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CXCL13 Responsiveness but Not CXCR5 Expression by Late Transitional B Cells Initiates Splenic White Pulp Formation

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Secondary lymphoid organs (SLO) provide the structural framework for coconcentration of Ag and Ag-specific lymphocytes required for an efficient adaptive immune system. The spleen is the primordial SLO, and evolved concurrently with Ig/TCR:pMHC-based adaptive immunity. The earliest cellular/histological event in the ontogeny of the spleen’s lymphoid architecture, the white pulp (WP), is the accumulation of B cells around splenic vasculature, an evolutionarily conserved feature since the spleen’s emergence in early jawed vertebrates such as sharks. In mammals, B cells are indispensable for both formation and maintenance of SLO microarchitecture; their expression of lymphotixin $\alpha_1\beta_2$ (LT$\alpha_1\beta_2$) is required for the LT$\alpha_1\beta_2$:CXCL13 positive feedback loop without which SLO cannot properly form. Despite the spleen’s central role in the evolution of adaptive immunity, neither the initiating event nor the B cell subset necessary for WP formation has been identified. We therefore sought to identify both in mouse. We detected CXCL13 protein in late embryonic splenic vasculature, and its expression was TNF-$\alpha$-dependent. A substantial influx of CXCR5+ transitional B cells into the spleen occurred 18 h before birth. However, these late embryonic B cells were unresponsive to CXCL13 (although responsive to CXCL12) and phenotypically indistinguishable from blood-derived B cells. Only after birth did B cells acquire CXCL13 responsiveness, accumulate around splenic vasculature, and establish the uniquely splenic B cell compartment, enriched for CXCL13-responsive late transitional cells. Thus, CXCL13 is the initiating component of the CXCL13:LT$\alpha_1\beta_2$ negative feedback loop required for WP ontogeny, and CXCL13-responsive late transitional B cells are the initiating subset.

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The spleen is the primordial secondary lymphoid organ, which evolved concurrently with Ig/TCR:pMHC-based adaptive immunity (1). It provides the structural framework necessary for the coconcentration of Ag and Ag-specific lymphocytes required for an efficient adaptive immune system (2). The spleen is unique among secondary lymphoid organs in its functional and histological segregation into two discrete areas: the red pulp (RP) and the white pulp (WP) (3). The RP is tasked with filtration of the blood, including removal of effete erythrocytes and free heme for iron recycling as well as bacterial capture and clearance; the WP is the spleen’s lymphoid component. The early events in the ontogeny of the splenic WP are conserved since the appearance of the spleen itself in early jawed vertebrates ~500 million years ago; B cell accumulation around splenic vasculature marks the onset of WP ontogeny in the neonatal nurse shark *Ginglymostoma cirratum* (4). In the spleen of the adult nurse shark, B cells remain vasculature associated, with T cells peripheral to the follicle (H. Neely, Y. Ohta, and M. Flajnik, unpublished observations). This is also the case in the adult African clawed frog *Xenopus laevis* (common ancestor with humans ~350 million years ago) (5).

In the mouse, the WP comprises a central arteriole, a periarteriolar lymphoid sheath (PALS) of T cells (the T cell zone), one or more adjacent B cell follicles, and a surrounding marginal zone populated by a specific subset of B cells and two distinct populations of macrophages (3, 6). Although the microarchitecture of the mature mammalian splenic WP does not retain the early developmental features like in cold-blooded vertebrates, mouse WP ontogeny also begins with the accumulation of B cells around splenic vasculature within 48 h after birth and their subsequent contraction into a nascent follicle (7). This is followed by an accumulation of T cells around the splenic vasculature central to the nascent follicle and the appearance of the marginal zone within 96 h of birth and ultimately the displacement of the B cell follicle from the vasculature by the PALS.

