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by

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Voor mijn Liesje

CONTENTS

GENERAL INTRODUCTION

Development of human lymph nodes and Peyer's patches

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INTRODUCTION

The human immune system harbors the potential to generate novel lymphoid organs within inflamed tissues during disease. These tertiary lymphoid organs (TLOs) resemble lymph nodes and can contain segregated T and B cell areas, germinal centers, high endothelial venules and follicular dendritic cells (FDCs)[1,2]. While TLO formation is likely to have evolved as a means to locally create an optimal environment for local eradication of infections, evidence is culminating that during chronic or auto-immune inflammation TLOs contribute to sustaining aberrant inflammation, and serve as a site for activation of auto reactive lymphocytes[3,4]. TLO-like structures can also contribute to the survival of malignancies, since disseminated follicular lymphoma in the bone marrow induces TLO-like structures to acquire a survival supportive microenvironment[5,6].

In animal models, the development of TLOs with a higher degree of cellular organization, i.e. the segregation of T and B cells and the formation of B cell follicles, is guided by mechanisms similar to those guiding formation of murine lymph nodes during embryogenesis[1,2,7,8]. A crucial event in organized TLO formation is lymphotoxin-β-receptor (LTβR)-dependent initiation of homeostatic chemokine production[9]. TLO formation is preceded by the accumulation of activated lymphocytes, a process guided by inflammatory chemokines. These chemokines will in most cases be produced by activated cells of the innate immune system[10]. Subsequently, two criteria are thought to be needed for TLO development. First, expression of the LTβR on tissue-resident stromal or reticulum cells and second, a persistent stimulus such as an auto antigen to ensure the prolonged influx of activated, lymphotoxin-expressing lymphocytes[2,8]. The subsequently achieved continuous signaling via the LTβR will lead to a switch in chemokine production, in which homeostatic chemokines will eventually be favored over the inflammatory chemokines mainly produced during the initial acute phase of the response[9]. Eventually, this will result in the influx of naïve lymphocytes and organization into T and B cell areas[11,12]

Lymph node development in mice

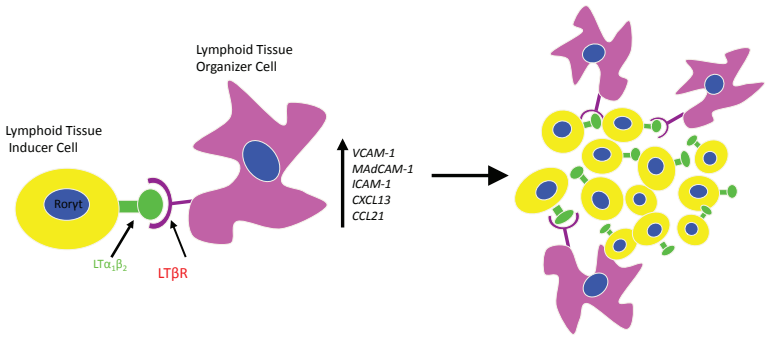

Figure 1. **LTi cell – LTo cell interactions in LN development.** Mature lymphoid tissue inducer cells (LTic) ligate the lymphotoxin-beta receptor (LTβR) expressed by LTo cells which are triggered to express adhesion molecules and chemokines in order attract and retain more LTic: a positive feedback loop.

TLO formation in experimental systems was shown to display a high degree of similarity with murine lymph node development. In mice, lymph node development starts between days 12 and 14 gestation, depending on the location of the lymph node[13,14]. This process is initiated via paracrine interactions between at least two cell types: sessile stromal cells and specialized circulating immature lymphoid tissue inducer (LTi) cells. At prospective lymph node locations retinoic acidprobably produced by adjacent nerve fibers, induces the expression of the homeostatic CXC-chemokine ligand 13 (CXCL13) by local stromal cells of mesenchymal origin and this attracts $CXCR5^+$ pre-mature LTi cells [15].Upon clustering, immature LTi cells start to express surface lymphotoxin induced by signaling through the IL-7Rα and RANK[16-18]. As depicted in Figure 1, mature LTi cells subsequently ligate the lymphotoxin-β receptor expressed by stromal cells, which are now designated lymphoid tissue organizer (LTo) cells, and this event leads to the production of the homeostatic chemokines CCL19/CCL21,elevated levels of CXCL13 and the expression of the adhesion molecules VCAM-1, ICAM-1 and MAdCAM-1[13,19,20]. The up-regulation of the homeostatic chemokines and adhesion molecules allows for the initiation of a positive feedback mechanism by which additional lymphotoxin-expressing cells are recruited and retained, reciprocally ensuring the continuous triggering of the LTβR.

In 2008, Katakai and colleagues identified the adult equivalent of LTo cells in fully developed SLOs and designated these cells Marginal Reticular Cells (MRCs) [21] They showed that these cells are developmentally programmed and depend on LT-signaling for their phenotypical integrity. The role of these cells has not been identified yet. Besides lymphotoxin- and the LTβR several other genes were proven to be indispensable for murine lymph node development, mostly through the generation of gene targeted mice. These include knockouts for members of the TNF-superfamily (RANK and RANKL)[22-24], chemokines and their receptors (CXCL13/CXCR5, CCL21,CCL19/CXCR7)[25,26] and cytokine receptor signaling molecules (common-γ-chain, IL7Rα)[27-29] (for an overview see [2,8]). In subsequent developmental stages, high endothelial venules (HEVs) are formed, enabling the entry of cells from the circulation (reviewed in[2,8]). Postnatally,

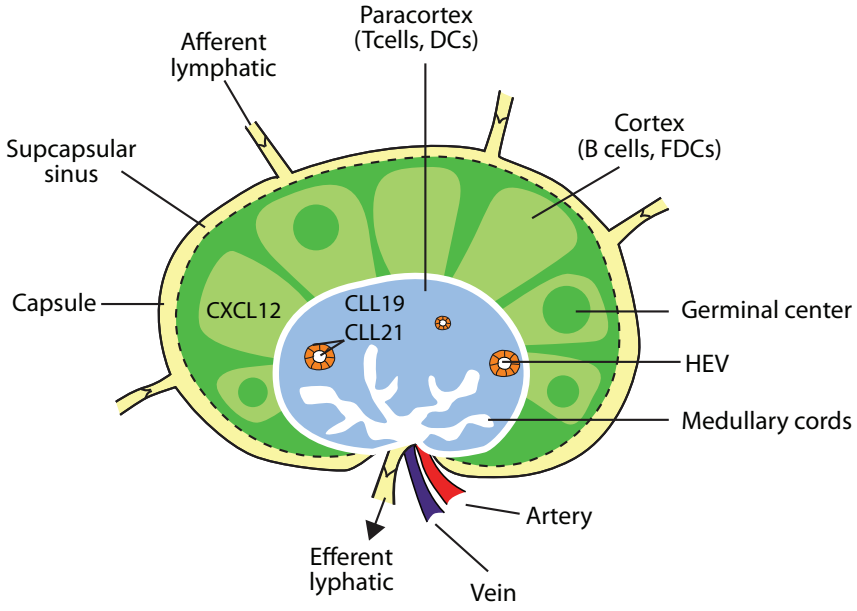

Figure 2. Schematic overview of an adult lymph node. Cortex is green, the paracortex and medulla blue. *Adapted from Drayton et al., Nature Immunology, 2006.*

the typical lymphoid architecture starts to become apparent from day 2, when mature T cells exit the thymus, flood the periphery and enter the developing lymph nodes. At day 4, T and B cells in the lymph node have segregated, although no B cell follicles have formed yet. This occurs at day 7, when clearly discernable follicles containing FDCs can be readily detected[30]. Figure 2 depicts a schematic overview of an adult lymph node. In green the B cell zone with FDCs, follicles and germinal centers. In blue the T cell zone where TRC produce CC21 and HEVs end. Blood vessels enter and leave the lymph node at the hilum. Lymphocytes in the blood enter the LN via the HEV. In the cortex (green area) B cells reside and are organized in follicles. When an immune response is underway, focal points of B cell proliferation known as germinal centers (GC) are localized in some follicles. T cells are located within the cortex (blue area) and paracortical area. Lymph enters the LN via afferent lymphatic vessels that open into the subcapsular sinus. The lymph drains towards the medulla and subsequently into a network of medullary chords that converge at the hilum and drain into the efferent lymphatic vessel.

RORC⁺ innate lymphoid cells

LTi cells belong to the family of RORC⁺ innate lymphoid cells (ILCs)[31] and are derived from a common lymphoid progenitor-like precursor that resides in the fetal liver and the bone marrow in adult mice and are among the first cells to cluster at developing lymph node anlagen[18,32,33]. Crucial steps in murine Rorc⁺ ILC development are the expression of the inhibitor of DNA binding 2 (Id2) and nuclear retinoid-acid receptor related orphan receptor γt (Rorγt). Mice deficient for either of these two genes lack LTi cells and all lymph nodes and Peyer's patches [34-37]. For all

functional purposes, murine LTi cells strongly resemble activated lymphocytes, only without an antigen receptor. Based on this observation, we and others have put forward the idea that a close relationship exists between the formation of organized inflammatory infiltrates and the formation of lymph nodes, with LTi cells creating an inflammatory-like response in the developing fetus without the need for antigen[38,39].

