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Development of secondary lymphoid organs

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Abstract

Secondary lymphoid organs develop during embryogenesis or in the first few weeks after birth according to a highly coordinated series of interactions between newly emerging hematopoietic cells and immature mesenchymal or stromal cells. These interactions are orchestrated by homeostatic chemokines, cytokines and growth factors that attract hematopoietic cells to sites of future lymphoid organ development and promote their survival and differentiation. In turn, lymphotoxin-expressing hematopoietic cells trigger the differentiation of stromal and endothelial cells that make up the scaffolding of secondary lymphoid organs. Lymphotoxin signaling also maintains the expression of adhesion molecules and chemokines that govern the ultimate structure and function of secondary lymphoid organs. Here we describe the current paradigm of secondary lymphoid organ development and discuss the subtle differences in the timing, molecular interactions and cell types involved in the development of each secondary lymphoid organ.

Keywords

lymphotoxin; homeostatic chemokine; lymphoid tissue inducer

Summary points

1. The development of secondary lymphoid organs is initiated during embryogenesis by interactions between hematopoietic cells ($CD4^+CD3^-IL\text{-}7R\alpha^+$ α $4\beta7^+$ LTi cells and $CD4^-CD3^-IL\text{-}7R\alpha^-CD11c^+$ LTin cells) that arise in the fetal liver and mesenchymal cells that are located at sites of future lymphoid organ development.

2. The development of LTi cells from precursors in the fetal liver and their local differentiation into LTαβ-expressing cells requires the activities of cytokines, such as TRANCE or IL-7. IL-7 seems to be most important for the differentiation of LTi cells at mucosal sites, such as the Peyer's patches, while TRANCE is most important at sites of peripheral lymph node development.

3. LTi cells are attracted to sites of lymphoid organ development by homeostatic chemokines, including CXCL13, CCL19 and CCL21. Like TRANCE and IL-7, these chemokines also maintain surface LTαβ expression on LTi cells.

4. The lymphotoxin signalling pathway plays a central role in the development of secondary lymphoid organs due to its ability to trigger mesenchymal cell differentiation, elicit homeostatic chemokine expression and to promote the differentiation of HEVs, stromal cells and dendritic cells.

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5. Signalling through the LTβR initiates both the canonical and alternative NFκB signalling pathways.

6. The vascular addressins, MAdCAM and PNAd are sequentially expressed during lymph node development so that LTi cells and mature lymphocytes are recruited in a temporally ordered fashion.

7. $CD4^+CD3^-$ cells can be found in adults and seem to have a function in facilitating follicular T cell responses. However, the lineage relationship between embryonic $CD4^+CD3^-$ LTi cells and those found in adults remains uncertain.

8. The developmental pathways for peripheral lymph nodes, mesenteric lymph nodes and Peyer's patches differ in their requirements for chemokines, cytokines and growth factors. However, the pathways that govern the development of ILFs and NALT are dramatically different than those that govern conventional lymph nodes and Peyer's patches.

Future issues

1. What is the relationship between $CD4^+CD3^-$IL-7Rα^+ LTi cells and $CD4^-CD3^-$IL-7Rα^-CD11c^+ LTin cells?

2. Does each secondary lymphoid organ use slightly different populations of LTi and LTo cells for their development and does this differential utilization of cell types explain why each lymphoid organ uses subtly different combinations of chemokines, growth factors and cytokines for their development?

3. Other than the lymphotoxin signalling pathway, what pathways are used by LTi cells to trigger the differentiation of mesenchymal cells and initiate secondary lymphoid organ development?

4. Why is the development of most secondary lymphoid organs (lymph nodes, Peyer's patches and NALT) restricted to embryogenesis, when the development of ILFs can occur in adults?

5. Why does the development of NALT occur in the absence of lymphotoxin signalling and what pathways substitute for lymphotoxin signalling in this process?

Introduction

Primary immune responses are initiated in secondary lymphoid organs, including spleen, regional lymph nodes, Peyer's patches, Isolated Lymphoid Follicles (ILFs), tonsils and Nasal Associated Lymphoid Tissue (NALT). These tissues are situated throughout the body at strategic sites where antigens from pathogens are most likely to be encountered. Regional lymph nodes are found along lymphatic vessels that collect antigen and antigen presenting cells from non-lymphoid organs, whereas mucosal lymphoid organs, such as Peyer's patches, ILFs, tonsils and NALT, lack afferent lymphatics and acquire antigen directly across the mucosal epithelium. The spleen is also a secondary lymphoid organ and has evolved a unique structure to sample blood-borne antigens. All of these secondary lymphoid organs have specialized architecture and microenvironments that promote the controlled interactions of immune cells in order to elicit a rapid and appropriate immune response to infectious agents (reviewed in (1–3)).

Most infectious agents gain entry to the body via the skin or mucosal epithelium, which comprise an extremely large surface area. Moreover, many infectious agents target non-lymphoid tissues and organs to complete their life cycle. Since the frequency of naïve T or B

lymphocytes specific for particular epitopes is in the order of 1 in 10^5–10^6, it would be very difficult for these few cells to monitor every inch of each peripheral tissue and organ for foreign antigens and pathogens. This problem is elegantly solved by secondary lymphoid organs, which recruit naïve lymphocytes from the blood and attract activated, antigen-bearing antigen presenting cells from regional tissues. In essence, lymphocytes recirculate from lymph node to lymph node and allow antigen to be delivered to them. As a result, the process of immune surveillance is much more efficient and adaptive immune responses to infectious agents are initiated more quickly (reviewed in (1,4,5)).

Secondary lymphoid organs develop during embryogenesis or in the early post-natal period. This process occurs independently of antigen or pathogen recognition at pre-determined sites throughout the body as a result of complex interactions between various hematopoietic, mesenchymal and endothelial cells. Many, but by no means all, of the cellular and molecular interactions that are involved in secondary lymphoid organ development are understood and some of these mechanisms are also involved in the maintenance of secondary lymphoid organ architecture in adults as well as the function of these organs during immune responses (previously reviewed in (6–11)). Moreover, similar mechanisms govern the formation of tertiary lymphoid tissues, which develop in adults at sites of persistent infection or chronic inflammation. Since the formation and function of tertiary lymphoid organs has been recently reviewed (12,13), this review will focus exclusively on the pre-programmed organogenesis of secondary lymphoid organs.