The microarchitecture of both the mouse B cell follicle and the WP as a whole are dependent on a positive feedback loop in which B cell–derived lymphotixin (LT) $\alpha_1\beta_2$ promotes CXCL13 production by follicular dendritic cells (FDC) via the LT$\alpha_1\beta_2$:CXCL13, in turn, induces LT$\alpha_1\beta_2$ expression on B cells via CXCR5 (8). This CXCL13/LT$\alpha_1\beta_2$-positive feedback loop is also necessary for proper T cell zone (9) and marginal zone establishment (10). Lymphoid tissue inducer cells are also a significant source of LT$\alpha_1\beta_2$, and although they are necessary for the formation of lymph nodes and Peyer’s patches, lymphoid tissue inducer cells are dispensable for establishment of the splenic WP (11,12). In addition to LT$\alpha_1\beta_2$, B cell–derived TNF-$\alpha$ is required for both WP microarchitecture and maintenance of FDC networks within the follicle (13-15), although the precise role and timing of TNF-$\alpha$ are yet to be elucidated (16,17). Genetic ablation of any member of this pathway results in an inability of the WP to form properly (18,19) (although it has recently been reported that in the absence of LT$\alpha_1\beta_2$, overexpressed TNF-$\alpha$ alone is sufficient to promote WP ontogeny and microarchitecture (20)), and disruption...
of this pathway results in a loss of established WP integrity (21, 22).

Dramatic changes in B lymphopoiesis occur at birth, in parallel with the onset of WP ontogeny. The primary site of B lymphopoiesis shifts from the fetal liver, which, along with the yolk sac and paraaortic splanchnopleura, preferentially produces B−I B cells, to the bone marrow, which preferentially produces conventional (B−2) B cells (23). As B cells, because of their ability to express LTR1β2 in response to CXCL13 stimulation, are indispensable for the formation and maintenance of the WP, a fundamental question arises: which lineage and/or subset of B cells is responsible for the initiation of WP ontogeny? In this paper, we seek to identify the B cell subset that seeds the splenic WP as well as the initiating member of the CXCL13/LTR1β2-positive feedback loop required for the WP’s ontogeny and maintenance. We also synthesize recent and long-standing data into a coherent and progressive model for the early events in the ontogeny of the mammalian splenic WP.

Materials and Methods

Mice

Adult female (12–16 wk) and timed-pregnant C57BL/6J mice were purchased from The Jackson Laboratory for arrival in our facility at 6.5. Mice were housed under specific pathogen-free conditions at the University of Maryland until indicated developmental time points. All animal experiments were conducted under the guidelines and approval of the Institutional Animal Care and Use Committee. Spleens from TNF−/− embryos and pups (C57BL/6 background) were provided by G. Trinchieri (National Institutes of Health, Bethesda, MD), and spleens RAG2−/− embryos and pups (C57BL/6 background) were provided by K. Wilson (University of Maryland, Baltimore, MD).

Immunohistochemistry

Spleens were excised, immediately frozen in Tissue-Tek OCT Compound (Sakura) and sectioned at 6 μm on a CM3050S microtome (Leica). Sections were fixed in acetone, blocked in 5% nonfat milk in PBS-T, and stained for 2 h at 4°C with indicated Ab: IgM (R6-60.2), CD23 (B3B4) (BD Biosciences), smooth muscle actin (SMA) (1A4) (Sigma-Aldrich), CXCL13 (polyclonal), goat IgG (polyclonal) (R&D Systems), and IgM (eB121-159) (eBioscience). Sections were analyzed on an Eclipse E800 microscope (Nikon) using a Spot RT3 camera (Diagnostic Instruments) and analyzed with Spot Advanced software. Images were adjusted for brightness and contrast using Adobe Photoshop Elements (Adobe Systems).

Flow cytometry

Single-cell suspensions were prepared from pooled embryonic or neonatal spleens (three to five spleens per sample) or adult spleen by mechanical dissociation in PBS + 2% FCS, penicillin/streptomycin, sodium pyruvate, L-glutamine, and 2-ME. Erythrocytes were lysed by hypotonic shock. One million cells per sample were loaded with Fluo-5FAM (Life Technologies) and incubated at 37°C, 5% CO2 for 15 min. Ab was added to each sample, IgM (R6-60.2) (BD Biosciences), and samples were incubated an additional 15 min at 37°C, 5% CO2. Cells were analyzed for 30 s on an LSRII flow cytometer using FACSDiva software (BD Biosciences) prior to addition of 1 μg/ml (R&D Systems) and then analyzed for an additional 2 min. Data were analyzed using FlowJo software (Tree Star).