Human LTi cells are characterized as lineage$^-$ (lin$^-$; CD3$^-$CD20$^-$CD14$^-$CD34$^-$) cells that are CD45^{int}, IL7Rα$^+$, c-Kit$^+$ cells expressing Id2, RORc, LTA, LTB, RANK, RANKL and CCR7 in analogy to their murine equivalents. Similar to murine LTi cells, human LTi cells induce the expression of adhesion molecules on mesenchymal cells through lymphotoxin and TNF signaling. [40]. Human fetal RORC$^+$ ILC produce the inflammatory cytokine interleukin-17A (IL-17) which is mainly known to be produced by RORC$^+$ Th-17 cells[41-43]. IL-17 is associated with a inflammatory response against mucosal infections and was shown to play a role in several autoimmune diseases where its effects are not only beneficial but also adverse[44,45]. In contrast to fetal RORC$^+$ ILC postnatal RORC$^+$ ILCs in tonsils and intestines produce the IL-10 family member IL-22, which signals through the IL-22R$_1$/IL-10R$_2$ heterodimer (IL-22R)[46]. Triggering of the IL22-R expressed by intestinal epithelial cells initiates the JAK-STAT 3 pathway[47,48] and induces the production of anti-microbial proteins (AMPs) such as β-defensin-2, RegIIIβ, RegIIIγ and s-100 calcium binding proteins such as S-100A7[49-51]. IL-22 was shown to be important in the early defence against intestinal bacterial infection with *Citrobacter rodentium.* Mice deficient for IL-22 died within the first week after infection with *Citrobacter rodentium* [52].

Cultured human fetal RORC+ ILCs express several NK cells related molecules, including CD56 and the natural cytotoxity receptors (NCRs) NKp46, NKp44 and NKp30[40]. While Nkp46 and NKp30 are constitutively expressed by NK cells, NKp44 is only expressed by NK cells upon activation. In the murine genome only NKp46 is present[53]. NCRs are type-I transmembrane proteins that associate with ITAM bearing molecules for their intracellular signal transduction and are involved in NK cell mediated killing via granzyme and perforin excretion, and the induction of IFN-γ and TNFα production[53,54]. So far, several exogenous ligands for NCRs are known. In both mice and human the influenza virus hemaglutinin and the Sendai virus hemaglutin-neuraminidase can bind to NKp46[55,56]. In mice, NKp46 plays a role in the eradication of the influenza virus in vivo[57]. Furthermore, it has been shown that NKp46 contributes to the lysis of monocytes infected with *Mycobacterium tuberculosis* [58]. The involvement of NCRs in the killing of murine and human cancer cells has also been described [59,60].

Human NKp44$^+$RORC$^+$IL-22$^+$ cells share features with both LTi cells and immature conventional natural killer (cNK) cells. However, a recent study concluded that this lineage$^-$ c-Kit$^+$ cell population is a functional and developmental distinct lineage from cNK cells[61]. In contrast to cNK precursors, NKp44$^+$RORC$^+$IL-7Rα$^+$ cells maintain RORc expression when cultured in vitro. In addition, these cultured cells develop low cytotoxicity and IFN-γ production and killer inhibition receptors. In alignment with these data on human NKp44$^+$RORC$^+$ cells, murine NKp46$^+$Rorγt$^+$

cells in the intestines of adult mice also resemble LTi cells and cNK cell precursors. In contrast to cNK precursors, NKp46$^+$Rorγt$^+$ IL-7Rα$^+$ cells still can develop in the absence of IL-15 signaling and develop no cytolytic activity and IFN-γ production[62]. Like LTi cells, NKp46$^+$Rorγt$^+$ cells are absent in either Rorγt$^{-/-}$ or Id-2$^{-/-}$ mice, and strongly IL-2rg$^{-/-}$ mice.

Together, these data suggest that a developmental relationship between LTi cells and mucosal IL-22 producing NKp44$^+$RORC$^+$ ILCs might exist.

RORC$^+$ ILC in the human fetal intestine

In 1986, Spencer and colleagues made an observation that has led to a great deal of speculation in the ensuing years[96,97]. Before the apparent onset of Peyer's patch formation, at approximately 11 weeks gestation, the lamina propria was found to contain infiltrates of CD45$^+$CD4$^+$CD3$^-$HLA-DR$^+$ cells, which either had a macrophage-like morphology, or presented as spindle shaped cells. The extent of these infiltrates increased concomitantly with the age of the fetus examined. At the time, the authors assumed that these cells likely represented macrophages and dendritic cells[96,97], both of which had just recently been reported as expressing the CD4 antigen[99]. Currently, we know that there is a population of Rorc$^+$ ILC present in the fetal murine intestine that expresses CD4, the IL7-Rα and c-Kit[100,101]. As described in chapter two of this thesis, in human fetal intestines of 11 gw RORC$^+$ ILCs are also already present. However, these cells lack expression of CD4, suggesting that Spencer and co-workers were most likely right in their assumption that the CD4$^+$ cells were macrophages or dendritic cells. During fetal development, human intestinal ILC gradually acquire NKp44 expression and contain low levels of IL22 transcripts. The NKp44$^+$RORc$^+$IL-7Rα$^+$ innate lymphocytes that reside in the lamina propria of adult intestines produce IL22 levels comparable to tonsils[40,102 and chapter 2].

Peyer's patch development in mouse

Development of the murine Peyer's patches is extensively documented[63]. Three clearly defined steps have been identified, the first of which, at E15.5, marks the beginning of Peyer's patch development. At this time, VCAM-1 starts to be expressed by distinct clusters of stromal cells located on the anti-mesenteric side of the small intestine[63]. These VCAM-1 positive stromal cells also express Artemin, the ligand for the tyrosine kinase receptor RET[64]. During the second stage, VCAM-1 positive cells recruit RET$^+$CD11c$^+$cKit$^+$lymphotoxin$^+$ cells as well as IL7R$^+$lymphotoxin$^+$CD4$^+$CD3$^-$ LTic[63-65]. The VCAM-1-positive stromal cells express the LTβR, and upon ligation of this receptor produce IL-7 and homeostatic chemokines such as CXCL13[66]. This will reciprocally lead to increased expression of surface lymphotoxin on LTic, forming a self-sustaining Peyer's patch primordium[16]. From E17.5, during the third phase of Peyer's patch development, circulating lymphocytes are attracted, start to enter the developing organs and fill up the predetermined T and B cell niches[65].

Knowledge on the mechanisms of human lymph node development is still much less detailed when compared to mouse. However, careful studies were performed in which the morphological

and cellular changes during human lymph node and Peyer's patch development were recorded. In the following paragraphs we will compare these data on human organogenesis with experimental data from murine studies.

Development of the human fetal immune system

Definitive hematopoiesis in humans is initiated in the Aorta-Gonad-Mesonephros region[67]. Subsequently, at 6 weeks gestation, stem cells make their way to the fetal liver, the primary site for hematopoiesis during fetal development. From approximately 16 weeks onward, hematopoietic cells also appear in the bone marrow which will function as the main hematopoietic organ postnatally[68]. B- and NK cells are the most abundant immune cells found in the fetal liver from week 8 through 12, while the end of week 12 marks the appearance of the first thymus-derived αβT cells in the periphery. These will rapidly increase in numbers during the following weeks[68]. This sequence of hematopoietic events is largely similar during murine hematopoietic development. However, the timing is critically different. Murine T cells do not leave the thymus until day 2 post-natal[69], whereas the human immune system seems to have all cellular components available by week 13 and is able to mount an antibody response from the 20th gestational week. This means that the human immune system comes of age in an environment made up almost solely of self-antigens. To prevent activation of T cells, and the possible generation of an autoimmune response, the developing human lymph nodes contain an unusually high proportion of regulatory T cells. These cells get activated in the fetal lymph nodes, and subsequently prevent activation of T cells by (self) antigens[70,71].

Lymph node development in man

The exact number of lymph nodes in the human body remains unknown, but is estimated to be as much as 400-600[72]. Most of our knowledge on human lymphoid organ development stems from a series of pioneering studies performed in the 1970's and 1980's, employing light and electron microscopy to study morphology of the developing organ and its cellular constituents as well as immunostainings to identify these cells.

Formation of lymph nodes and lymph vessels in man is initiated, as it is in mice, by the budding of endothelial cells from the jugular and cardinal veins, forming a lymph sac[73,74]. At approximately 8-11 weeks gestation, the first lymph node anlage, or primordium, is formed when mesenchymal cells that condensed at the base of the lymph sac, subsequently invaginate into the lymph sac, moving the endothelium inwards[75,76]. Besides mesenchyme, the tissue invading the lymph sac contains capillaries, vascular loops, fibroblastic reticulum cells (FRCs) and extracellular matrix[75,76]. By 13 weeks gestation, the mesenchyme fills nearly the whole lymph sac, while the remaining space between the endothelial lining of the lymph sac and mesenchymal core gives rise to the marginal sinus. In addition, the number of capillaries increases and intermediate sinuses begin to develop. The outer side of the marginal sinus is surrounded by connective tissue which will eventually form the capsule of the lymph node[75,76].Lymph node generation in mice is temporally controlled by the need for ligation of the LTβR during a distinct window in

time. By blocking lymphotoxin signaling in-utero, Rennert et al. were able to show that murine lymph nodes develop in sequence, roughly head to tail, with brachial nodes being among the earliest formed, followed by axillary, inguinal, and popliteal[13]. In humans, similar observations were made by Kyriazis and Esterly, who performed a study in which 50 fetal specimens were analyzed[77]. The gestational age of each specimen in this study was estimated from the crown-rump length and the maternal menstrual history. The first peripheral lymph node structures that were found were located in the cervical and retroperitoneal regions of the embryo in specimens of approximately 8 weeks gestation (measuring 2.5 to 4.0 cm). In the next group of specimens measuring 4 to 6 cm (approximately 9 to 10 weeks gestation), peribronchial, mediastinal, celiac, pelvic, axillary, inguinal and popliteal nodes were detectable. Omental and mesenteric lymph nodes were only found in the oldest samples analyzed, measuring 10-14 cm (approximately 18 weeks gestation)[77]. While this study was performed using light microscopy, and small lymph node anlagen could obviously have been missed (mesenteric lymph nodes can be found at 13 weeks gestation ([70] and our unpublished data), the overall picture is very similar to that distilled from experimental data in the mouse. It is therefore very well possible that in humans a similar temporal control of lymph node development exists as in mice. Whether such control is also regulated by LTβR signaling is still undetermined.

