assess the degree of maturity of the B cell pool in the neonatal LNs. As shown in Fig. 4A, phenotypically mature IgM+ IgD− B cells were found on the day of birth in MLNs (Fig. 4A). These data were confirmed by the fact that ~40% of CD19+ cells at birth expressed the κ-L chain (Fig. 4B), indicative of progression beyond the pre-B cell stage. Therefore, a large part of the neonatal MLN B cells appeared phenotypically mature despite their low expression of CXCR5.

In the spleen, induction of surface LTαβ2 on B cells is the second feature attributed to CXCR5 signaling. To test whether the newborn CXCR5low B cells from MLNs had initiated expression of LTαβ2, MLNs from mice on the day of birth and those from adult mice were analyzed for the expression of LTαβ2 (Fig. 4C). Spleen cells from LTαβ2−/− mice were used as negative controls. In conjunction with their hampered migratory response to CXCL13, day 0 MLN B cells were also surface LTαβ2 negative. The expression of LTαβ2 became apparent from day 2 (data not shown) and reached adult levels on day 4 (Fig. 4C). This shows that although newborn B cells appear phenotypically mature, their low levels of CXCR5 lead to CXCL13 unresponsiveness as well as lack of surface LTαβ2.

B cell-independent induction of CXCL13 and CCL21

CXCL13 expression in newborn LNs was regulated in an LTαβ2-dependent manner (Fig. 2B), whereas B cells were LTαβ2 negative at this time (Fig. 4C). As a consequence, during LN ontogeny, CXCL13 is likely to be induced independently of B cell-derived signals. To further address this issue, protein expression of CXCL13 as well as the other homoeostatic chemokine, CCL21, was analyzed in LNs of mice devoid of B cells. Peripheral LNs (inguinal, axillary, and brachial) from SCID mice, which lack mature T and B cells, and control C57BL/6 mice were dissected on days 3 and 10 as were those from adult animals (6–8 wk old), and CXCL13 as well as CCL21 were determined by immunofluorescence (Fig. 5). Visualization of chemokine protein by this method is not as sensitive as detection of mRNA so situ hybridization, and in C57BL/6 mice, CXCL13 protein is detected no earlier than day 10 on, whereas CCL21 can be visualized from days 1–2 on (Fig. 5). Remarkably, similar to wild-type mice, lymphocyte-deficient SCID mice showed the first detectable CXCL13 protein expression on day 10 (Fig. 5B). In LNs from adult SCID mice, CXCL13 was expressed in a ring-like pattern in the outer cortex of the lymph nodes (Fig. 5C). Notably, in the absence of lymphocytes, CXCL13 expression failed to become organized in a follicular pattern. This indicates that induction of CXCL13 occurred independently of B cells, yet follicular organization was still B cell dependent. The kinetics of expression of CCL21 in LNs devoid of B cells was similar to those in C57BL/6 mice (Fig. 5); it was already abundantly present on day 3. Induction of these chemokines in LNs therefore occurs independently of B cells.

Migration of LTαβ2−/−CD45+CD4+CD3− cells in neonatal LNs

Because induction of CXCL13 was regulated in an LTαβ2-dependent, B cell-independent manner, newborn LNs were studied to identify an alternative LTαβ2-expressing cell population (Fig. 6A). Staining of MLNs on the day of birth for B220 and surface LTαβ2 led to the identification of a single discrete LTαβ2+ population, lacking expression of B220. Analysis of this population showed that the predominant cell type in the newborn LN that expresses LTαβ2 is CD4+ and CD3−. These CD4+CD4−CD3− cells have previously been shown to be the inducer cells of PPs and nasal-associated lymphoid tissue organogenesis, and are the putative inducers of LN formation during embryonic development (27–31). At birth, CD4+CD4−CD3− cells make up ~40–50%
of all hemopoietic cells, and upon adoptive transfer, these cells home to the B cell follicles (32, 33). These features make LTα1β2+CD45+CD4+CD3− cells likely candidates for inducing LTα1β2-dependent chemokines in the developing LN. SCID mice contain a normal population of LTα1β2+CD45+CD4+CD3− cells (32), and in Fig. 5, A and B, the predominant localization of LTα1β2+CD45+CD4+CD3− cells at the outer cortex of the SCID lymph nodes could clearly be seen due to the absence of CD4+TCR+ lymphocytes. This cortical distribution was apparent around day 2 after birth in both SCID and C57BL/6 mice (32) and coincided with the migration of B cells to this area during stage II of follicle formation. Furthermore, similar to the migration of B lymphocytes, this migration of CD45+CD4+CD3− cells occurred independently of CXCL13, because a predominant cortical localization of these cells was seen in CXCL13−/− mice (Fig. 6B). However, in contrast to neonatal B cells, CD45+CD4+CD3− cells showed a very potent migratory response to CXCL13 in vitro (Fig. 6C) (34). Therefore, LTα1β2+CD45+CD4+CD3− cells display many of the features attributed to B cells in the adult spleen for induction of the LTα1β2-CXCL13 feedback loop, indicating that LTα1β2+CD45+CD4+CD3− cells may very well be responsible for the induction of homeostatic chemokines during postnatal development and initiation of the lymphoid microdomains.