Statistical analysis

Statistical analyses were performed using Prism 6.0 (GraphPad Software).

Results

Cellular/histological onset of WP ontogeny after birth

To precisely determine the timing of the cellular/histological onset of WP ontogeny, we analyzed splenocytes and splenic cryosections from E17.5 through P0.5 C57BL/6J mice (partum E19.25). At all time points analyzed, two distinct populations of CD19+ B lineage cells were detected by FACs: an IgM+ population consisting of CD43+ IgD− (data not shown) pro-/pre-B cells and an IgM+ population (Fig. 1A); all subsequent analyses focus exclusively on the latter population of IgM+ B cells. Between E17.5 and E18.5, splenic B cell numbers increased ~10-fold (Fig. 1B) and then remained relatively constant between E18.5 and P0.5. This increase in splenic IgM+ B cells was accompanied by a reduction in the proportion of IgM− B cells in the liver (data not shown).

At E17.5, the spleen consists entirely of red pulp, throughout which the few B cells detected were scattered randomly (Fig. 1C). After the influx of B cells at E18.5, the cells remained randomly distributed throughout the spleen (Fig. 1C). Although B cell numbers did not increase significantly between E18.5 and P0.5 (Fig. 1B), by the early neonatal time point, the majority of splenic B cells had aggregated around the splenic vasculature. As such, aggregation of B cells around the splenic vasculature at P0.5 marks the cellular/histological onset of WP ontogeny.

Perivascular CXCL13 expression precedes perivascular B cell aggregation

B cell homing to and retention in lymphoid follicles is dependent on the chemokine CXCL13 and its B cell–expressed receptor, CXCR5 (25). Although CXCL13 mRNA has been detected in extracts from whole embryonic spleen (7), CXCL13 protein production has not been observed previously. Because perivascular B cell aggregation did not occur until birth, we predicted that CXCL13 protein would be undetectable until birth. However, CXCL13 protein was detectable around the splenic vasculature as early as E17.5 (Fig. 2A). To determine whether the perivascular CXCL13 protein was produced locally by perivascular cells or had accumulated in the perivascular extracellular matrix after production elsewhere, we analyzed E18.5 splenic sections by in situ hybridization.
and detected CXCL13 mRNA-expressing cells at the splenic vasculature (Fig. 2B). Interestingly, we also observed CXCL13 mRNA-expressing cells in the subcapsular region of several (three of seven) spleens. However, subcapsular CXCL13 protein was not readily detectable by immunohistochemistry (IHC) (because of high fluorescent background at the tissue edges). We are currently endeavoring to identify these cells and determine their ability to produce CXCL13 protein.

The precursors of FDC recently have been identified as perivascular mural cells that coexpress the PDGFRβ and low levels of CXCL13 (26). In situ hybridization analysis of serial sections from E18.5 spleen revealed that the CXCL13-expressing cells coexpressed PDGFRβ, demonstrating that the low levels of CXCL13 in the late embryonic spleen are produced by pre-FDC (Fig. 2C). Therefore, expression of both CXCL13 mRNA and protein by perivascular splenic pre-FDC precede aggregation of B cells around the splenic vasculature.

Embryonic CXCL13 expression is independent of TNF-α and rearranging lymphocytes

LTα1β2 and TNF-α are both necessary for maximal CXCL13 production in the splenic WP (27), and both are produced by radiosensitive hematopoietic lineage cells (14). LTα1β2 is not detectable in the spleen until birth (Ref. 7 and data not shown); as we detected CXCL13 mRNA and protein in the late embryonic spleen are produced by pre-FDC (Fig. 2C). Therefore, expression of both CXCL13 mRNA and protein by perivascular splenic pre-FDC precede aggregation of B cells around the splenic vasculature.