Development of the lymphoid architecture

From the 12th week gestation onward, when T cells start to flood the periphery, the number of circulatory cells that enter the lymph node increases rapidly and includes, besides lymphocytes also cells from the erythropoietic and granulopoietic lineages (Figure 1). As a result of this influx the lymph node grows in size considerably [76-78]. At 14 weeks, CD4$^+$ T cells are scattered throughout the axillary lymph node anlage, but CD8$^+$ cells are scarce[78]. At the same time, B cells are found mainly in the outer cortex, although not yet arranged in lymphoid follicles. During subsequent weeks, loosely aggregated primary follicles (PF) emerge[78,79]. These PF also contain precursors of follicular dendritic cells, as indicated by their expression of CAN.42 and Ki-Mp4[80]. However, CD35 and CD21, hallmarks of functional FDCs, are still absent [78]. Clearly demarcated B cell follicles are found from 17 to 22 weeks onward. Coinciding with the appearance of these densely packed follicles is the expression of CD35 and CD21 on FDCs[79,80].

Similar kinetics are seen when the non-hematopoietic cells in the lymph node were analyzed. Using light and electron microscopy, precursors of interdigitating cells (IDCs), identified based on morphology, are mainly located near the marginal sinus of the axillary lymph nodes analyzed[76]. During the 2nd trimester, precursors of IDCs become more differentiated and are located in the paracortical region[76]. From the 16th week gestation, the first fibroblastic reticulum cells are found in the paracortical regions of the lymph node, marking the formation of a T cell zone.

The demarcation of cortex and medulla become more distinct with the increase in gestational weeks. As observed by light and electron microscopy, the marginal, intermediate and medullary sinuses become more developed[76]. Compared to the cortex, the medulla has a looser structure of mainly mesenchymal cells and contains more hematopoietic cells. The T cell regions are located

in the cortex with almost fully differentiated IDCs[76,79] In the B-cell regions, primary follicles are clearly defined but contain no germinal centers (GCs)[76,77,79]. This will occur in the first postnatal weeks till months, when their formation is driven by antigenic stimuli[81].

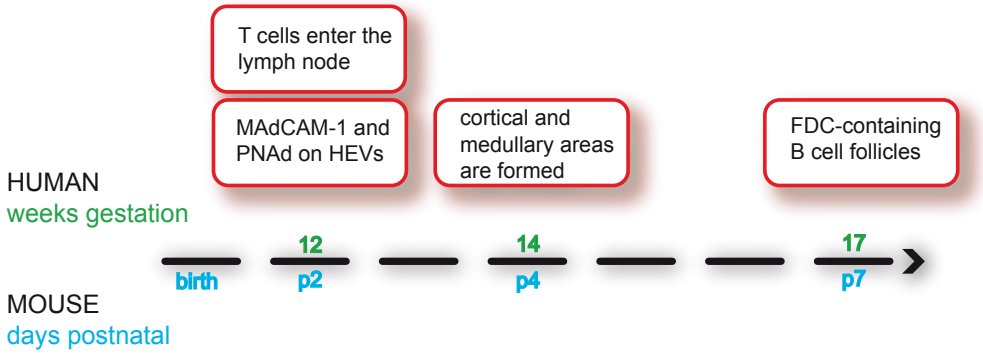

Figure 3. Temporal alignment of lymphoid architecture development in mouse and man. T cells leave the thymus and enter the periphery at day 2 postnatal in the mouse, and by week 12 gestation in humans. By day 4 postnatal, or week 14 gestation, distinct T and B cell areas are visible, without B cell follicles. Formation of FDC-containing follicles is completed in mouse and man by day 7 postnatally or week 17 gestation respectively. The dates given for human are averages and will vary between individuals as well as between lymph node locations.

Adressins regulating fetal lymph node homing

To facilitate the interactions between circulating lymphocytes and the local stroma that will drive lymph node development, the correct cells need to be recruited from the circulation at the right time. To this end, expression of addressins on HEVs is crucial. In adult mice, theperipheral node addressin PNAd is expressed on HEVs in all peripheral lymph nodes[82]. PNAd is the ligand for the homing receptor L-selectin, which is expressed on the vast majority of circulating lymphocytes. On the other hand, lymph nodes draining the mucosa, as well as the Peyer's patches express MAdCAM-1, the ligand for α4β7 on their HEVs resulting in the selective homing of α4β7 positive cells to the mucosal lymphoid organs[83]. In contrast, during fetal murine development HEVs in both peripheral and mesenteric lymph nodes exclusively express MAdCAM-1. At this time, 40-70% of circulating cells expresses L-selectin, and only about 1-2% is α4β7 positive. As a result, this minor portion of fetal α4β7 positive cells, including murine LTic, is selectively recruited to the developing lymph nodes and Peyer's patches[84]. From approximately 24 hours after birth onward, PNAds start to be synthesized and are expressed on HEVs in both peripheral and mesenteric nodes[85,86]. After the second postnatal week, peripheral MAdCAM-1 expression starts to decline and will eventually only be present in the mucosa draining lymphoid tissues.

In the developing lymph nodes of the human fetus, the first HEVs can be identified at 15 weeks gestation[87]. In contrast to the mouse, both peripheral and mesenteric nodes co-express PNAd and MAdCAM-1 on their HEVs prenatally and this is maintained throughout fetal development. Postnatally, the number of HEVs expressing PNAd in peripheral lymph nodes keeps increasing for several years. Similar to mouse development, MAdCAM-1 positive HEVs gradually disap-

pear from the peripheral lymph nodes after birth, although MAdCAM-1 positive vessels are occasionally found in adults[87]. Even though the fetal co-expression of peripheral and mucosal addressins in humans is different than in mice, it needs to be determined whether this leads to functional differences in colonization of the developing lymph nodes. Thymic-derived αβ-T cells leave the mouse thymus at 2 days after birth and the human thymus at week 12[68,69]. In both cases, when reaching the developing lymph node, these cells will encounter HEVs decorated with MAdCAM-1 and PNAd and in both cases they will be able to enter the organ using either α4β7 or L-selectin. However, the most striking difference with mouse development is the promiscuous expression of MAdCAM-1 in embryonic and fetal lymphoid and non-lymphoid tissues. From 7 weeks onward, this addressin is present on endothelial cells in the pancreas, gallbladder, kidney, skin and intestines, as well as in muscle tissue. In the intestines MAdCAM-1 expression outside of the lymphoid areas is of such prominence, that, based on addressin expression, the developing Peyer's patches cannot be distinguished from the surrounding tissue[87]. Non-lymphoid organ-restricted MAdCAM-1 expression peaks around week 20-23 and subsequently disappears quite rapidly. During the second trimester, MAdCAM-1 is also transiently expressed in the spleen and thymus[87]. It is at present unclear whether the transient broad expression of this addressin has functional implications in terms of hematopoietic cell migration to these non-lymphoid organs. However, during human fetal development, it is not uncommon to find transient lymphoid infiltrates containing distinct T and B cell areas in organs such as the pancreas and lung[88,89].

Signaling pathways driving human lymph node development

In the mouse, signaling cascades downstream of the IL7R (consisting of the IL7Rα chain and the IL2Rβ or common-γ-chain) were shown to be instrumental in regulating surface expression of lymphotoxin on LTic and to control their proliferation[16,90]. As a consequence, IL7 receptor signaling is essential for murine lymph node-development. Mice with defects in this signaling route, including deficiencies for the common-γ-chain or Jak3, have a severe impairment of lymph node development[27-29]. Interestingly, one of the very few clues on signaling cascades essential for human lymph node development also implicate the common-γ-chain/Jak3 pathway as essential. Upon postmortem examination of 46 patients who died as a result of various Severe Combined Immunodeficiency Diseases (SCIDs), patients who had suffered from SCID due to mutations in the common-γ-chain (Xq13.1) or Jak3 (19p13.1) presented with an absence of lymph nodes[91]. In addition, one patient not having had either common-γ-chain or Jak3 mutations also had no detectable lymph nodes. The underlying mutation in this patient has however not been determined. These findings imply that signaling via the common-γ-chain and Jak3 is also in humans indispensable for normal lymph node development. Recent work has shown that IL7R mutations are the third most common cause of SCID in the United States[92] and therefore the possibility remains that the un-typed lymph node-deficient SCID patient described by Fachetti et al. indeed harbored such a mutation. In addition, Vonarbourg and colleagues found that in patients with an IL7R deficiency IL-22 producing $CD117^{+}RORc^{+}$ were virtually absent[93].

Peyer's patch development in man

The fetal human small intestine contains on average 60 Peyer's patches before 30 weeks gestation, but their number steadily increases to reach an astonishing average of almost 240 at puberty[94]. To our best knowledge the earliest study on the formation of lymphoid organs in the human intestine dates back to 1882, when Adolf Baginsky described the presence of clusters of "nice round cells" in the sub-mucosa of the terminal ileum of a 4 month old fetus[95]. Detailed analysis of fetal human small intestines by light microscopy led several groups to confirm and extend Baginskys original observation with the identification of clearly distinct clusters of T and B cells in the small intestine at 14-16 weeks gestation[89,94,96-98]. By week 19, these aggregates have matured into clearly recognizable Peyer's patches containing FDCs, although no germinal centers are present. The latter rapidly develop after birth, when the intestines become colonized by commensal bacteria. It is not until 24 weeks gestation that the Peyer's patches have sufficiently increased in size to become discernable macroscopically[94].

Spleen development

Development of the spleen differs in the requirement for LT signals compared to lymph node development. Mice deficient for Rorγt and/or Id2, lack LTi cells and no LNs and PPs develop. However, these mice do have spleens with segregated B and T cell compartments[103,104]. The specific role of LTi cells in the development of the lymphoid tissue of the spleen, the white pulp (WP), is still not fully elucidated. Data show the lack of requirement of lymphotoxin (signaling) for a proper development of the WP is mostly obtained in spleens from newborn mice from day 4 and onwards in which B cells that express lymphotoxin colonize the spleen[105]. Lymphotoxin^{+} B cells are capable of inducing CCL21 production by resident stromal cells to induce the development of the T zone, the PALS, prior to B cell follicle formation. RAG$^{-/-}$ mice lack B and T lymphocytes and have very low expression of CXCL13 and CCL21 which is necessary for T and B cell segregation[106]. However, grafting experiments of E 15 LTα$^{-/-}$ spleens in adult RAG$^{-/-}$ hosts followed by transfer of LTα$^{-/-}$ splenocytes revealed no requirement of lymphocyte derived LTα in the induction of CCL21[107]. These data suggest that interactions between adult LTi cells and embryonic stromal cells occur. More recent data show that LTi cells in the murine neo-natal spleen do not express membrane-bound lymphotoxin and that segregation of the RP and WP is primed in a LT-independent manner before E 15.5[108].