Early cellular interactions in Peyer's patch development

Many of the cellular interactions that initiate secondary lymphoid organ development are well characterized, particularly with regards to Peyer's patch development (11). Hematopoietic cells derived from precursors in the fetal liver first begin to colonize the gut between days E12.5 and E15.5 (14,15). Although these cells are evenly distributed throughout the gut on day E15.5, they rapidly form clusters at sites of Peyer's patch development (16). This is step 1 as shown in Figure 1. These hematopoietic cells include a population of $CD4^+CD3^-IL\text{-}7R\alpha^+c\text{-}kit^+$ Lymphoid Tissue inducer (LTi) cells as well as a population of $CD4^-CD3^-IL\text{-}7R\alpha^-c\text{-}kit^+CD11b^+CD11c^+$ Lymphoid Tissue initiator (LTin) cells (16,17). Both the LTin and LTi cells cluster together with non-hematopoietic $VCAM\text{-}1^+ICAM\text{-}1^+$ Lymphoid Tissue organizer (LTo) cells on the anti-mesenteric side of the small intestine and form the primitive anlagen of the Peyer's patch (15,16).

The available evidence suggests that both LTin and LTi cells are important for the initial stages of Peyer's patch development. For example, mice depleted of $CD11c^+$ cells during embryogenesis develop fewer Peyer's patches and mice lacking the tyrosine kinase receptor, RET, which is expressed by LTin cells, completely lack Peyer's patch anlagen (16). Interestingly, $Ret^{-/-}$ mice have normal numbers of LTin and LTi cells, although these cells do not aggregate and fail to trigger the differentiation of $VCAM\text{-}1^+ICAM\text{-}1^+$ LTo cells (16). One of the ligands for RET, ARTN, is produced by non-hematopoietic $VCAM\text{-}1^+$ cells - probably LTo cells, and promotes the clustering of LTin and LTi cells at sites of Peyer's patch development. In fact, the ectopic application of ARTN to embryonic gut tissue leads to the clustering of both LTin and LTi cells, the local appearance of $VCAM\text{-}1^+$ cells and the formation of an ectopic Peyer's patch anlagen (16).

LTi cells are also important for secondary lymphoid organ development, as mice deficient for the transcription factor, Id2 (18), and mice deficient for the nuclear hormone receptor, RORγ (19) fail to develop $IL\text{-}7R\alpha^+CD4^+CD3^-$ LTi cells and consequently lack lymph nodes and Peyer's patches (18,19). Furthermore, the adoptive transfer of purified LTi cells from normal mice into neonatal $CXCR5^{-/-}$ recipients promotes the development of Peyer's patches (20),

and the adoptive transfer of IL-7R3$^+$CD4$^+$CD3$^-$ cells into neonatal *Id2*$^{-/-}$ mice induces the development of Nasal Associated Lymphoid Tissue (NALT) (21). Mice lacking the transcription factor Ikaros also fail to develop lymph nodes (22,23), a fact that is attributed to the loss of LTi cells. However, given the defects in the dendritic cell lineage in Ikaros mutant mice (24), it is possible that the development of LTin cells is also compromised in these animals and may contribute to the loss of lymphoid organs. Additional evidence for the involvement of LTi cells in lymphoid organ development comes from transgenic mice that over-express IL-7 (25). These mice have abnormally high numbers of LTi cells and develop a much higher number of lymph nodes and Peyer's patches than normal mice (25). However, IL-7 by itself is not sufficient to drive the development of these lymphoid organs, since IL-7 transgenic mice crossed to *Rorc*$^{-/-}$ mice fail to produce LTi cells and do not develop any lymph nodes or Peyer's patches (25). Thus, IL-7Rα$^+$CD4$^+$CD3$^-$ cells appear to be essential for lymphoid organ development.

Despite the essential roles that LTin and LTi cells play in secondary lymphoid organ development, the lineage relationship between these cell types remains obscure. LTin cells seem to have some characteristics of dendritic cells, including CD11c, CD11b and MHC class II expression. However, they lack DEC205 and instead express NK1.1 and Gr-1 (16). Importantly, they lack IL-7Rα, which is important for the expansion of LTi precursors and for the expression of LTαβ on LTi cells, particularly at mucosal sites. On the other hand, LTi cells are not easily categorized into a particular lineage. They arise from common lymphoid progenitors in the fetal liver (26), but lack any features of B or T lymphocytes. Under the appropriate conditions, they mature into NK-like cells and CD11c$^+$ dendritic cells (27), suggesting that they may give rise to CD11c$^+$ LTin cells. However, it is currently unclear whether the development of LTin cells also requires RORγ or Id2, which would provide better evidence that the two populations are related.

Early events in lymph node development

The early stages of lymph node development are slightly different that those of Peyer's patch development. Unlike Peyer's patches, lymph nodes are encapsulated by lymphatic endothelium and their development occurs concurrently with the process of lymphatic vascularization. As first shown by ink injections and micro-dissection, lymph sacs form from endothelial cells, which bud from the veins during early development (28,29). These primitive lymph sacs then form buds that branch and form the lymphatic network. This process is controlled by the transcription factor, Prox1, which is expressed exclusively by lymphatic endothelial cells (30) and is essential for the formation of lymphatic vessels (31,32). In fact, the early lymph node anlagen is bordered by a mix of endothelial cells that have characteristics of both lymphatic and blood vascular endothelial cells (33). Analogous to what happens in developing Peyer's patches, CD4$^+$CD3$^-$IL-7Rα$^+$ LTi cells aggregate near endothelial cells at sites of future lymph node development and trigger the differentiation of VCAM-1$^+$ICAM-1$^+$ LTo cells to form the primitive lymph node anlagen (34). Although CD11c$^+$ cells are also observed in developing lymph node anlagen (34), it is currently unclear whether these cells are the equivalent of CD11c$^+$ LTin cells or whether RET/ARTN interactions are also important for the development of lymph nodes.

Differential role of TRANCE and IL-7 in Peyer's patch and lymph node formation

The differences in Peyer's patch and lymph node organogenesis are also illustrated by the differential roles of the TNF family member TRANCE and the cytokine IL-7 in these processes (34,35). TRANCE (also known as OPGL/ODF/RANKL/TNFSF11), its receptor, RANK (also known as TRANCE-R/ODFR/OFE) and a critical component of the TRANCE signalling

pathway, TRAF6, are all required for the formation of lymph nodes, but not Peyer's patches or NALT (35–38). Although *Trance*$^{-/-}$ mice do develop some cervical lymph nodes, those that form are small and poorly populated (35,36). Moreover, *Trance*$^{-/-}$ mice have relatively few LTi cells and those that are present fail to properly induce the differentiation of mesenchymal cells (35). TRANCE is expressed by mesenchymal LTo cells in sites of lymph node development and functions to promote the differentiation and survival of LTi cells and to upregulate LTαβ on their surface (33,34). In turn, the ligation of the LTβR on local mesenchymal cells triggers their differentiation and promotes the expression of CCL19, CCL21 and CXCL13 as well as VCAM-1 and ICAM-1 (34,39). This is step 2 as shown in Figure 1. These chemokines and adhesion molecules promote LTi clustering and continue to recruit newly emerging lymphocytes to the developing lymph nodes. Therefore, in the absence of TRANCE, VCAM-1^{+}ICAM-1^{+} cells fail to appear in the lymph node anlagen and lymph nodes fail to complete their program of development.