Differential responsiveness of E18.5 and P0.5 splenic B cells to CXCL13

The presence of CXCL13 protein around the late embryonic splenic vasculature prior to any localization of B cells to the CXCL13-expressing vasculature suggested a differential responsiveness of E18.5 and P0.5 splenic B cells to CXCL13. We therefore analyzed the relative levels of surface CXCR5 expression on splenic B cells from E18.5 and P0.5 mice (Fig. 4A). Surface CXCR5 levels were indistinguishable between the two perinatal time points, although both were significantly lower than those observed on adult splenic B cells. We then analyzed the relative abilities of E18.5 and P0.5 splenic B cells to mobilize intracellular calcium in response to CXCL13 stimulation (Fig. 4B) and found both populations competent to mobilize calcium.

Next, we analyzed the relative abilities of E18.5 and P0.5 splenic B cells to migrate toward CXCL13. We therefore analyzed the relative abilities of E18.5 and P0.5 splenic B cells to specifically migrate toward CXCL13 by Transwell assay (Fig. 4C). P0.5 splenic B cells migrated at approximately the same frequency as adult splenic B cells. However, E18.5 splenic B cells showed a 6.3-fold reduction in the frequency of specific migration, despite their surface expression of CXCR5 at levels comparable to those observed on P0.5 B cells. To determine whether the E18.5 cells’ chemotactic unresponsiveness is restricted to CXCL13 or representative of a general impairment in chemokine-driven migration, we repeated the Transwell assay with CXCL12, toward which B cells of all developmental time points have been reported to migrate (28). Both the E18.5 and P0.5 B cells robustly migrated toward CXCL12 (Fig. 4D), demonstrating that B cells from both developmental time points are capable of chemokine-driven mi-
gration and suggesting a functional uncoupling of CXCR5 from the G protein receptor kinase (GRK)/arrestin/MAPK signaling cascade driving cellular chemotaxis (29).

**Initiation of WP ontogeny by CXCL13-responsive late transitional B cells**

To identify potential differences among the perinatal splenic B cell populations that could explain their differential responsiveness to CXCL13, we further analyzed the IgM+ B cells from E17.5, E18.5, and P0.5 spleen. E17.5 splenic B cells were exclusively CD9+CD5lo B-1a cells (Fig. 5A) (30, 31), although both the E18.5 and the P0.5 splenic B cells were exclusively AA4.1/CD93+ transitional (T) B cells (Fig. 5B) (32, 33). At both E18.5 and P0.5, the vast majority of cells were CD23+ early transitional (T1) B cells, although a small proportion of CD23+ late transitional (T2) B cells was also detected, and between E18.5 and P0.5, the proportion of CD23+ T2 cells increased slightly but significantly (Fig. 5C). Histologically, both CD23+ and CD23-IgM+ B cells were detected surrounding the vasculature in the P0.5 spleen (data not shown).

To determine the relative abilities of the P0.5 T1 and T2 B cells to specifically migrate in response to CXCL13, we performed a Transwell assay and analyzed input and migrated cells for transitional phenotype (Fig. 5D). We observed a 2.3-fold enrichment of CD23+ T2 B cells in the migrated fraction relative to the input, which suggests that chemotactic responsiveness to CXCL13 is acquired by B cells during their maturation from T1 to T2.

Last, we compared the surface phenotypes of splenic and blood B cells from each developmental time point. At E17.5, the spleen and blood B cell compartments both exclusively contained CD5+CD9+ B-1a cells (Fig. 5A), suggesting that the B cell compartment observed in the spleen at this time point is representative of the blood B cell compartment, rather than a uniquely splenic compartment. At E18.5, blood and spleen contained equal proportions of T1 and T2 B cells (Fig. 5E). Taken in conjunction with the lack of chemotactic responsiveness to CXCL13 and the random distribution of IgM+ B cells throughout the spleen, these data suggest that the E18.5 splenic B cell compartment is also representative of the blood B cell compartment and not a uniquely splenic compartment. However, at P0.5 the proportion of T2 B cells in the spleen increased significantly relative to blood (Fig. 5E). This marks the point at which a uniquely splenic B cell compartment is established and therefore represents the cellular onset of WP ontogeny. Furthermore, the enhanced migratory capacity of the CD23+ T2 cells along with their overrepresentation in the P0.5 spleen (relative to P0.5 blood as well as to E18.5 spleen) demonstrate that CXCL13-responsive late transitional B cells initiate splenic WP ontogeny.