The development of the human fetal spleen has been characterized by performing electron microscopy and immunohistochemistry on paraffin embedded fetal spleens. Together, these studies give an overview of the cellular hematopoietic and non-hematopoietic organization in fetal spleens varying from the 11th gestational week (gw.) until newborn children[109-112]. However, no study has touched upon the presence of stromal LTo-like cells in WP development and the interaction of stromal cell subsets with B and T cells and LTi cells. Whether stromal LTo(-like) cells are present in the developing human spleen and if they play a role in the development of the WP is currently unknown (see chapter 5 of this thesis).

Models to study human lymphoid organ development

In addition to detailed observational studies on human fetal tissues of sequential stages, which were discussed in previous sections, few techniques have been developed that could allow functional studies on human lymph nodes. The most promising technique may very well be transplantation of individual human fetal lymph nodes in immuno-deficient mice. Several groups have shown that this is technically possible, without overt signs of rejection in the SCID hu model either combined with thymus and liver or as solitary lymph node transplant[113,114]. Depending on the pretreatment regiment, lymph nodes were shown to grow extensively, while maintaining their original architecture[114]. In an independent model, co-transplantation of human fetal bronchial tissue and the peribronchial lymph nodes in SCID mice, led to increased survival of the lymph node, again with preservation of T and B cell areas[115]. In this study, the authors speculate that similar co-transplantation approaches may also work for other mucosal tissues such as the intestines. If proven correct, such techniques could allow for the in-vivo monitoring of lymph node and Peyer's patch development. Finally, γc/RAG2-deficient mice reconstituted with human fetal liver or cord blood stem cells[116,117] could be a suitable model to study organization of human lymphocytes within the few endogenous murine lymph nodes still present in these animals. Using this system, the development of a near normal splenic architecture was described, although no data exist on lymph nodes[117].

Lymphoid malignancies recapitulating early lymphoid organogenesis

Tightly regulated bi-directional interactions between hematopoietic cells and local stromal cells are at the basis of lymphoid organogenesis[8,118]. Lymphotoxin expressed on LTic induces chemokines, cytokines and adhesion molecules in stromal cells, which in turn regulate retention, survival and proliferation of LTic. At later stages, in the postnatal lymph node, B cell derived signals maintain the B cell follicle through interactions with FDCs and the local mesenchyme[119,120]. Similar interactions are known to exist between malignant lymphocytes and their microenvironment. This is especially apparent for low grade B cell malignancies like chronic lymphocytic leukemia (CLL) and follicular lymphoma (FL) that originate from the lymph node[121]. Where high grade B cell malignancies are characterized by intrinsic abnormalities in proliferation and or differentiation, the low-grade malignancies often present with survival abnormalities that are in part mediated by external signals. CLL cells will go in apoptosis in vitro, unless cultured in the presence of IL-4 or IFN- α or γ[122-124]. However, culturing CLL cells in the presence of stromal cells from lymph node or bone marrow, rescues the leukemic cells from apoptosis and supports their survival[125,126]. CLL cells, together with $CD4^+$ T-cells, are present in a nodular fashion surrounding FDCs in lymph nodes and induce similar structures de-novo upon spreading to the bone marrow.[127,128]. Furthermore, CD40 expression is up regulated in many cases of CLL compared to healthy donors[5]. A pivotal role of cross-talk between the stromal microenvironment and the B cell tumor was also shown for FL. The role of the microenvironment as a prognostic factor for FL progression has gained appreciation[129-135]. FL cells have a close association with FDCs[136], as well as with the local stroma in the B cell follicle of the lymph node. Upon spreading to the

bone marrow, FL cells also arrange in follicular-like structures, inducing FDCs from the bone marrow-mesenchyme.[5]. Thomazy and colleagues (2003) used transglutaminase as a marker of the extracellular reticulum network and found it to be up regulated in areas surrounding FL nodules[137]. In addition to this, BM biopsies of FL patients show high expression of CD40 and VCAM-1 by adventitial reticulum cells[5]. Recent work has shown that FL cells arising in the LN depend on interactions with FRCs for their survival[138]. Strikingly, upon spreading to the BM, FL cells can locally induce the differentiation of BM-mesenchymal cells into LN-like FRCs, a process that can be mimicked in-vitro[139]. These in-vitro experiments indicate an involvement of Lymphotoxin and TNFα in this process, in line with the role of these molecules in normal lymph node development. In essence, both FL and CLL are mimicking normal development and differentiation of lymph node stromal cells in order to create a lymph node-like environment in the bone marrow that will ensure their survival. This suggests that remodeling of the non-malignant microenvironment in the bone marrow by malignant B cells might occur along similar lines as the generation of lymph node stromal cells during embryogenesis. Elucidating the molecular pathways involved in human lymph node development might therefore generate knowledge that will open up the non-malignant stromal microenvironment of the lymph nodes and the bone marrow as novel therapeutic targets in low-grade B cell malignancies.

Aim and outline of this thesis

During the second half of the last century, a substantial amount of knowledge has been gathered on the morphological changes in developing human lymph nodes during embryonic and fetal development **(chapter 1)**. Overall, human and mouse lymph nodes seem to develop via similar schemes, both during early organogenesis as well as during the generation of the lymphoid architecture. The major difference is that lymphoid architecture forms prenatally in humans, yet postnatally in mice (Figure 1). While human LTic in fetal LNs and intestine have been described, knowledge on stromal organizers in humans is very poor. Observations made in SCID patients indicate the common-γ-chain/Jak3 as essential for human lymph node development. Determining which of the common-γ-chain cytokines is responsible for the lack of lymph nodes in these patients is of great interest. Finally, basic knowledge on the early events in human lymph node development could be a first step towards an understanding of the manipulation of the stromal environment by malignant lymphocytes. The fact that impaired crosstalk between stromal cells and lymphocytes can lead to disturbed lymph node development in mice and presumably man, suggests that targeting the mesenchyme with which malignant cells interact could be a potential mechanism to restrain survival of malignant cells. The overall aim of the studies described in this thesis is to identify and characterize the cellular and molecular requirements for lymph node development in humans.

Human LTic were identified in fetal LNs by Cupedo et al. in 2009 and belong to the family of RORC$^+$ innate lymphoid cells (ILC) that play various roles in lymphoid tissue remodeling and innate and adaptive immune responses. In fetal LN and inflamed intestines RORC$^+$ ILC contain transcripts for IL-17 while these cells in mucosal tissues like tonsils and adult intestines

are producers of IL-22. The analysis of a potential role for RORC$^+$ ILC derived cytokines in homeostasis and disease is hampered by a poor characterization of cellular subsets and a lack of knowledge on the distribution of these cells in adults. We hypothesized the presence of subsets of RORC$^+$ ILC with distinct phenotypes and functions in fetal and adult lymphoid and mucosal tissues. In **chapter 2** we conduct a detailed analysis of RORC$^+$ ILC isolated from fetal and adult lymph nodes, intestines and tonsils in order to characterize functional cell subsets within the overall RORC$^+$ ILC population. We analyze by flow cytometry the differential expression of markers known from literature to be expressed by RORC$^+$ ILC. Next, we determine the cytokine producing capacity of various RORC+ ILC subsets in order to designate functional subsets.

As described in the previous section, human RORC$^+$ ILC are present in fetal and adult tissues however knowledge on stromal niches of these cells is very poor. In developing murine lymph nodes LTic interact directly with LTo cells. The latter being a distinct stromal cell subset that upon activation expresses RANKL, the adhesion molecules MAdCAM-1, VCAM-1 and produces both CXCL13 and CCL21. In 2008, Katakai and colleagues described in murine adult lymphoid tissues the Marginal Reticular Cells (MRC). This stromal cells subsets presents with a phenotype equivalent to LTo cells. The presence of MRC in human lymph nodes remains elusive. In **chapter 4** we analyze human fetal and adult lymph nodes for the stromal niches of RORC$^+$ ILC. We hypothesized that if MRC reside in human fetal and adult LNs, RORC$^+$ ILC might colocalize with this stromal subset. Therefore, we analyze frozen sections of fetal and adult lymph nodes for the localization of RORC$^+$ ILC. Subsequently we analyze the niches of these cells for the presence of stromal cells with an LTo / MRC-like phenotype. In addition, we investigate the gene expression profile of the stromal cell subset of the RORC+ ILC niche in adult LNs.

Since Katakai et al. identified MRC in all murine developmentally programmed lymphoid organs we set out to investigate the presence of MRC in human fetal spleens in **chapter 5.** Here, we analyze the developing white pulp of fetal spleens varying from 15 to 20 week for the presence stromal cells with a MRC-like phenotype. In addition, we determine whether the gene expression profile of the putative MRC is reminiscent of murine LTo cells. We also investigate whether RORC$^+$ ILC with the putative MRC.

