General Discussion
Organ development consists of several discrete stages. Initially cells will enter or differentiate at specific locations in the body to form inducer tissue. Interaction with adjacent responder tissue leads to cell aggregation and further growth of the rudiment. The next stage is characterized by patterning events that result in organization of a primordium with areas containing different cell subsets. In the last stage, these areas differentiate into functional structures with the cell types that are found in the fully matured organ.

During the development of lymphoid organs all these stages of induction and positioning, aggregation and patterning and final differentiation take place, but the developmental mechanisms that govern the transition from one stage into the next are still not fully unraveled. In this thesis several of the early cellular interactions that occur in the development of lymph nodes and splenic white pulp have been studied. For instance for LN formation the events that take place at these early time points are decisive for the positioning and subsequent development of lymphoid structures and can only ensue within a certain time window during embryonic development. Since these processes are thought to be recapitulated at sites of chronic inflammation, when ectopic lymphoid structures develop, more knowledge of these processes might increase our understanding of and ultimately treatment of to prevent tertiary lymphoid tissue formation in diseases such as rheumatoid arthritis (RA) and inflammatory bowel disease (IBD).

**Early steps in lymph node formation**

Several years ago it was shown that for the development of lymph nodes lymphoid tissue inducer (LTi) cells are crucial to initiate an alteration in gene expression in stromal organizer cells. The insight into this mechanism appeared relatively late when seen in the historical perspective of research in lymphoid organ development as described in chapter 2, but led to a rapid expansion of insight in this field, especially when the crucial role of LTβR triggering in the formation of lymph nodes was demonstrated by gene-targeting experiments.

After their positioning in the vicinity of the locations where the primordia further develop, LTi cells trigger lymph node stromal cells to express the factors that are necessary for the aggregation of more hematopoietic cells. This results in the accumulation of a second wave of LTi precursors and later in the attraction of B- and T-cells. In the primordium, the patterning events strongly depend on a variety of chemokines that direct B- and T-cells to the future cortical and paracortical areas.

During the last step of lymph node formation, cortical and paracortical areas undergo their terminal differentiation. In the paracortex medullary cords emerge, while in the cortex B-cell follicles become visible. In a resting lymph node these are primary B-cell follicles, but after antigen encounter, B-cells can further differentiate into plasma cells or memory cells that proliferate in the cortex upon a second antigen encounter. Thereby they form the secondary B-cell follicles, containing germinal centers with a typical dark zone, light zone and mantle zone. These structures, which emerge as a consequence of the adaptive immune response, can therefore be regarded as the ultimate differentiation of the lymph node. Finally, after clearance of the antigen they become senescent. The recurrent development of germinal centers can therefore
be seen as an active process involved in keeping the body's homeostasis. (Fig. 1).

For the attraction and the retention of lymphocytes in later phases of lymphoid organ development, the differentiation of stromal cells that build up the lymphoid organ primordia is crucial. The important role of stromal cells in the organization of the primordium was previously shown for Peyer's patches 21 - 23. In chapter 3 of this thesis, it is shown that also in lymph node anlagen, stromal organizer cells are present early in development. At stage E16.5 in murine lymph node development, these cells can be distinguished by the expression of the cell adhesion molecules (CAMs) ICAM-1, VCAM-1 and MAdCAM-1 and the chemokines CXCL13, CCL19 and CCL21, which are induced upon LTβR signaling 24.

Interestingly, at this time point in development, there are two populations of stromal organizer cells present in mesenteric lymph node anlagen. One population is ICAM-1^{hi}VCAM-1^{hi}MAdCAM-1^{hi} (IVM^{hi}) and the second population consists of ICAM-1^{int}VCAM-1^{int}MAdCAM-1^{lo/int} (IVM^{int}) cells. Both populations express chemokines that are important for the attraction of CD4^{+}CD45^{+}IL7r^{+}RORγ(t)^{+}CD3^{−} LTI cells and their precursors (CD4^{−}CD45^{+}IL7r^{+}RORγ(t)^{+}CD3^{−}), but the expression is highest on IVM^{hi} stromal cells. In mesenteric lymph nodes of newborn mice, these stromal cells can still be found, which could point at functional differences of these two populations that are important in later stages of development. It was striking to find that both populations are also present in peripheral lymph nodes (PLNs), but that in PLNs their distribution differs significantly. Especially the number of IVM^{int} cells is strongly reduced in PLN anlagen. Because these cells have the highest expression of LTβR, they might be an additional target for LTI cells early in development during the inductive event of lymphoid organogenesis. Consequently, a suboptimal triggering of the LTβR, as seen in mice with targeted deletions of LTβ, components of the IL7r signaling complex, or the CXCL13/CXCR5 pair disrupts the development of PLNs but has little or no effect on the development of MLNs.