**Migratory capacity of adult T1/T2 B cells mirrors that of neonatal B cells**

A preponderance of transitional B cells in the neonatal spleen has been reported as early as P3, and these transitional cells give rise to mature B-1a cells (34). As such, we predict that the transitional B cells we observe in the perinatal spleen are also of the B-1
lineage. Developmental differences between transitional B-1 and B-2 cells have been described previously (35); we therefore sought to determine whether the enhanced chemotactic response of late transitional (relative to early transitional) B cells to CXCL13 is also a characteristic of adult, bone marrow–derived B cells. To address this question, we repeated our Transwell analysis on adult splenic and blood-derived B cells (Fig. 6). Consistent with our data from the neonate, we observed a 1.75-fold increase in the ratio of T2 to T1 B cells from peripheral blood after migration toward CXCL13 (relative to input) (Fig. 6A), whereas no difference in the ratio of T2 to T1 B cells was observed after migration of splenic B cells toward CXCL13 (Fig. 6B). The similar rates of CXCL13-elicited migration by splenic early and late transitional B cells are likely the result of desensitization to the chemokine; the majority of adult splenic B cells will have encountered CXCL13 upon entry into the WP. The enhanced migratory capacity of blood-derived T2 B cells (relative to T1), however, suggests that acquisition of chemotactic responsiveness to CXCL13 during the maturation from the early to late transitional stages of B cell development is a characteristic of both B-1 and B-2 lineage B cells.

Discussion
These results demonstrate a stepwise and ordered progression of discrete events in the initiation and onset of splenic WP ontogeny:
1) production of CXCL13 protein by perivascular pre-FDC in the late embryonic spleen in an LToαβ2-, TNF-α−, and T/B cell–independent manner, which “primes” the spleen for WP ontogeny (7); 2) an increase in peripheral IgM+ B cell numbers, dominated by early transitional B cells, at E18.5, and then at P0.5; 3) the acquisition chemotactic responsiveness to CXCL13 by B cells; 4) aggregation of B cells around the splenic vasculature; and 5) establishment of the first, to our knowledge, uniquely splenic B cell compartment, defined by an increase in the proportion of late transitional B cells relative to peripheral blood.

The “priming” of the embryonic spleen for WP establishment has been demonstrated by transplantation of E15.5 spleen into the kidney of Rag2γc−/− mice, and the subsequent establishment of lymphoid architecture surrounding the graft (7). Moreover, these “primed” cells have recently been shown to be of a stromal origin (36). In addition, basal levels of CXCL13 transcription in peripheral lymph node anlagen (presumably stromal cells) have been detected and shown to be dependent on neuronally derived retinoic acid and the retinoic acid receptor β, and induction of CXCL13 transcription in the intestine can be controlled by stimulation of the Vagus (10th cranial) nerve in a retinaldehyde dehydrogenase 2–dependent manner (37). Because the spleen is innervated by the Vagus nerve, it is possible that this or a similar mechanism of CXCL13 regulation controls the initial expression of CXCL13 in the embryonic spleen.

How this basal CXCL13 expression (and the consequent induction of SLO ontogeny) is restricted to only a subset of Vagus-innervated organs warrants further investigation.

Although initial embryonic expression of CXCL13 is independent of LTαβ2 and TNF-α, its upregulation in the spleen, as well as the differentiation and maintenance of splenic FDC, requires physiological concentrations of both. Krautler et al. (7) have suggested that the “maintenance of pre-FDC relies on LTβR and their further maturation depends on TNFR1 signaling.” Our observation that perivascular B cell aggregation in the spleen is delayed by 24 h in the absence of TNF-α is in accordance with this prediction, particularly in light of the recent observation that defective WP ontogeny in the absence of LTαβ2 can be rescued by increased concentrations of TNF-α (20).

A reduced chemotactic responsiveness of neonatal B cells to CXCL13 has been previously described (38), but this report demonstrated a gradual acquisition of CXCL13 responsiveness by total B220+ cells (isolated from mesenteric lymph nodes), rather than exclusively IgM+ B cells, between P0 and P4. Our data from the spleen show a near absence of specific migration toward CXCL13 by IgM+ B cells at E18.5 but a frequency of specific migration comparable to that of adult splenic B cells at P0.5. The chemotactic unresponsiveness of the E18.5 B cells, despite their expression of CXCR5 and their ability to mobilize calcium in response to CXCL13 stimulation, suggests that a functional coupling of CXCR5 to cellular chemotaxis is absent in these cells, and the acquisition of CXCL13 responsiveness in the P0.5 cells suggests that this coupling takes place as the transitional cells mature.