While murine lymph node development has been characterized extensively, the molecular and cellular mechanisms involved in B cell follicle formation and ectopic germinal center formation are poorly understood. Follicular lymphoma (FL) cells are malignant germinal center (GC) B cells which have the capacity to remodel bone marrow (BM) in such a way it resembles a GC. The GC-like structure induced by FL cells in the BM is now capable of providing the FL cells with signals essential for survival. In **chapter 3** we set out to study the interactions between FL cells and BM stromal which eventually lead to the formation of a ectopic GC-like structure. Therefore, we set up and analyze a 3-D co-culture system conducted with cell line WSU-FSCCL en fetal BM derived stromal cells in order to create an *in vitro* model that can be applied for FL cell induced BM remodeling. To study at early stages the behavior of ectopic FL cells in an environment which can be manipulated we use an *in vivo* xenograft model in RAG-2$^{-/-}\gamma c^{-/-}$ mice as experimental model. Furthermore, we investigate the genetic alterations induced in the non-hematopoietic

compartment of lymph nodes upon entry of B cells. We inoculate RAG-$1^{-/-}$ mice for seven days with B cells and isolate and subsequently analyze by micro-array the non-hematopoietic compartment of isolated lymph nodes.

Chapter 6 discusses the conclusions of the previous chapters from a broader and translational perspective.

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FUNCTIONAL DIFFERENCES BETWEEN HUMAN NKP44^{-} AND NKP44^{+} RORC^{+} INNATE LYMPHOID CELLS

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ABSTRACT

Human RORC$^+$ lymphoid tissue inducer cells are part of a rapidly expanding family of innate lymphoid cells (ILC) that participate in innate and adaptive immune responses as well as in lymphoid tissue (re) modeling. The assessment of a potential role for innate lymphocyte-derived cytokines in human homeostasis and disease is hampered by a poor characterization of RORC$^+$ innate cell subsets and a lack of knowledge on the distribution of these cells in adults. Here we show that functionally distinct subsets of human RORC$^+$ innate lymphoid cells are enriched for secretion of IL-17a or IL-22. Both subsets have an activated phenotype and can be distinguished based on the presence or absence of the natural cytotoxicity receptor NKp44. NKp44$^+$ IL-22 producing cells are present in tonsils while NKp44$^-$ IL-17a producing cells are present in fetal developing lymph nodes. Development of human intestinal NKp44$^+$ ILC is a programmed event that is independent of bacterial colonization and these cells colonize the fetal intestine during the first trimester. In the adult intestine, NKp44$^+$ ILC are the main ILC subset producing IL-22. NKp44$^-$ ILC remain present throughout adulthood in peripheral non-inflamed lymph nodes as resting, non-cytokine producing cells. However, upon stimulation lymph node ILC can swiftly initiate cytokine transcription suggesting that secondary human lymphoid organs may function as a reservoir for innate lymphoid cells capable of participating in inflammatory responses.

INTRODUCTION

There is an increasing awareness that early innate response involve several types of RORC^{+} and RORC^{-} lymphoid-like cells that combine aspects of innate and adaptive immunity including the production of cytokines traditionally associated with adaptive immune cells. These innate cells are collectively referred to as innate lymphocytes (ILC) and are on the one hand important in early immunity to pathogenic bacteria and helminthes as well as tissue integrity after infection, while these cells on the other hand can be part of pathology during experimental colitis and are specifically expanding in Crohn's disease patients and allergic rhinosinusitis patients [1-7]

The best studied population of RORC^{+} ILC are lymphoid tissue inducer cells (LTi cells) that are present in secondary lymphoid organs and whose prime function is to control fetal development of lymph nodes and Peyer's patches in mouse and man [8,9]. Even though lymphoid organogenesis occurs in utero, cells that phenotypically resemble LTi cells have been found in adult mice [10,11]. Similar to fetal LTi cells, adult cells express molecules involved in LN development such as lymphotoxin$\alpha 1\beta 2$, (LT) and TNFα and depend on expression of Rorγt and Id2 for their development [11-14].

Although it remains to be determined to what extent adult LTi cells are similar to their fetal counterparts in terms of the ability to induce lymphoid organs, adult LTi cells were shown to have additional functions in the support of adaptive immune responses by facilitating memory T cell generation in the spleen [15,16]. In humans, ILC that resemble the mouse LTi cells are present in developing fetal lymph nodes [8]. In adult mice and humans, RORC^{+} ILC are found in secondary lymphoid organs, in the skin and in the intestines. In mice, RORC^{+} ILC are essential for the first wave of defense against attaching and effacing intestinal pathogens like *Citrobacter rodentium* [4,17]. IL-22 produced by ILC activates the epithelium via the IL-22 receptor to produce antimicrobial products [4,17,18]. In humans, several mucosal ILC subsets that produce IL-22 were described. We have previously identified a population of CD56^{+}RORC^{+} ILC in the tonsil that constitutively produce IL-22 [8]. At the same time, a population of NKp44^{+}RORC^{+} ILC were described in tonsils, Peyer's patches and small intestines and named NK22 [17]. Clonal analysis of tonsil-derived RORC^{+} ILC has since shown that CD56^{+}RORC^{+} ILC and NKp44+ NK22 cells are the same population of ILC [19].

NKp44 belongs to a family of activating receptors found on human NK cells. These receptors are collectively termed Natural Cytotoxicity Receptors (NCRs) and next to NKp44 include NKp30 and NKp46 [20]. All three are Ig-like transmembrane receptors that convey activating signals to NK cells. NKp46 and NKp30 are expressed constitutively, while expression of NKp44 is induced upon NK cell activation. NKp46 is also expressed on mouse NK cells, as well as on mouse ILC in the intestines. NKp30 and NKp44 are not expressed in Mus. Musculus [20,21].

Besides production of IL-22, RORC^{+} ILC in mice and man have been reported to produce IL-17a. In man, RORC^{+} ILC in fetal lymph nodes [8] and inflamed intestines [22] contain IL-17a transcripts and in mice, stimulated splenic RORC^{+} ILC and RORC^{+} ILC from the small intestines were shown to produce IL-17a [23,24].

In sum, RORC$^+$ ILC from tonsils and fetal lymph nodes differ strikingly in cytokine production: tonsil ILC produce IL-22 while fetal lymph node ILC contain only transcripts for IL-17a [8]. Whether in vivo IL-17 and IL-22 are produced by the same population of cells or by different ILC populations is still subject to debate [25,26]. Clarifying the cellular sources of these functionally distinct cytokines will increase our understanding of ILC biology and activation. In this report we determined the cellular source of ILC-derived IL-17a and IL-22 and show that these cytokines are preferentially transcribed by distinct subsets of ILC located in peripheral or mucosal lymphoid organs respectively.

RESULTS

Differential NCR expression on tonsil- and fetal LN-derived ILC

We hypothesized that expression of Natural Cytotoxicity Receptors could be used to distinguish ILC subpopulations biased towards production of either IL-17a or IL-22. To test this we first compared the ex-vivo expression of NKp30, NKp44 and NKp46 on freshly isolated human ILC from fetal lymph nodes versus tonsils. The following phenotypic definition was used for ILC: lineage (CD3; CD19; CD14; CD34) negative cells expressing intermediate levels of CD45 and high levels of both CD127 (IL7Rα) and CD117 (c-Kit). Cells within this gate were uniformly RORC positive **(Figure 1a)**. In fetal lymph nodes, the majority of RORC$^+$ ILC lacked expression of NKp46 **(Figure 1b)**.

Approximately half of the cells expressed NKp30 and a very small fraction of these NKp30$^+$ ILC co-expressed NKp44 **(Figure 1b)**. In contrast, the majority of tonsil-derived RORC$^+$ ILC expressed NKp44 as well as NKp30. In addition, a substantial fraction of tonsil RORC$^+$ ILC expressed NKp46 **(Figure 1b)**. The biggest differences in NCR expression between fetal lymph node and tonsils were observed for NKp44 and upon enumeration the percentage of NKp44-expressing RORC$^+$ ILC was indeed significantly increased in tonsils compared to fetal lymph nodes **(Figure 1c)**. These data show that the relative distribution of NCR-positive versus NCR-negative ILC is different between tonsils and fetal lymph nodes and follows the pattern described for IL-22 [8].

Next, we assessed the proportion of CD56 expressing ILC in fetal lymph nodes and tonsils. The percentage of CD56$^+$RORC$^+$ ILC was low in fetal lymph nodes, but was increased in tonsils where on average half of the RORC$^+$ ILC expressed CD56 **(Figure 2a)**. This implies that CD56 expression follows a pattern similar but not identical to NKp44, and indeed in tonsils only half of the NKp44$^+$ ILC population expressed CD56 **(Figure 2b)**. In summary, NKp44, and not CD56, is the most prominent cell surface marker differentiating between RORC$^+$ ILC from fetal lymph nodes and adult tonsils.