On the other hand, IL-7Rα, IL-2Rγ and JAK3 are required for the organogenesis of Peyer's patches, but not lymph nodes (14,15). This was initially surprising, since the LTi cells in developing lymph nodes express IL-7Rα just like the LTi cells in developing Peyer's patches (34). In fact, LTi cells in developing lymph nodes are capable of responding to IL-7, as the ectopic application of IL-7 to developing *Traf6*$^{-/-}$ embryos (which cannot signal through TRANCE) promotes the development of lymph nodes (34). Like TRANCE, IL-7 upregulates LTαβ on the surface of LTi cells (40), which leads to the expression of chemokines and adhesion molecules by LTo cells. Other cytokines that signal through the IL-2Rγ chain, such as IL-4, are also capable of triggering LTαβ expression on LTi cells. However, there is no evidence that these cytokines are involved in secondary lymphoid organ development, probably because these cytokines are not expressed in the developing embryo. Thus, TRANCE and IL-7 perform similar functions and the reason that TRANCE is required for lymph node development and IL-7R is required for Peyer's patch development is due to differential expression of these cytokines in lymph nodes and Peyer's patches.

Although these data would suggest that the role for IL-7 in lymph node genesis is minimal, other data demonstrate that mice doubly deficient in CXCL13 and IL-7Rα completely fail to form lymph nodes (40), including mesenteric lymph nodes, which are normally present in *Cxcl13*$^{-/-}$ mice (40,41). Moreover, the transgenic over-expression of IL-7 promotes the development of extra lymph nodes as a result of much higher numbers of LTi cells (25). Thus, IL-7 probably also plays a role in the expansion or development of LTi cells from common lymphoid progenitors as well as being a critical inducer of LTαβ expression in some locations.

The counterparts to LTi cells in the development of secondary lymphoid organs are LTo cells, which are are mesenchymal in origin and form the stromal cell matrix of the developing lymphoid organ (14,33,39,42). Just as there seem to be multiple types of hematopoietic cells that trigger lymphoid organ development, there are also multiple types of mesenchymal LTo cells, with different expression patterns of adhesion molecules, cytokines and receptors (43). In fact, the VCAM-1^{+}ICAM-1^{+} LTo population can be divided into VCAM-1^{int}ICAM-1^{int} and VCAM-1^{hi}ICAM-1^{hi} populations (33). Whereas both of these populations are present in peripheral and mesenteric lymph nodes, the frequency of VCAM-1^{int}ICAM-1^{int} cells is dramatically lower in peripheral lymph nodes (33). Differences between these populations are also found in developing Peyer's patches and mesenteric lymph nodes. In fact, the LTo cells found at sites of mesenteric lymph node and Peyer's patch development express very different gene programs (43). For example, LTo cells at sites of mesenteric lymph node development express much higher levels of TGFβ and stem cell factor (the ligand for c-kit), whereas LTo cells at sites of Peyer's patch development express higher levels of IL-6, TRANCE, CXCL1, CCL7 and CCL11 (43). These differences in gene expression may provide an explanation as to why various lymphoid organs have different requirements for cytokines and growth factors,

such as the requirement for IL-7 in Peyer's patch development and the requirement for TRANCE in lymph node development. However, it remains an open question whether all secondary lymphoid organs have slightly different populations of LTo cells and whether the differences in these populations are intrinsic or are caused by the local environment.

Lymphotoxin signaling and secondary lymphoid organ development

One of the key events in the development of secondary lymphoid organs is lymphotoxin signalling, which promotes the differentiation of mesenchymal LTo cells and leads to the expression of various chemokines and adhesion molecules that are necessary for secondary lymphoid organ development. The essential role of LT is illustrated by *Lta*$^{-/-}$ mice, which lack Peyer's patches and most lymph nodes, except for rudimentary mesenteric lymph nodes that occasionally appear in some mice (44,45). This was initially somewhat surprising, since LTα is a soluble cytokine that is structurally and functionally related to TNF (46). In fact, LTα binds to both TNFR1 and TNFR2, suggesting that the biological functions of LTα and TNF should be very similar (47). However, *Tnf*$^{-/-}$ mice have normal numbers of lymph nodes and Peyer's patches, albeit with some structural alterations (48,49). Moreover, *Tnfr1*$^{-/-}$ and *Tnfr2*$^{-/-}$ mice also have normal numbers of secondary lymphoid organs (48,50), demonstrating that LTα signalling through TNFR1 or TNFR2 is not responsible for secondary lymphoid organ development.

This discrepancy was resolved by the observation that LTα also forms heterotrimers with LTβ, which is expressed as a transmembrane protein (51,52). The LTαβ heterotrimer is expressed on the cell surface of lymphocytes (53) as well as on LTi and LTin cells (16) and binds to the LTβR. Since the organogenesis of secondary lymphoid organs is disrupted in *Lt3*$^{-/-}$ mice as well as *Ltbr*$^{-/-}$ mice (54,55), it is clear that the surface form of LTαβ must be primarily responsible for triggering secondary lymphoid organ development. However, *Ltb*$^{-/-}$ mice still retain cervical and mesenteric lymph nodes (54), whereas *Lta*$^{-/-}$ mice lack these organs (44). Therefore, the soluble form of LTα must cooperate with surface LTαβ to contribute to the development of some lymph nodes. Furthermore, since all lymph nodes and Peyer's patches are absent from *Ltbr*$^{-/-}$ mice (55), it is likely that another TNF family member, such as LIGHT (56,57), acts through LTβR to facilitate the development of cervical and mesenteric lymph nodes.

In fact, TNF, LTα, LTβ and LIGHT all participate in secondary lymphoid organ development to some degree. For instance, mice treated in utero with soluble LTβR fused with the Fc protion of immunoglobulin (LTβR-Ig) retain mesenteric and cervical lymph nodes (58,59), whereas mice treated in utero with both LTβR-Ig and TNFR1-Ig do not develop these organs (60). Moreover, mice treated with LTβR-Ig and blocking antibodies against TNF also lack all lymph nodes and Peyer's patches (60), indicating that TNF signaling through TNFR1 plays some role in mesenteric lymph node development. As discussed above, LIGHT also contributes to mesenteric lymph node development. Although both *Light*$^{-/-}$ and *Lt3*$^{-/-}$ mice have mesenteric lymph nodes (57), a portion of *Light-Lt3*$^{-/-}$ mice lack mesenteric lymph nodes (57), suggesting that LIGHT and LTβ cooperate to induce mesenteric lymph node formation. Furthermore, since all *Ltbr*$^{-/-}$ mice lack mesenteric lymph nodes (55), these results also suggest that there may be another ligand for the LTβR that facilitates mesenteric lymph node development. Thus, although LTαβ signaling through the LTβR is the major pathway through which secondary lymphoid organ development is triggered, other TNF family members also contribute to lymph node development.