CXCR4-mediated chemotaxis toward CXCL12 is dependent upon β-arrestin2 and GRK6 in T cells and, to a lesser extent, B cells (39); the β-arrestin and GRK linking CXCR5 to cellular migration have not yet been identified. Our observation that the B cells from both E18.5 and P0.5 are capable of migration toward CXCL12—and migrate toward CXCL12 at similar frequencies—demonstrates that cells from both developmental time points are capable of chemokine-elicted migration and suggests the uncoupling of CXCR5 from chemotaxis at the level of the undefined CXCR5-associated GRK/β-arrestin. This raises an intriguing and novel mechanism for the regulation of chemokine-driven migration in chemokine receptor-expressing cells—differential regulation of G-protein–coupled receptor–associated signaling intermediates—and transitional B cells should provide a valuable system in which to elucidate this phenomenon.

Hayakawa and colleagues have reported an intimate, physical association of B-1a cells with WP FDC in the mature, adult spleen (40). Given that the transitional B cells we observe colonizing the P0.5 spleen and initiating WP ontogeny are likely of a B-1 lineage (34), we propose that these cells mature into canonical B-1a cells, and that they continue to support the maintenance of follicular microarchitecture.

The transitional stages of B cell development are commonly described as occurring in the spleen (41,42), but it has been suggested that these stages are, in fact, a blood phenomenon. Our data suggest a compelling refinement of the latter theory: early T B cells are effectively excluded from the splenic WP (and are therefore maintained in the blood and/or splenic RP) by their chemotactic unresponsiveness to CXCL13 and only after the window of peripheral tolerance has closed do B cells acquire the ability to migrate toward CXCL13 and are thus allowed egress from the blood/RP and entry into the splenic WP (and other SLO). Teleologically, the lack of chemokine responsiveness by the early transitional B cells affords them the opportunity for tolerance induction to peripheral self-Ags not encountered in the fetal liver during their sojourn throughout the body. As such, acquisition of chemotactic responsiveness to CXCL13 represents a discrete step in the maturation of early to late transitional B cells, and we are currently in-
vestigating whether susceptibility to BCR-induced tolerance is lost as responsiveness to CXCL13 is acquired. As the differential CXCL13 responsiveness of T2 and T1 B cells is a characteristic of adult bone marrow–derived B cells as well as neonatal, fetal liver–derived B cells, these data have significant implications for the regulation of humoral peripheral tolerance throughout life.

In lower vertebrates such as frog and shark, the mature splenic WP retains the architecture seen early in development, with the B cell zone remaining associated with the central arteriole (5). We plan to determine whether the developmental progression we have uncovered in mice, with CXCL13 expression at the vasculature and CXCR5 responsiveness of developing B cells, extends to all jawed vertebrates. Ultrastructural and some functional data suggest that FDC and germinal centers do not form in lower vertebrates (43), despite the presence all of the basic features of adaptive immunity such as MHC restriction of T cells, and somatic hypermutation and some level of affinity maturation of Ab responses (44). Although LTα and LTβ exist in lower vertebrates (45), these cytokines have not been co-opted for FDC generation and maintenance. Thus, further studies of immune responses in ectotherms may uncover primitive features of immunity that have been overlooked in mammals.

**FIGURE 6.** Enhanced CXCL13 responsiveness of adult blood-derived but not spleen-derived T2 B cells. (A) Ratios of blood-derived T2 to T1 B cells before (input) and after (migrated) specific migration toward 1 μg/ml CXCL13 by Transwell migration assay. (B) Ratios of splenic T2 to T1 B cells before (input) and after (migrated) specific migration toward 1 μg/ml CXCL13 by Transwell migration assay. **p ≤ 0.05.
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Disclosures
The authors have no financial conflicts of interest.

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