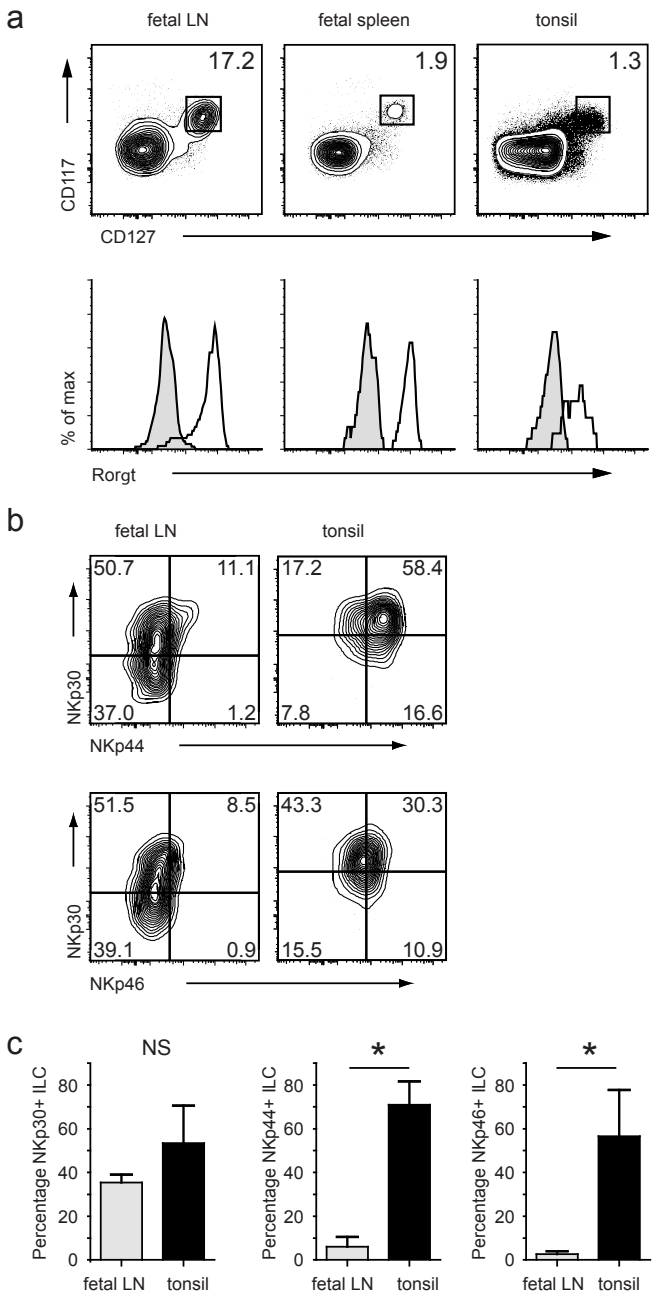

Figure 1. NCR expression on ILC from fetal LN and tonsil

a. Lineage-negative cells in fetal lymph nodes, fetal spleen and tonsil labeled for CD117 and CD127 are RORC positive. Shaded histogram indicates isotype control staining (representative example of 3 independent experiments). **b.** Flow cytometric analysis of NKp30 ($p = 0.07$), NKp44 ($p = 0.00$) and NKp46 ($p = 0.03$) expression on ILC and fetal lymph nodes and tonsil **c.** Percentage of NKp30, NKp44 and NKp46 expressing RORC⁺ ILC in fetal lymph nodes and tonsil (n>5; Average +/- SD.)

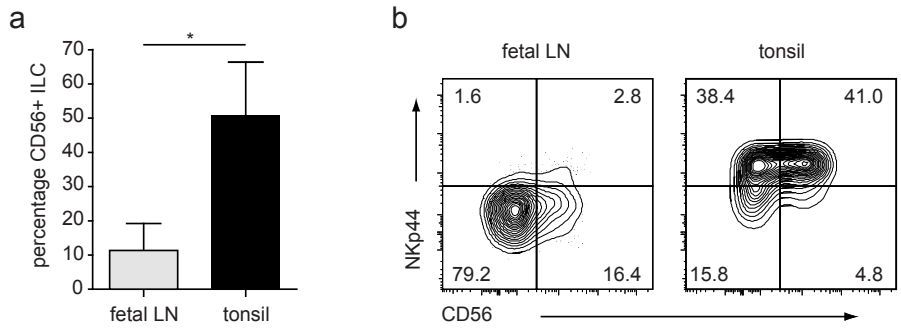

Figure 2. CD56 expression on ILC from fetal LN and tonsil

a. Percentage of CD56 expressing RORC$^+$ ILC in fetal lymph nodes and tonsil (n > 5; Av +/- SD, p = 0.007) **b.** Flow cytometric analysis of CD56 and NKp44 expression on RORC$^+$ ILC in fetal lymph nodes and tonsil. (representative example of 5 independent experiments)

NKp44 expression allows identification of IL17 and IL22 biased ILC

On NK cells, NKp44 is only expressed upon activation. In addition, production of IL-17a in T cells is restricted to activated or memory cells [27]. This led us to hypothesize that ILC which contain transcripts for either *IL17A* or *IL22* might also be cells with an activated phenotype. To test this hypothesis, we analyzed the expression of surface proteins known to be regulated during cellular activation on RORC$^+$ ILC cells from fetal lymph nodes and tonsils. The early activation marker CD69 was expressed on part of RORC$^+$ ILC, and the combination of CD69 with NKp44 revealed discrete ILC subpopulations **(Figure 3a)**. In fetal lymph nodes, where the majority of cells lack expression of NKp44, CD69 divided the ILC population into a CD69$^-$ and a CD69$^+$ population. In tonsils, NKp44$^+$ ILC were most abundant, and all NKp44$^+$ cells co-expressed CD69. In addition, a small population of CD69$^+$NKp44$^-$ cells was present **(Figure 3a)**.

We next set out to determine the presence of *IL22* and *IL17A* transcripts in these ILC subpopulations. To this end, the NKp44$^-$ ILC were sorted from fetal lymph nodes and separated in CD69$^-$ and CD69$^+$ populations. From tonsils, NKp44$^+$ ILC were purified and divided in CD69$^-$ and CD69$^+$ fractions. Transcripts for *IL17A* and *IL22* were determined without ex-vivo stimulation.Within the fetal lymph nodes, *IL17A* transcripts were enriched within the CD69$^+$NKp44$^-$ population **(Figure 3b, top right)**. *IL22* transcripts were very low to absent in the fetal lymph nodes **(Figure 3b, top left)**. In tonsil RORC$^+$ ILC, *IL22* transcripts were easily detectable within the CD69$^+$NKp44$^+$ population as expected [17] but were much lower in the NKp44$^-$ population **(Figure 3b, bottom left)**. Transcripts for *IL17A* were undetectable in tonsil ILC **(Figure 3b, bottom right)**.

In order to assess whether the divergent cytokine patterns observed directly ex-vivo are maintained after in-vitro stimulation we isolated tonsil NKp44$^+$ and NKp44$^-$ ILC and stimulated these overnight with PMA and ionomycin. Similar to freshly isolated ILC, overnight stimulation induces IL-22 secretion from NKp44$^+$ ILC, but not NKp44$^-$ ILC **(Figure 3c)**, confirming the cytokine bias seen directly ex-vivo. The*IL22* induction in NKp44$^+$ ILC by PMA/ionomycin stimulation was observed in all donors analyzed, but did however show a high degree of variability.

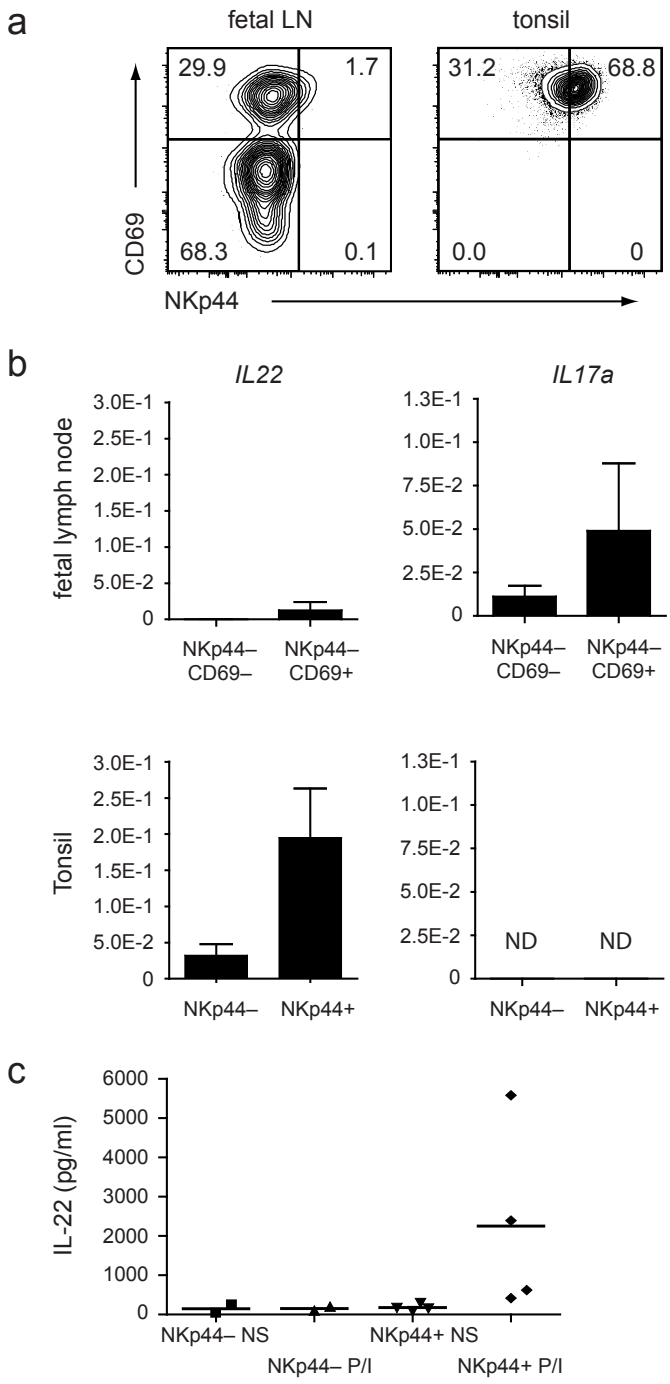

Figure 3. Differential cytokine production by NKp44+ and NKp44⁻ ILC
a. Flow cytometric analysis of CD69 and NKp44 expression on RORC⁺ ILC from fetal LN and tonsil (representative example of 3 independent experiments). **b.** Expression of *IL22* and *IL17A* mRNA in sorted ILC populations from tonsils and fetal LN (Representative of 2 individual experiments with 3 – 5 donors per experiment). **c.** IL-22 protein production by NKp44⁻ and NKp44⁺ ILC from tonsils stimulated overnight with PMA + ionomycin (n=3).