Despite the essential nature of interactions between LTαβ-expressing LTi cells and LTβR-expressing LTo cells, this interaction alone is not sufficient to promote secondary lymphoid organ development. For example, although the administration of an agonistic anti-LTβR

antibody in utero promotes lymph node development in *Lta*$^{-/-}$ mice, the administration of this same antibody in *Rorc*$^{-/-}$ mice, which lack LTi cells, does not promote lymph node development (19). Furthermore, the administration of anti-LTβR to *Cxcl13*$^{-/-}$ mice also fails to promote lymph node development. These data demonstrate that LTi cells or possibly LTin cells provide additional signals to mesenchymal or stromal cells that are necessary for secondary lymphoid organ development. The identification of these additional signals is key to our future understanding of secondary lymphoid organ development.

Most TNFR family members are coupled to the NFκB signaling pathway (61) and not surprisingly, molecules important in this signalling pathway are involved in LTβR signaling and lymphoid organ development. In particular, triggering of the LTβR induces the canonical pathway of NFκB activation that involves IKKβ and IKKγ/NEMO and leads to the phosphorylation of IκB and the translocation of p50/RelA or p52/RelA complexes to the nucleus (62). In turn, these complexes control the expression of inflammatory proteins, including VCAM-1, MIP1β and MIP2 (62). This pathway also promotes an increased production of the NFκB2/p100 precursor (62). However, LTβR signalling also induces a second pathway that leads to the sequential activation of NIK and IKKα and leads to the processing of p100 to p52 (62,63). In association with RelB, p52 translocates to the nucleus and activates transcription of molecules involved in secondary lymphoid organ development and homeostasis (62). These molecules include the homeostatic chemokines, CCL19, CCL21 and CXCL13. Both pathways appear to be important in lymphoid organ development, since mice homozygous for a natural mutation in NIK (alymphoplasia or *aly/aly* mice)(64,65) as well as *Tnfr1-Rela*$^{-/-}$ mice lack lymph nodes and Peyer's patches (66). Furthermore, RelB is essential for Peyer's patch organogenesis and aspects of splenic architecture (67–69). These results partly explain why LTβR and NIK signaling pathways are essential for lymphoid organ development and also explain why signaling through TNFR1 can facilitate lymph node development under some circumstances.

Lymphotoxin signaling elicits chemokine and adhesion molecule expression by stromal and endothelial cells

As mentioned above, interactions between LTαβ-expressing LTi and LTin cells promote the differentiation of LTβR expressing mesenchymal and endothelial cells (7,11,34). As part of this differentiation pathway, the mesenchymal cells express adhesion molecules, such as VCAM-1, ICAM-1 and MAdCAM as well as homeostatic chemokines, including CXCL13, CCL19 and CCL21. LTi cells, and probably LTin cells express chemokine receptors, such as CXCR5 - the receptor for CXCL13, and CCR7 - the receptor for CCL19 and CCL21 (40). LTi cells also express the integrins α4β7 and α4β1 (27,70), which bind to the vascular adhesion molecule, MAdCAM and VCAM-1 respectively. Homeostatic chemokines, particularly CXCL13, are expressed in Peyer's patch anlage during development (39). This low level expression attracts CXCR5-bearing IL-7Rα^{+}CD4^{+}CD3^{-} cells to the Peyer's patch anlage and activates α4β1 integrin in order to facilitate adhesion to the local stroma (20). Once IL-7Rα^{+}CD4^{+}CD3^{-}cells have arrived at the future sites of Peyer's patch genesis, they provide surface LTαβ (27), which induces the expression of higher levels of chemokines as well as adhesion molecules on the mesenchymal cells (39,71,72). These newly expressed molecules then recruit lymphocytes, which provide a sustained source of surface lymphotoxin and induce the differentiation of local stromal cells (73). The chemokine receptors and integrins expressed by LTin cells are currently unknown. However, since LTin cells cluster with LTi cells in the developing Peyer's patch at the same time, it is probably safe to assume that they express similar chemokine receptors and adhesion molecules.

Homeostatic chemokines do more than simply attract LTi cells to sites of secondary lymphoid organ development. Like IL-7 and TRANCE, they promote the expression of LTαβ on the

surface of LTi cells and lymphocytes (40,41). For example, CXCL13 promotes the expression of LTαβ on the surface of LTi cells as well as on mature B cells (41), whereas CCL21 and CCL19 promote the expression of LTαβ on LTi cells as well as mature CD4 T cells (40). In fact, the expression of homeostatic chemokines and lymphotoxin is co-dependent in a positive feedback loop (41), in which lymphotoxin signalling triggers the expression of homeostatic chemokines by mesenchymal/stromal cells and chemokine signalling maintains expression of LTαβ on the surface of LTi cells and lymphocytes. The essential role of homeostatic chemokines in secondary lymphoid organ development is demonstrated by *Cxcl13*$^{-/-}$ mice, which fail to develop Peyer's patches and most lymph nodes (41). *Cxcr5*$^{-/-}$ mice also lack Peyer's patches and most lymph nodes (74). Although mice with a natural mutation (paucity of lymph node T cells - *plt*) that disrupts CCL19 and CCL21 generally form a full complement of secondary lymphoid organs (75–78), the lymph nodes and Peyer's patches that do develop in *plt/plt* mice are small and poorly organized (75–77). In part, this is due to the reduced capacity of these organs to recruit mature lymphocytes from the blood and activated antigen presenting cells from regional tissues (75–77,79–81). However, CCL19 and CCL21 also play a role in the development of secondary lymphoid organs as shown by mice triply deficient in CXCL13, CCL19 and CCL21, which exhibit a more frequent absence of facial and cervical lymph nodes than mice lacking CXCL13 alone (40). The loss of these additional lymph nodes is attributed to poor recruitment of LTi cells as well as the failure of LTi cells to upregulate LTαβ and to promote the differentiation of local mesenchymal cells (40).

Programmed colonization and development of follicular structures in developing lymphoid organs

Although IL-7Rα^{+}CD4^{+}CD3^{-} LTi cells and VCAM^{+}ICAM^{+} mesenchymal cells are initially arranged in homogenous clusters, they begin to segregate into follicular structures around day E18 (82). CD11c^{+} cells also segregate into follicular structures in the developing Peyer's patches at this time (82), although it is not clear whether these cells are CD11c^{+} LTin cells or whether they are CD11c^{+} dendritic cells that have differentiated in situ from LTi cells (27, 70). Regardless, the initial follicular formation requires LTαβ and occurs independently of B and T cells (82). Similar events are thought to take place during lymph node development (34). This is step 3 as shown in Figure 1.