**Discussion**

The shaping of the cellular architecture within developing LNs requires an intricate interplay among several cell types and soluble factors. Postnatal segregation of lymphocytes and arrangement into functionally and anatomically distinct microdomains is a multistep process, in which several distinct stages can be discerned (Figs. 1 and 7). During the first stage (stage I), from day of birth until day 2, LNs are still devoid of αβTCR+ T cells, and the main hemopoietic cell types present are B cells, LTα1β2+CD45+CD4+CD3− cells, and γΔT cells (25). During this time, no cellular organization is apparent. The transition from stage I to stage II is marked by an influx of the first αβTCR+ T cells. In conjunction with the influx of T cells, which accumulate in the paracortex of the LN, B cells and CD45+CD4+CD3− cells start to migrate toward the outer cortex. In this study CXCL13 expression was upregulated, in an LTα1β2-dependent manner, most likely induced by LTα1β2+CD45+CD4+CD3− cells. During stage II, the pattern of CXCL13 mRNA expression indicates the creation of a microenvironment permissive for follicle formation, culminating on day 4 in the organized follicular expression of this chemokine. Stage III is initiated when B cells become responsive to CXCL13, and CXCL13-dependent migration can occur, leading to the clustering of B cells in the outer cortex and the expression of surface LTα1β2.

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**FIGURE 4.** Newborn LNs contain phenotypically mature, but LTα1β2-negative, B cells. On the day of birth, B cells from MLNs were analyzed for the expression of surface Igs. A, Gated on CD19+ cells. ~33% of the B cells in newborn LNs express both IgM and IgD; B, roughly the same percentage of CD19+ B cells expresses the κ-L chain, indicative of maturation beyond the pre-B cell stage. C and D, Surface expression of LTα1β2 was evaluated by staining with a soluble LTβ-R-IgG fusion protein. Splenic B cells from LTα1−/− mice were used as a negative control, and B cells from either day 0 MLNs or day 4 MLNs were compared with B cells from adult C57BL/6 MLNs. At birth, no surface LTα1β2 could be detected on B cells. Expression was instigated on approximately day 2 (data not shown) and reached levels comparable to adult values on day 4. The percentage LTα1β2-expressing B cells of the total B cells is shown.
The latter enables B cells to contribute to the sustained production of CXCL13 in a similar way as described for the spleen (21). The LN architecture is completed on day 7, when FDCs become apparent, and B cells are clustered in anatomically distinct, tightly packed follicles (Fig. 7).

The observed discrepancy between CXCL13 mRNA and protein levels indicates that low levels of CXCL13 protein are likely to be present before day 10. However, because development of the lymphoid architecture in C57BL/6 mice and that in CXCL13−/− mice are completely identical until day 4, this low level of expression is obviously not essential for the early phases of T/B segregation. Furthermore, differences in the sensitivity of the CCL21 and CXCL13 Abs might exist, which could hamper determination of the exact onset of protein expression.

Recently, the importance of B cells in maintenance of the architecture in spleen and LNs of adult mice was questioned (22). Tumanov et al. (22) showed that mice with a B cell-specific LTβ deletion display normal chemokine levels, FDC, and follicular structures in the LN. Furthermore, it was shown that T cell-derived LTβ1/2 is sufficient to maintain the lymphoid architecture. This
CXCL13

**Stage I**
Day 0-2

**Stage II**
Day 4

**Stage III**
Day 7

B cell independent

B cell dependent

CXCL13 independent

CXCL13 responsive

CXCL13 independent

CXCL13 dependent

CD45^-/CD4^-CD3^- cell

B cell

FDC

CXCL13

**FIGURE 7.** Model for the initiation of lymphoid segregation and follicle formation in LNs. During the segregation of T and B cells and the formation of lymphoid follicles, three distinct stages can be perceived. At birth, no αβTCR^+ cells are present, and B cells and CD45^-/CD4^-CD3^- cells make up the main hematopoietic cell types (stage I). During stage I, CXCL13 is expressed in the outer cortex (left panel). Organization is initiated on approximately day 2, when the first T cells enter the LN. B cells and CD45^-/CD4^-CD3^- cells now start to localize to the outer cortex, forming a ring-like pattern on day 4 (stage II). This migration is regulated in a CXCL13-independent manner, whereas expression of CXCL13 is organized in an follicular constellation on day 4. From day 4 on, B cells become CXCL13-responsive and start to cluster in follicular structures. At 7 days after birth, the first anatomically distinct B cell follicles can be detected as well as the first FDCs (stage III). The migrational events during stage III are CXCL13-dependent, and abundant CXCL13 expression can be found within the follicles.