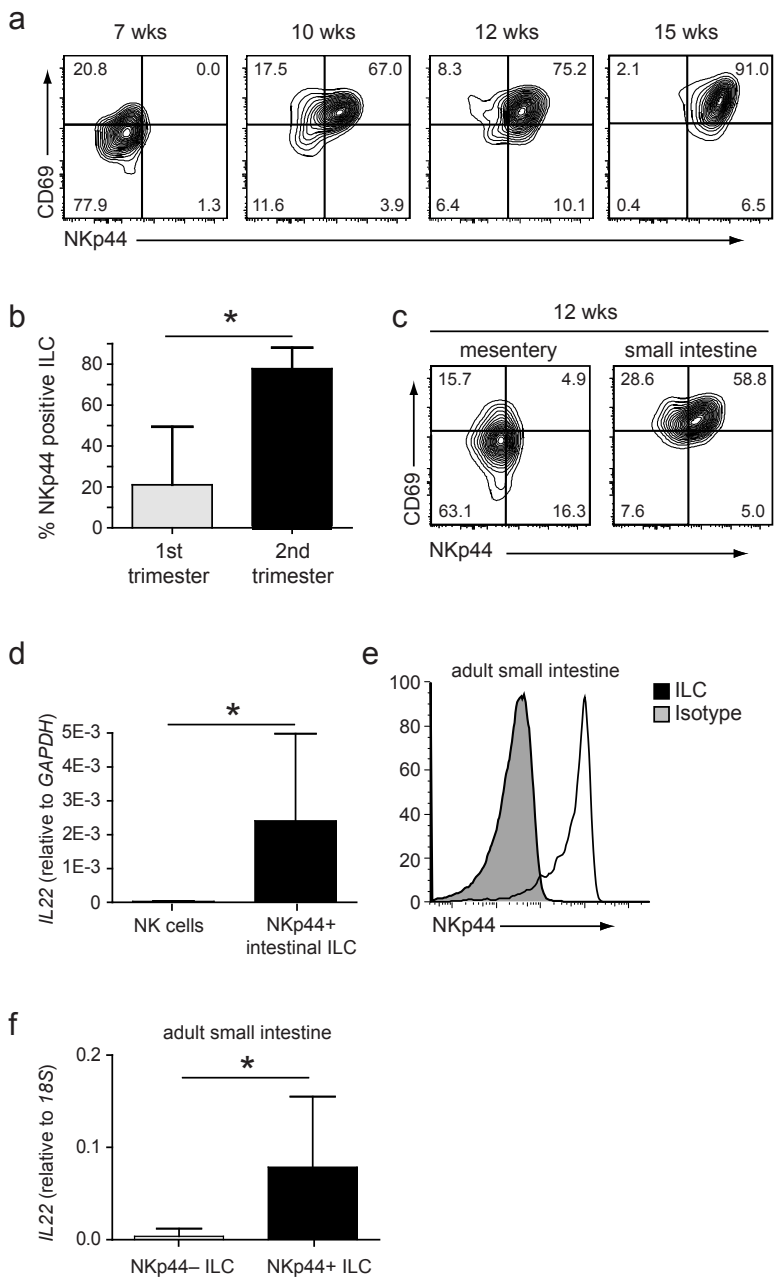

Figure 4. Human NKp44⁺ ILC are developmentally programmed

a. Flow cytometric analysis of ILC from fetal intestines of indicated ages (weeks gestation) (representative examples of at least 3 independent experiments per time point). **b.** percentage of ILC expressing NKp44 in the fetal small intestine during 1st trimester (< 12 wks) or 2nd trimester (>12wks) (n>6; Average +/- SD; p = 0.003). **c.** Flow cytometric analysis of ILC in fetal small intestines and mesentery of the same donor at 12 weeks gestation (representative example of 3 independent experiments). **d.** Expression of *IL22* transcripts in NKp44⁺ ILC from fetal intestine compared to NK cells from fetal lymph node (5 independent samples per condition, Average +/- SD; p = 0.009). **e.** Flow cytometric analysis of NKp44 expression on ILC from adult intestine. Filled histogram shows isotype control staining (representative example of 5 independent experiments). **f.** Expression of *IL22* transcripts in NKp44⁺ and NKp44⁻ ILC from adult intestine (n=7, Average +/- SD; p = 0.005)

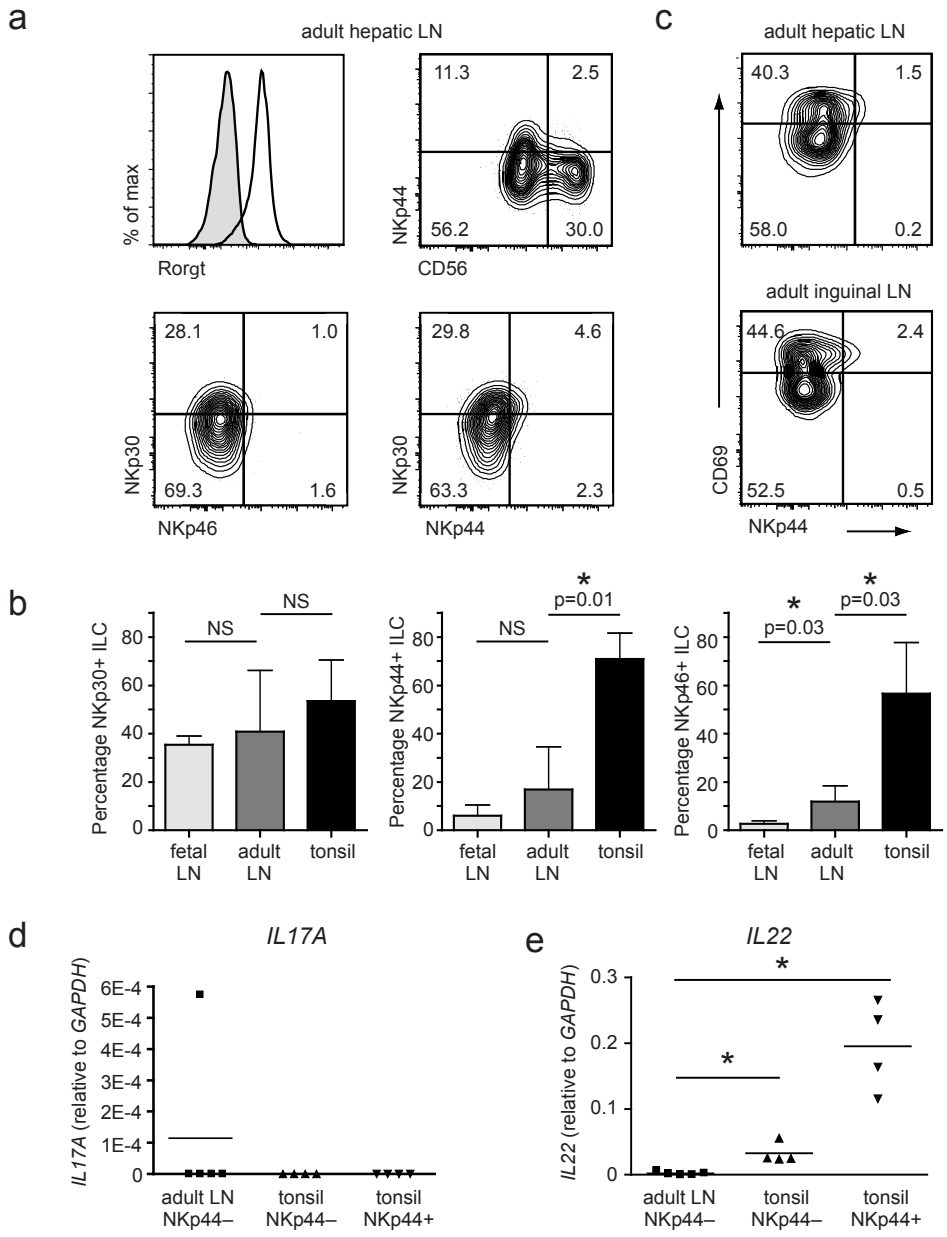

Figure 5. NKp44⁻ ILC in adult non-inflamed lymph nodes

a. Flow cytometric analysis of ILC from adult hepatic lymph nodes for expression of RORC, NKp44, NKp30, NKp46 and CD56 (representative example of 3 independent experiments). **b.** Flow cytometric analysis of CD69 and NKp44 expression on ILC from adult hepatic and inguinal lymph nodes (representative example of at least 3 independent experiments). **c.** Percentage of NKp30, NKp44 and NKp46 expressing RORC⁺ ILC in fetal lymph nodes, adult lymph nodes and tonsil (representative example of at least 3 independent experiments) **d.** PCR analysis for *IL17a* and **e.** *IL22* transcripts of sorted ILC from adult hepatic lymph nodes compared to NKp44⁻ and NKp44⁺ ILC from tonsil (Adult LN vs NKp44⁻ p=0.02; adult LN vs NKp44⁺ p=0.02; n=4).

These findings imply that tonsil-derived NKp44 positive ILC preferentially transcribe IL-22, while IL-17 is preferentially transcribed by fetal lymph node-derived NKp44^{+}CD69^{+} ILC, enriched within the ILC that lack expression of NKp44, but do express CD

Human mucosal NKp44^{+} ILC are developmentally programmed

In the mouse, NKp46^{+} ILC develop in the absence of microbiota**,** and appear in the intestines after birth [5,25], [28]. Since fetal human intestines are not yet colonized by bacteria or exposed to ingested antigens [29-31], this allowed us to assess the development of human fetal NKp44^{+} ILC in the absence of external stimuli. We analyzed ILC in first and second trimester fetal small intestines and determined their expression of CD69 and NKp44. First trimester small intestinal-derived ILC (7 wks gestation) did not express CD69 or NKp44 and resembled putative LTi cells **(Figure 4a)**. CD69^{+}NKp44^{+} ILC appeared during late first trimester, between weeks 7 and 10 of gestation and with increasing age replaced the LTi-like cells. From week 12 onwards, the majority of ILC in the small intestines were expressing both CD69 and NKp44 **(Figure 4b)**. This was specific for the fetal small intestines and was not found in paired mesentery samples containing developing lymph nodes **(Figure 4c)**. Since our analysis of NKp44^{+} ILC from tonsils had shown *IL22* transcription in these cells, we also probed fetal intestine-derived NKp44^{+} ILC for *IL22* transcripts. We consistently found low levels of IL22 transcripts in NKp44^{+} ILC from second trimester fetal small intestines (**figure 4d)**. It was technically impossible to purify sufficient NKp44^{-} ILC from fetal intestines as a comparison.