The last steps in the development of fully mature secondary lymphoid organs involve the recruitment of lymphocytes, the segregation of B and T cell areas and the formation of mature B cell follicles. In mouse lymph nodes this process is delayed until day 3 after birth (83) due to the sequential expression of MAdCAM and PNAd on HEVs (83,84). Prior to day 3, MAdCAM-1 is the only vascular addressin expressed on HEVs (83). As a result, cells entering developing lymph nodes during this time must express α4β7, the receptor for MAdCAM. Cells that use this pathway include CD4^{+}CD3^{-} LTi cells and γδT cells (27,70,84). However, around day 3, MAdCAM expression is downregulated on the HEVs of lymph nodes and PNAd expression is upregulated, allowing the entry of mature naïve B and T cells (83). This developmental switch is controlled in part by the de novo expression of CD34 and GlyCAM-1 (83), which are substrates for the sulfation reaction that generates the PNAd epitope.

After B and T cells start arriving in the developing lymph node, they begin to take over the role of CD4^{+}CD3^{-} LTi cells by expressing LTαβ and maintaining the differentiation and survival of LTβR-expressing mesenchymal and stromal cells. When B cells first arrive in the developing lymph node, they do not respond to CXCL13 and lack surface LTαβ expression (85). At this time, CXCL13 expression is still dependent on LTαβ expressed by CD4^{+}CD3- LTi cells (85). The lymph node becomes more organized upon the entry of T cells, when B cells begin to segregate in the outer cortex. Interestingly, the initial segregation of T and B cells occurs independently of CXCL13. This is step 4 as shown in Figure 1. However, from

day 4 on, the architectural changes are dependent on CXCL13 and CXCL13-responsive B cells. B cells also express LTαβ at this time (85), suggesting that LTαβ-expressing B cells play a major role in maintaining the architecture of secondary lymphoid organs. Consistent with the idea that LTαβ-expressing lymphocytes take over the role of LTαβ-expressing LTi cells, mice that lack NK, B and T cells develop the initial lymph node anlagen, which persists for a while after birth before dissipating (86). Interestingly, the adoptive transfer of IL-7Rα^{+} T cells or NK cells, but not B cells, restores the final stages of lymph node development (86), suggesting that these cells have the ability to replace LTi cells as the source of LTαβ at this later stage. Although it is not totally understood why adoptively transferred NK and T cells would be more efficient at promoting lymph node maturation than B cells, the authors of this study speculate that IL-7-driven homeostatic expansion of NK and T cells may play a role (86).

Role of LT in the maintenance of lymphoid architecture

Lymphotoxin and TNF expression are also required for the maintenance of lymphoid architecture in adults as well as the appropriate homing and segregation of B and T cells within secondary lymphoid organs (44,45). For example, the spleens of *Lt3*$^{-/-}$ mice lack organized B and T cell areas (44,45), marginal zones (45,87) and germinal centers (87,88). Moreover, follicular dendritic cells fail to develop in the absence of lymphotoxin or TNF signaling (73, 89,90). As in the developmental stages of secondary lymphoid organs, the expression of lymphotoxin and homeostatic chemokines are co-dependent in a positive feedback loop in which lymphotoxin promotes the differentiation and survival of the stromal and dendritic cells that express CXCL13, CCL21 and CCL19 (72), whereas chemokine receptor signalling controls the steady state recruitment and positioning of naïve B and T cells and maintains LTαβ expression on B and T cells(41,72). Most evidence suggests that the expression of LTαβ and TNF on mature B cells is essential for the maintenance of CXCL13 expression and follicular dendritic cells in the B cell follicle (73,91). However, mice in which LTβ is genetically deleted specifically in the B cell lineage have normal levels of chemokines, follicular dendritic cells and B cell follicles in the lymph nodes (92,93). In this case, LTαβ expression by T cells is sufficient to maintain these structures. Together, these data suggest that LTαβ expression on both B and T cells is important for the proper maintenance of lymphoid architecture in adults.

In addition to the maintenance of B cell follicles and the segregation of B and T cells areas, lymphotoxin signalling is also important to maintain dendritic cell numbers via homeostatic proliferation (94) and for the activation and maturation of dendritic cells during immune responses (95). The development of gp38-expressing stromal cells in the spleen is also dependent on lymphotoxin-expressing B cells (96). Furthermore, the continued expression of the vascular addressins, PNAd and MAdCAM, on high endothelial venules is dependent on lymphotoxin signaling (60,97,98). Interestingly, soluble LTα and membrane bound LTαβ are responsible for different aspects of lymphoid architecture. For example, the defects in B and T cell separation are less severe in the spleens of *Lt3*$^{-/-}$ mice(54) than they are in the spleens of *Lt3*$^{-/-}$ mice (45) and *Ltbr*$^{-/-}$ mice (55), suggesting that LTα signaling through TNFR1 as well as LTαβ signaling through LTβR is important for lymphocyte organization. In contrast, the presence of dendritic cells in the spleen as well as the expression of MAdCAM and PNAd on high endothelial venules and in marginal zone sinuses is controlled by surface LTαβ and the LTβR but not by TNF, LTα or TNFR1 (59,94). Finally, the differentiation of follicular dendritic cells requires TNF, LTα and LTβ (49,73,90,99). Thus, various aspects of lymphoid architecture are controlled by the overlapping, yet distinct, activities of soluble LTα, membrane LTαβ and TNF.

Secondary Lymphoid Organogenesis development is strictly confined to a developmental window

The development of conventional secondary lymphoid organs occurs during a temporal window in embryogenesis that varies depending on the particular lymphoid organ (58). For example, mesenteric lymph nodes develop first (around day E9–E10), followed by brachial (day E13), axillary (day E15) inguinal (day E16) and popliteal lymph nodes (day E17)(58). Mucosal lymphoid organs, such as Peyer's patches and NALT appear to develop last, as disruption of lymphotoxin signaling just before birth blocks Peyer's patch formation (58) while the adoptive transfer of LTi cells can restore the development of NALT in neonatal *Id2*$^{-/-}$ mice (21) and the development of Peyer's patches in neonatal *Cxcr5*$^{-/-}$ mice (20). In fact, there is a strict developmental window within which the organogenesis of lymph nodes and Peyer's patches must occur (58). Once this window has passed, the development of these organs cannot take place, regardless of whether lymphotoxin signalling is restored. There also seems to be a developmental window that restricts the formation of lymphoid tissues at ectopic sites, as demonstrated by the intradermal transfer of cells from dissociated lymph nodes to recipient mice (100). When given to adults, the transferred cells formed disorganized clumps. However, when given to neonates, the transferred cells organize into follicular structures with segregated T cell zones and B cell follicles that contain follicular dendritic cells (100). In contrast, there does not seem to be a fixed developmental window for the formation of ILFs. Although ILF development is dependent on lymphotoxin signalling (101,102), these structures can be restored in adult *Lta*$^{-/-}$ mice by reconstitution with normal hematopoietic cells (102). Although it is unclear why these particular tissues lack a fixed developmental window, it is known that RORγ-expressing CD4^{+}CD3^{-} LTi-like cells are found in the cryptopatches of adults (10). Thus, the developmental window for each lymphoid organ may be restricted by the availability of LTi cells in that location.