It remains to be investigated whether IVM^{hi} and IVM^{int} stromal cells can still be distinguished in mature lymph nodes and whether these populations contribute to the immunological differences of mesenteric versus peripheral lymph nodes, such as induction of immune tolerance versus activation. IVM^{int} might then especially be important for mechanisms involved in tolerance.

As mentioned before, the LTβR is the key component that regulates the expression of adhesion molecules and chemokines 24. During the aggregation phase adhesion molecules might facilitate the migration and subsequent retention of LTI cells and LTI precursors within the lymph node primordium, ensuring a rapid growth of the primordium. In chapter 4 it is shown that LTβR signalling also induces expression of cytokines such as IL7 and TRANCE. For differentiation and survival of LTI precursors, the cytokine IL7 is crucial 25. Upon triggering of the IL7r, lymphotoxin expression is upregulated 26. Thus, the triggering of the LTβR and thereby the upregulation of IL7 in lymph node stromal cells, also contributes to the positive feedback loop in which novel LTI precursors are attracted to and retained at the primordium. The IL7 production by stromal cells ensures local differentiation of
Figure 1: A schematic overview of sequential steps in lymph node development. A) Attraction of LTi cells and positioning of these cells to the area of the future lymph node primordium location. B) Clustering of LTi cells results in induction of LTαβ expression on LTi cells. These cells can now signal through the LTβR on mesenchymal cells, which mediates their differentiation into stromal organizer cells that express cytokines, chemokines, adhesion molecules and growth factors necessary for the attraction of additional hematopoietic cells. C) Attraction and accumulation of pre LTi cells and local differentiation of these precursors into mature LTi cells. Expansion of the stromal organizer cells and encapsulation of the cluster of LTi cells and stromal cells by lymphatic endothelial cells. Sprouting of blood vessels into the lymph node primordium (not shown). D) Differentiation of small blood vessels in the lymph node primordium. First appearance of high endothelial venules (HEVs). Attraction of B- and T-cells to the primordium. Separation of B and T cells into distinct areas. E) Terminal differentiation of cortical and paracortical areas. In the last step in lymph node development, stromal organizer cells further differentiate into different subpopulations located at distinct sites in the lymph node. In the cortex, B-cell follicles can be distinguished. In this phase, B-cells can differentiate into plasma cells and memory cells. The latter proliferate in the cortex upon a second antigen encounter, forming secondary B-cell follicles. These contain germinal centers with a typical dark zone, light zone and mantle zone. In the secondary B-cell follicles, follicular dendritic cells (FDCs; not shown) are present. Furthermore, medullary cords emerge in the paracortex. At the final step of lymph node development, macrophages can be found in the medulla, and different subsets of dendritic cells are present at various locations in the lymph node (not shown).
Figure 1, continued.
precursor cells resulting in an increase in lymphotoxin expressing LTi cells.

Also TRANCE is likely to be involved in this differentiation, since mice deficient for TRANCE or its receptor lack lymph nodes, most likely because mice with deficient TRANCE-signaling have severely diminished numbers of LTi cells in lymph node anlagen. The finding that LTβR signaling controls the expression of both TRANCE and IL7 places this receptor even more central to lymphoid tissue formation. This is further stressed by the fact that also factors for lymphangiogenesis are upregulated upon ligation of LTβR. And thus, signaling via this receptor ensures the attraction, retention and further differentiation of accumulating numbers of LTi precursor cells during the aggregation phase of lymph node development, while the developing lymph nodes become encapsulated by lymphatic endothelial cells (LECs) ensuring their drainage function. We can therefore state that LTβR is truly the key component of the aggregation phase in lymph node development.

In one of the first models of lymph node development it was postulated that the earliest events in this process were characterized by the formation of lymph sacs; sinus-like structures made up by lymphatic endothelial cells from which novel lymph vessels would sprout and that would grow out to lymph nodes in later stages. When combined with the model in which LTi cells induce stromal cells to express all the factors required for lymph node development, this raised the question whether these two processes occurred in parallel or whether lymphatic endothelial cells were responsible for the attraction of the very first LTi cells to predilect sites of lymph node anlagen. This would imply that LECs would be the true inducer cells in lymphoid organogenesis and would control this process before the LTβR is triggered by LTi cell during the aggregation phase of lymph node development. Since Prox-1 signaling is crucial for LEC formation we studied the effects in Prox-1 deficient mice. Assuming that LECs are indeed the initiators of the whole process, deficiency in Prox-1 would then completely abolish the aggregation of LTi cells and thereby the formation of lymph nodes.

To our surprise however, this appeared not to be the case. In chapter 5 we show that, although the formation of the lymph node primordium is abnormal in Prox-1-/- fetuses, LTi cells can still aggregate at the locations where normally lymph nodes develop. This shows that the initial aggregation of LTi cells does not depend on factors expressed by LECs, such as CCL21. However, subsequent to the initial clustering of LTi cells, LEC do play an important role, since analysis of E17.5 conditional Prox1–mutant embryos revealed that stromal organizer cells do not fully differentiate in the absence of LECs. Our results suggest a role for CCL21 produced by LECs in further attracting LTi cells to a more tight cluster, allowing better triggering, and thus differentiation, of mesenchymal cells. Our data thus indicate that the first aggregation can still occur in Prox-1 knockout mice. We also observed that these first clusters of LTi cells are lacking in CXCR5-/- mice, and therefore we speculate that the initiation phase of LN development is controlled by CXCL13-CXCR5 signalling (our unpublished observations).

**The role of vascularization on lymph node development**
The development of lymphoid tissue primordia not only depends on the cross
General Discussion

talk between hematopoietic cells and mesenchymal stromal cells. Also the vascularization of the primordium is important for further development. Signaling molecules that direct these processes, such as chemokines, cytokines and angiogenic factors, can bind to heparan sulphate proteoglycans (HSPGs)\textsuperscript{33}. In chapter 6 we showed that during lymph node development, the binding of signaling molecules is important for normal lymph node organogenesis. In C5 epi\textsuperscript{-/-} animals, displaying disturbed processing of HS-chains and altered binding of a variety of factors, the attraction and retention of hematopoietic progenitors was not completely blocked, but lymphoid tissue formation was severely affected. In some but not all C5 epi\textsuperscript{-/-} littermates, inguinal lymph nodes were lacking, while the major vessels with which they are normally associated, did not develop normally. It is tempting to speculate that abnormal blood vessel development and failure of LN formation are linked.

The dependency of lymph node formation on the proper location of major blood vessels is striking. Indeed, in the adult, all lymph nodes are found in close proximity to major vessels. But even lymph node primordia at E14.5 in murine lymph node development, are found in close proximity to a VEGFR1\textsuperscript{-}VEGFR2\textsuperscript{+}MECA32\textsuperscript{-} and a VEGFR1\textsuperscript{-}VEGFR2\textsuperscript{+}MECA32\textsuperscript{+} blood vessel as we describe in chapters 4 and 5. These vessels are most likely arteries or arterioles as well as venules, since the same expression pattern is found on the aorta and the inferior vena cava, determined by their position in the fetus. Because also during the development of the splenic white pulp LTi cells are found back adjacent to blood vessels with a similar expression pattern, while during human fetal spleen development expression of CXCL13 could be observed in arterial smooth muscle cells as well as cells around these arterioles\textsuperscript{34}. Therefore, also in the developing spleen CXCL13 could regulate the attraction to this area. A future study on the special properties of the stroma around these vessels and how chemokine expression is controlled could give further insight in spleen development.

\textit{LT\textbeta R independent splenic white pulp development}

Based on the fact that LT\textbeta R triggering is indispensable for normal white pulp development it was questioned whether LTi cells would also play a role in white pulp ontogeny. However, in chapter 7 we clearly show that splenic LTi cells lack cell surface expression of LT\textbeta R ligand in the murine fetus, whereas at day 4 after birth B cells express LT\alpha\textsubscript{1}\beta\textsubscript{2} and LTi cells do not. This is the same time point when T cell areas start to emerge, demonstrating that B cells, and not LTi cells give the LT\alpha\textsubscript{1}\beta\textsubscript{2} dependent inductive signal during white pulp development. This signal is needed for the aggregation of lymphocytes in later stages of white pulp formation. While the organization of the white pulp into functional T and B cell areas depends on LT\textbeta R signalling, the separation of white and red pulp areas occurs before birth and is independent of LT\alpha\textsubscript{1}\beta\textsubscript{2} expressing cells.