Interestingly, there is even a developmental window that restricts the formation of some splenic structures, such as the gp38-expressing stromal cells in the T cell areas of the spleen (96). The development of these stromal cells requires LTαβ-expressing B cells during the neonatal period (96). As a result, gp38^{+} stromal cells cannot be restored in the spleens of adult B cell deficient or *Lta*$^{-/-}$ mice by reconstitution with normal bone marrow (96). On the other hand, most of the other defects in splenic architecture, including the differentiation of follicular dendritic cells, expression of homeostatic chemokines, formation of the marginal zone and the development of germinal centers after immunization, can be reversed upon reconstitution of *Lt3*$^{-/-}$ mice with normal bone marrow (73,87,90,99).

A role for CD4^{+}CD3- LTi cells after lymphoid organogenesis

One of the most intriguing questions in lymphoid organogenesis is whether the sole purpose of CD4^{+}CD3^{-} LTi cells is to promote secondary lymphoid organ development during a narrow temporal window in fetal and neonatal development or whether these cells are also present and functional in adults (103). Although LTi cells are easily located with relatively high frequency within the early anlagen of developing Peyer's patches and lymph nodes (14,34), they are difficult to locate in adults. In fact, the presence of LTi cells in adults remains a controversial point (104). However, RORγ^{+} LTi-like cells can be observed within the cryptopatches of the adult gut (105), where they are postulated to promote the development of ILFs in response to microbial stimulation (106). CD4^{+}CD3^{-} LTi cells are also proposed to regulate Th2 responses in the spleen (107). CD4^{+}CD3^{-} cells in adult spleens appear to have a program of gene expression similar to that in CD4^{+}CD3^{-} cells found at sites of embryonic lymph node development. For example, CD4^{+}CD3^{-}cells in adults express high levels of LTαβ and TNF. (104). Moreover, the adoptive transfer of normal CD4^{+}CD3^{-} cells from either embryonic or adult tissues into *Lt3*$^{-/-}$ mice leads to the segregation of B and T cell areas and the expression

of CCL21 (108). However, unlike embryonic LTi cells, adult $CD4^+CD3^-$cells express high levels of OX40 ligand and CD30 ligand (109). Therefore, the precise lineage relationship between embryonic LTi cells and adult $CD4^+CD3^-$ cells remains unclear.

To make matters even more complicated, there is evidence that LTi cells in embryo are not a single homogenous population. For example, the development of NALT occurs in the absence of RORγ (110), whereas it is completely dependent on Id2 (21). Since both RORγ and Id2 are thought to be essential for the differentiation of LTi cells (18,19,111) and since normal LTi cells restore NALT development in *Id2*$^{-/-}$ mice (21), these data suggest that there must be some populations of LTi cells that develop independently of RORγ. These different populations of LTi cells may express different patterns of chemokine receptors that preferentially attract them to particular sites of secondary lymphoid organ development. This would fit with the idea that there are also different populations of mesenchymal LTo cells at sites of Peyer's patch, mesenteric and peripheral lymph node development that express different arrays of chemokines (43). Thus, the $CD4^+CD3^-$ LTi-like cells found in the spleens and cryptopatches of adults may simply be a few examples of the multiple types of these intriguing cells.

Exceptions to the paradigm of secondary lymphoid organ development

Although Peyer's patch organogenesis serves as an instructive model for the organogenesis of all lymphoid organs (11), the molecular requirements for the development of Peyer's patches and other lymphoid organs are not identical. This is illustrated by the differential requirement of lymph nodes and Peyer's patches for TRANCE and IL-7 (34,35). Although these two cytokines seem to share a similar purpose in secondary lymphoid organ development (34), their differential expression at sites of Peyer's patch and lymph node development leads to their differential importance in the development of these organs.

In contrast, the development of some mucosal lymphoid tissues differs much more substantially from that of Peyer's patches. For example, although NALT and Peyer's patches share an architectural similarity, the development of these organs is very different. The organogenesis of NALT occurs during the first week or two after birth (21,112), which is significantly later than that of Peyer's patches. As in Peyer's patch development, $CD4^+CD3^-$ LTi cells are among the first to appear in the developing NALT anlagen (21,113). However, the development of NALT does not require RORγ-dependent LTi cells (110). On the other hand, NALT development does require Id2-dependent LTi cells (21). In fact, the organogenesis of NALT can be restored in *Id2*$^{-/-}$ mice by the adoptive transfer of normal LTi cells to neonatal recipients (21). Most importantly, unlike the development of lymph nodes and Peyer's patches, the development of NALT is not dependent on the lymphotoxin signalling pathway (21,110). In fact, NALT is present in *Lt3*$^{-/-}$, *Lt3*$^{-/-}$, *Tnf-Lt3*$^{-/-}$, *Ltbr*$^{-/-}$ and *Tnfr1*$^{-/-}$ (21,110). Moreover, NALT develops normally in mice treated in utero with both soluble LTβR and soluble TNFR1 (21), even though these mice do not develop any lymph nodes or Peyer's patches. These data argue that none of the ligands that bind to either the LTβR or the TNFR1, including the LTα homotrimer, the LTαβ heterotrimer, TNF or LIGHT, are essential for NALT development. Furthermore, mice with mutations in the NFκB signalling pathway, including *aly/aly*, *Nfkb1*$^{-/-}$, *Nfkb2*$^{-/-}$ and *Relb*$^{-/-}$ mice, all have NALT to some degree (114). Together, these data argue that the central feature in the paradigm of secondary lymphoid organ development - the interaction between LTαβ-expressing LTi cells and LTβR-expressing mesenchymal LTo cells - is not a part of the NALT developmental program.

Although it is not clear why NALT development requires IL-$7R3^+CD4^+CD3^-$ cells in *Id2*$^{-/-}$ mice but not in *Rorc*$^{-/-}$ mice, the difference may reflect the activities of additional cell types that facilitate NALT development. Since *Id2*$^{-/-}$ mice lack NK cells as well as multiple populations of dendritic cells (18,115,116), and have defects in the CD8 T cell lineage (117),

these cells may play important, although currently undefined, roles in NALT development in the absence of RORγ-dependent LTi cells. This would be consistent with other data showing that NK cells participate in the stabilization of lymph nodes during development (86). However, if $CD4^+CD3^-$ cells are a central component in NALT developmental pathway, even in the absence of lymphotoxin signalling, then what signals might these cells provide during NALT organogenesis? It is clear from other studies that additional signals from LTi cells do play a role in lymph node development, since agonistic antibodies to the LTβR do not promote lymph node development in *Rorc*$^{-/-}$ mice (19). Defining these interactions will be key to our future understanding of secondary lymphoid organ development.

The discrepancy between *Rorc*$^{-/-}$ and *Id2*$^{-/-}$ mice in the relative importance of $CD4^+CD3^-$ cells in NALT development may also reflect diversity in the LTi population - with some LTi cells being formed independently of RORγ. Consistent with this idea, the LTi cells found in NALT express very low levels of CCR7 and CXCR5 relative to their levels on LTi cells from Peyer's patches (113). Moreover, NALT develops in the absence of CXCL13, CCL19 and CCL21 (113,118) and normal numbers of LTi cells migrate to the NALT of *Cxcl13*$^{-/-}$ or *plt/plt* mice just after birth (113). These data demonstrate that conventional LTi cells are probably not involved in NALT development and further suggest that additional, as yet undefined chemokines attract LTi cells to the NALT anlage. One of these chemokines might be CCL20, which is normally expressed in the dome epithelium of NALT in a lymphotoxin dependent manner (118). If CCL20 is required for NALT formation, possibly by recruiting CCR6-expressing LTi cells, then NALT development might be more analogous to the development of ILFs, which are highly dependent on CCR6 (119).

Despite the fact that the initial stages of NALT development occur independently of the lymphotoxin signalling pathway, the structure of NALT is severely compromised in *Lt3*$^{-/-}$, *Tnf-Lt3*$^{-/-}$ and *Lt3r*$^{-/-}$ mice (110,118). In fact, the B and T cells are not segregated into separate areas, follicular dendritic cells and $BP3^+$ stromal cells do not develop, high endothelial venules cannot be found and the normal array of homeostatic chemokines are not expressed in the NALT of *Lta*$^{-/-}$ mice (110,118). However, the architectural defects in the NALT of adult *Lta*$^{-/-}$ mice can be repaired by the restoration of normal lymphotoxin-expressing hematopoietic cells (110). Again, this is dramatically different than other lymphoid organs, which cannot be reconstituted in *Lta*$^{-/-}$ mice, even after the transfer of normal bone marrow into irradiated recipients (120). Thus, the structural defects in the NALT of *Lta*$^{-/-}$ mice are primarily due to the failure of lymphotoxin to promote the differentiation of stromal and endothelial cells and the loss of homeostatic chemokine expression.

The development of ILFs is also different than that of Peyer's patches, despite the similarity of their locations and presumably their functions in the small intestine. For example, the formation of ILFs is clearly dependent on lymphotoxin signalling, since ILFs are absent in *Lt3*$^{-/-}$ and *aly/aly* mice (101,102,121). However, like NALT, ILFs are completely restored in *Lta*$^{-/-}$ mice after reconstitution with normal bone marrow (102,122). Moreover, even though the treatment of mice in utero with LTβR-Ig or anti-IL-7R blocks Peyer's patch development, it does not prevent the development of ILFs (101,122). These data suggest that lymphotoxin signalling is important for the maintenance of ILFs after embryonic development rather than for the developmental formation of ILFs. This is probably analogous to role of lymphotoxin in the formation of NALT, in which lymphotoxin is essential for the expression of chemokines and the differentiation of stromal and endothelial cells (118). An alternative explanation is that lymphotoxin is required for ILF development, but that there is no temporal window in development, during which ILF formation must occur.

As it is in NALT, CCL20 is highly expressed in the dome epithelium of ILFs and seems to attract $CCR6^+$ dendritic cells as well as B and T cells to the dome region (119,123). The central

importance of CCR6/CCL20 interactions in the development of ILFs is demonstrated in *Ccr6*$^{-/-}$ mice, in which ILFs fail to develop properly (119). Like other homeostatic chemokines, CCL20 expression is dependent on LTα (118),j suggesting that the lymphotoxin-induced expression of CCL20 may be the essential step in ILF formation. While this might imply that LTi cells responsible for ILF formation must express CCR6 (124), it reflects instead a requirement for CCR6 on B cells (119). Given that LTαβ expression on B cells and not T cells is also required for the maturation of ILFs (125), one could speculate that there is a positive feedback loop between CCL20 expression in the dome epithelium of ILFs and LTαβ expression on B cells. Interestingly, B cells in ILFs do not have to be antigen specific to mediate their effects. In fact, normal ILFs develop in mice in which all B cells express a transgenic BCR specific for lysozyme (125). Despite the central role of LTαβ^{+}CCR6^{+} B cells in ILF formation, ILF development is compromised in *Rorc*$^{-/-}$ mice (106), suggesting that LTi cells are important for ILF formation. However, it is unclear at this time whether LTi cells are attracted to sites of ILF development via CCL20 or other homeostatic chemokines, like CXCL13, which is also highly expressed in mature ILFs.

Another unusual feature of ILF development unlike that of any other secondary lymphoid organ is that it is linked to microbial exposure (102,125–127). For example, ILF formation is barely detectable in germfree mice (102) and those ILFs that do develop have only small clusters of c-kit^{+} cells and essentially lack T cell zones or B cell follicles. However, other investigators find that ILFs (also known as Solitary Intestinal Lymphoid Tissue - SILT) are present in normal numbers in germfree mice, but are very small and immature and lack identifiable B cell follicles (126,127). Importantly, ILFs return to normal upon restoration of intestinal flora (102). These data argue that the location and number of ILFs is determined developmentally and cannot be altered by inflammation or infection. However, the final maturation and organization of ILFs is dependent on microbial stimuli and can even be enhanced by infection with pathogenic bacteria (128). Together, these data demonstrate that the development of ILFs has some characteristics in common with the development of ectopic lymphoid follicles, but other characteristics of secondary lymphoid organs.

Concluding remarks

Our understanding of the programmed development of secondary lymphoid organs has progressed remarkably over the last decade. We now appreciate that interactions between newly emerging populations of LTi and LTin with immature mesenchymal LTo cells are instrumental in this process and that these interactions are orchestrated by a variety of homeostatic chemokines, cytokines and growth factors. In particular, the lymphotoxin signalling pathway is centrally important for the initial differentiation of mesenchymal LTo cells and for the maintenance of architectural elements that make up the scaffolding of secondary lymphoid organs. However, it is now apparent that the developmental pathways governing each secondary lymphoid organ are often subtly and sometimes dramatically different. These differences are reflected in the variety of cytokines and chemokines that are required for the development of each organ and in the various types of LTi cells and LTo cells that are present in each site. Understanding these differences and determining how they impact the ultimate structure and function of each secondary lymphoid organ will be the challenge for the next decade.