The fact that LTi cells do not express LT\alpha\textsubscript{1}\beta\textsubscript{2} on their cell surface but nevertheless accumulate around splenic vessels and associate with VCAM-1\textsuperscript{-}ICAM-1\textsuperscript{-} stromal cells is intriguing. We do not exclude the possibility that LTi cells contribute to splenic white pulp development in an LT\textbeta R independent manner, but it is possible that the surface expression of LT\alpha\textsubscript{1}\beta\textsubscript{2} by LTi cells is actively suppressed in the
spleen to delay triggering of the LTβR at this location. In this way, other locations in
the body, such as lymph nodes and Peyer’s patches could have a small advantage
in the attraction and retention of lymphocytes at early time points in development.
This would ensure that these lymphoid structures are able to emerge at the time
point in development in which interaction of lymphocytes and stromal cells of
lymph node and Peyer’s patches is possible. In addition, the suppression of LTβR
triggering in the spleen could also prevent the expression of lymphangiogenic
factors such as VEGF-C, which is upregulated upon ligation of the LTβR.

We showed that the spleen attracts and retains LTi cells by expression of chemokines
such as CXCL13, CCL19 and CCL21, but that these cells cannot contribute to the
LTβR signaling. This implies that there is a necessity to ‘capture’ circulating LTi cells
that are not retained in other lymphoid tissues such as lymph nodes or Peyer’s patches.
In this way, the number of circulating LTi cells can be tightly regulated, preventing
other tissues to be activated via the LTβR by the LTαβ2 expressing LTi cells.

The capability of the spleen to attract and retain LTi cells was maintained in C5 epi
−/− spleens, in which splenic white pulp anlagen were still present. This could be a
consequence of CXCL13, CCL19 and CCL21 being less dependent on C5epi for HSPG
binding than CXCL12 (our unpublished observations). And in fact our observation that
LTi cells were significantly elevated in spleens from mutant mice suggest that LTi cells
which can not be retained at sites of LN development will collect in the spleen.

As mentioned previously, blood vessels are an important component of lymphoid
tissue primordia already early in development, because they allow migration
of precursor cells into the primordium necessary for their differentiation. The
importance of blood vessel formation for the fetal spleen (FS) is evident, because
a major part of the red pulp compartment is constituted by blood vessels. The
effect of a deficiency in C5 epimerase, which affects blood vessel formation, as
well as spleen size suggest a correlation between these two processes. It will be
interesting to see whether the specialized venous system of the red pulp, which
has the unique capacity to filter the blood and remove old erythrocytes, as well
as the specialized marginal zone, can function properly in adult C5epi−/− spleens.
Since C5epi−/− mice die around birth, transplantation of C5 epi−/− spleens into wild
type mice could allow further maturation of these spleens, and clarify whether
structures such as the marginal zone, are blocked in their formation.

Implications for clinical research
In rheumatoid arthritis patients development of germinal centers was shown
to depend on the combined action of lymphotoxins expressing B cells and the
chemokine CXCL13, implicating a high degree of similarity between secondary
and tertiary lymphoid structure formation 35. Further evidence of LTβR triggering
in inflamed joints comes from the observation of increased Il7 expression in tissue
biopsies from RA patients, since Il7r signaling induces lymphotoxin expression. In
addition we showed in chapter 4 that signaling through the LTβR on stromal cells
leads to an increased production of Il7. Taking these findings together, in inflamed
tissue in pathological conditions, the previously described positive feedback loop
probably also occurs. Thus under these circumstances, it is likely that the interaction
between hematopoietic and stromal organizer cells results in the accumulation and local differentiation of newly attracted hematopoietic cells and thus in the growth of the lymphoid tissue. The list of pathological conditions that show the development of tertiary lymphoid structures becomes longer, thus suggesting that in all these diseased tissues stromal cells are permissive to inductive signals from inducer type cells, and respond with differentiation towards organizer cells.\(^{36, 37, 38}\)

It is evident that the circumstances under pathological conditions are not identical to the situation in embryonic and fetal development because of the existence of cell types that are not yet formed in early developmental stages. But comparing the morphology and cellular make up of tertiary lymphoid tissue with different developmental stages of lymph node anlagen might narrow down the collection of genes that could have a key function in lymphoid tissue development, especially for genes that are important for early events in this process.

**Concluding remarks**
The development of lymphoid organs during ontogeny is based on a complex interplay between various cell types that interact at different time points. With the present knowledge of the molecular requirements that underlie the very first phases of lymph node development further studies are warranted to try and interfere with the development of tertiary structures as seen in autoimmune disease.