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Terms/Definitions

1. Homeostatic chemokine

Homeostatic chemokines, including CXCL12, CXCL13, CCL19, CCL21 and CCL20 are constitutively expressed in secondary lymphoid organs and control the steady state recruitment and positioning of lymphocytes and dendritic cells. The expression of these chemokines is often regulated by lymphotoxin

2. Lymph node anlagen

The lymph node or Peyer's patch anlagen is the unstructured collection of hematopoietic and mesenchymal cells that initially forms at sites of secondary lymphoid organ development

3. Developmental window

The development of most secondary lymphoid organs occurs during a defined period in embryogenesis known as a developmental window. Certain developmental steps must be completed during this time period or the ability of these organs to develop is permanently disabled

Acronyms

1. Lti

Lymphoid Tissue inducer

2. Ltin

Lymphoid Tissue initiator

3. Lto

Lymphoid Tissue organizer

4. NALT

Nasal Associated Lymphoid Tissue

5. ILF

Isolated Lymphoid Follicle

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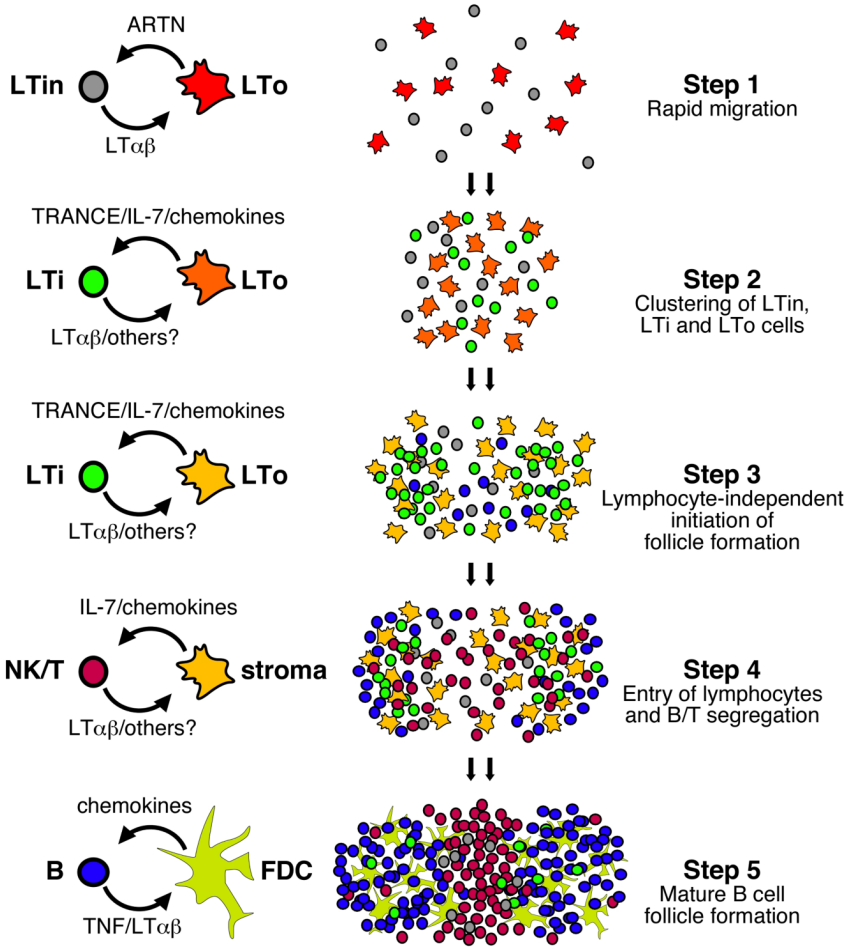

Figure 1. General scheme of secondary lymphoid organ development

As noted in the text of the review, the details in the developmental pathways of Peyer's patches and individual lymph nodes are often slightly different. Therefore, this scheme incorporates elements of both Peyer's patch and lymph node development and outlines major steps that are likely to be in common in these pathways. Importantly, this scheme implies several events that are purely speculative. For example, it is unknown whether LTo cells are the direct precursors of mature stromal cells and follicular dendritic cells (FDCs). It is also unclear whether LTi cells and LTin cells are maintained in mature secondary lymphoid organs in adults.

Table 1

Mutations that impair secondary lymphoid organ development.

Mutation	Signalling pathway	Cells affected	mLNs[a]	pLNs[b]	PPs[c]	NALT	References
Lta$^{-/-}$	LTβR	stroma	±[d]	−	−	±[e]	(21,44,45,110)
Ltb$^{-/-}$	LTβR	stroma	±	−	−	±	(21,54,110)
Ltbr$^{-/-}$	LTβR	stroma	−	−	−	±	(21,55,110)
Light$^{-/-}$	LTβR	stroma	+	+	+	ND[f]	(57)
Light/Ltb$^{-/-}$	LTβR	stroma	−	−	−	ND	(57)
Aly/aly	LTβR	stroma	−	−	−	±	(21,65,110)
Il7ra$^{-/-}$	IL-7R	LTi	+	+	−	±	(15,21,34,110)
Il2rg/rag$^{-/-}$	IL-7R	LTi	+	+	−	−	(86)
Jak3$^{-/-}$	IL-7R	LTi	+	+	−	ND	(15,34)
Trance$^{-/-}$	TRANCE-R	LTi	−	−	+	+	(35,37,110)
Rank$^{-/-}$	TRANCE-R	LTi	−	−	+	ND	(36)
Traf6$^{-/-}$	TRANCE-R	LTi	−	−	+	ND	(38)
Rorc$^{-/-}$		LTi	−	−	−	+	(10,19,110)
Id2$^{-/-}$		LTi	−	−	−	−	(18,21)
Ikaros$^{-/-}$		LTi	−	−	−	ND	(23)
Cxcl13$^{-/-}$	CXCR5	LTi/B	−	−	−	±	(40,41)
Cxcr5$^{-/-}$	CXCR5	LTi/B	−	−	−	ND	(74)
Plt/plt	CCR7	LTi/B/T	+	+	+	+	(40,65)
Ccr7$^{-/-}$	CCR7	LTi/B/T	+	+	+	ND	(129)
Cxcl13/Ccl19/21$^{-/-}$	CXCR5/CCR7	LTi/B/T	−	−	−	±	(40)
Ret$^{-/-}$	RET	LTin	ND	ND	−	ND	(16)
Graf$^{51/51}$	RET	LTin	ND	ND	±	ND	(16)

[a] mesenteric lymph nodes

[b] peripheral lymph nodes

[c] Peyer's patches

[d] small mLNS developed in a few mice

[e] small, disorganized NALT developed

[f] Not determined