Therefore, CD56^{+}CD127^{+} NK cells sorted from fetal lymph nodes were used as a negative control. These data show that human NKp44^{+} ILC develop in-utero in the absence of microbial colonization and can be found in the small intestine.

To be able to compare intestinal NKp44^{+} and NKp44^{-} ILC we next isolated these cells from non-affected adult illial mucosa acquired during resection procedures for colon cancer. In the adult human intestine, the majority of ILC express NKp44 **(figure 4e)**. In line with our data from tonsil-derived ILC, intestinal NKp44^{+} ILC preferentially transcribe IL22, while NKp44^{-} ILC only contain very low levels of IL22 trancripts **(figure 4f)**.

Resting ILC are present in adult non-inflamed human lymph nodes

The presence of IL-17 transcribing ILC in fetal human lymph nodes raised the question whether these cells might also be found in adult lymph nodes. Therefore we determined the presence of RORC^{+} ILC in adult peripheral non-inflamed lymph nodes. Lymph nodes were collected during multi-organ donation procedures and were liver-draining hepatic lymph nodes unless stated otherwise. Donor age ranged from 24 — 68 years. In adult lymph nodes a population of Lineage^{-}CD45^{int}CD117^{+}CD127^{+} ILC was found that uniformly expressed RORC **(Figure 5a)**. Based on expression of NCRs, adult LN-derived RORC^{+} ILC resembled ILC from fetal lymph nodes rather then from tonsils in that they were mostly negative for NKp46 and NKp44 and only a part of the cells expressed CD56 and NKp30 **(Figure 5a and 5b)**. There was no difference in the percentage of NKp30 or NKp44 expressing ILC between fetal and adult LN, while slightly more

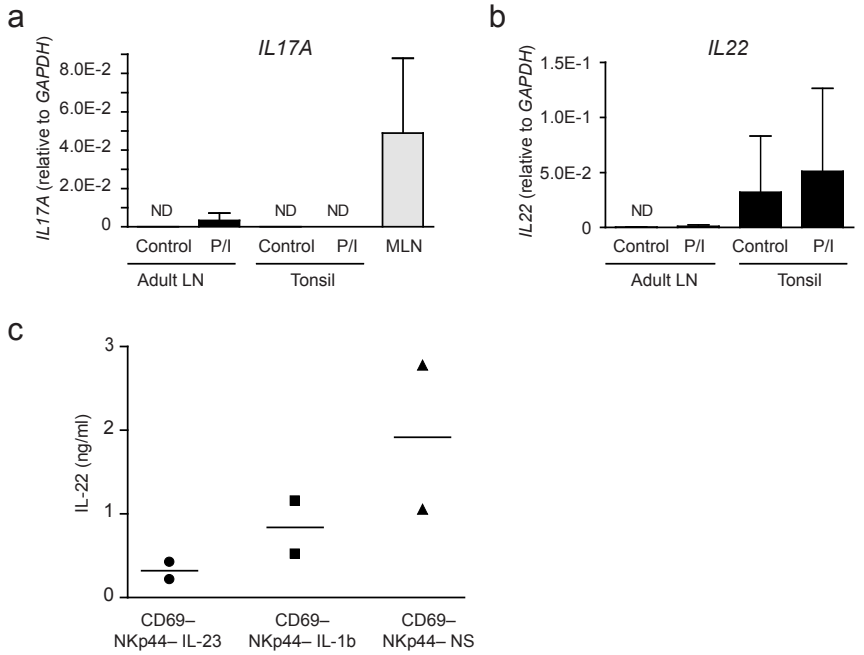

Figure 6. Stimulated adult CD69⁺NKp44⁻ ILC produce IL-22
a. *IL17A* transcripts in NKp44⁻ ILC from adult hepatic lymph nodes and in NKp44⁺ ILC from tonsils stimulated overnight with PMA + ionomycin as well as in freshly isolated fetal LN-derived CD69⁺ ILC (n=3) **b.** *IL22* transcripts NKp44⁻ ILC from adult hepatic lymph nodes and in NKp44⁺ ILC from tonsils stimulated overnight with PMA + ionomycin (n=3) **c.** IL-22 protein production by CD69⁻NKp44⁻ ILC from adult hepatic lymph nodes after 3 day culture with IL-23 or IL-1β (n=2)

adult ILC expressed NKp46 **(figure 5b)**. These data show that NKp30 is the only NCR that is consistently expressed on approximately half of all ILC, regardless of whether they were isolated from fetal LN, adult LN or tonsil.

Upon staining for CD69, the presence of CD69⁺ and CD69⁻ ILC became apparent, very much alike the fetal LN-derived ILC. This phenotype was not restricted to hepatic lymph nodes as a similar distribution of NCR was seen in adult inguinal nodes **(Figure 5c)**. To determine cytokine production by the ILC from adult lymph nodes, ᵗotal NKp44⁻ ILC were purified and analyzed for *IL22* and *IL17A* transcripts directly ex-vivo. Transcript levels for these cytokines were compared to the levels found in NKp44⁻ and NKp44⁺ ILC from tonsil. Strikingly, adult lymph node ILC lacked detectable *IL22* or *IL17A* transcripts **(Figures 5d)**. These data suggest that, in contrast to fetal lymph node-derived ILC, adult non-inflamed lymph node–derived ILC are not actively transcribing detectable levels of *IL17A* in-vivo.

To test whether these peripheral ILC were biased towards the production of either *IL17A* or *IL22* upon activation, we stimulated adult lymph node ILC overnight with PMA and ionomycin **(Figure 6)**. After overnight stimulation we could detect low levels of both *IL17A* and *IL22* transcripts in adult ILC. Induction of IL-17 was specific for adult lymph node ILC as tonsil-derived ILC did not contain *IL17A* transcripts after stimulation **(Figure 6a)**. However, the levels of *IL17A*

transcripts were much lower then those found in freshly isolated fetal LN-derived CD69^{+} ILC. Even more so, the *IL22* transcripts found in adult ILC after overnight stimulation were very low in comparison to the levels found in either stimulated or unstimulated tonsil ILC. These findings indicate that throughout adulthood human lymph nodes contain a resting ILC population that is characterized by the absence of NKp44 and the absence of active transcription of *IL17A* or *IL22*.

Finally, IL-17a and IL-22 protein levels produced by adult lymph node-derived NKp44^{-} ILC stimulated with physiological stimuli were determined by ELISA. Unfortunately, the scarcety of adult human tissue precluded the analysis of sufficient donors to draw statistical conclusions. This notwithstanding, in two independent donors 3 day culture in the presence of either IL-23 or IL-1β did induce detectable levels of IL-22 protein **(Figure 6 c).** These culture conditions did not induce IL-17a secretion to levels detectable by our ELISA (detection limit = 31 pg/ml)(data not shown). This suggests that even though low level transcription of *IL17A* ican be initiated, no detectable protein is secreted. The reason for this is presently unknown. These data suggest that resting adult lymph node-derived ILC retain the capacity to secrete IL22 upon in vitro stimulation with IL-23 or IL-1β. Further work is needed to substantiate these findings.

DISCUSSION

RORC⁺ ILC are increasingly recognized as important mediators of early innate immunological defenses against mucosal pathogens and as essential for safeguarding tissue integrity during infections. Both effects are mediated by ILC-derived cytokines, and especially IL-22 and IL-17a have been studied in this respect. In mice, IL-22 secreting ILC mediate the innate response to *Citrobacter rodentium* and IL-22 is an important mediator of epithelial homeostasis [32]. Conversely, IL-17a producing ILC are pathogenic in a T cell-independent mouse model of intestinal inflammation [1]. Most importantly, the balance between IL-22 and IL-17a secreting ILC was shown to be skewed towards IL-17a in patients with Crohn's disease, suggesting that ILC-derived cytokines might be involved in human disease [22].

The systematic analysis of ILC subsets in humans and the detection of any changes in subset distribution as a result of disease are hampered by the poor characterization of functionally distinct human ILC. In this study we show that expression of the NCR NKp44 is a good predictor for ILC that are actively transcribing IL-22, and that these cells are mainly found in mucosal tissues. In contrast, our data suggest that throughout adulthood, non-inflammed lymph-nodes may function as a reservoir of resting, RORC⁺ non-cytokine secreting ILC that lack NKp44 expression, yet retain the capacity to secrete IL-22 after appropriate cytokine activation in vitro.

Production of IL-17a by T cells is normally tightly controlled to avoid unwanted or excessive inflammation [33] and the fact that freshly isolated CD69⁺NKp44⁻ ILC in non-inflamed lymph nodes lack *IL17A* transcripts suggests that similar levels of restraint are operational for ILC. Unraveling the exact signals that can either induce or inhibit ILC activation is needed to understand regulation and control of innate IL-17 secretion.

IL-17a has been associated with several human diseases [18] and ILC-derived IL-17a has been reported in experimental models of colitis [1]. In addition, an increase in IL-17 producing ILC has now also been found in the intestines of some Crohn's disease patients [22]. The IL-17 producing ILC in Crohn's disease patients were mainly found within the CD56⁻ ILC fraction, while the CD56⁺ ILC were more prominent producers of IL-22 [22]. Our current data would predict that a dissection based on NKp44 expression, rather than expression of CD56, could further enhance the exact enumeration of IL-22 vs. IL-17a producing ILC in humans.

The biological function of IL17a produced by fetal LN-derived ILC remain enigmatic. Development of lymphoid organs has many similarities to a controlled inflammation and the inflammation-related cytokine IL-17a would fit that concept [34,35]. However, IL-17a is unlikely to have an essential function during organogenesis as IL-17Rα deficient mice display no gross abnormalities in LN development (KH and TC, unpublished observations), suggesting that IL-17a serves another yet to be determined function.

In-vivo, distinct subsets of RORC⁺ ILC seem biased towards either *IL17A* (CD69⁺NKp44⁻ ILC) or *IL22* (CD69⁺NKp44⁺ ILC) transcription. It is not clear whether these subsets belong to different ILC lineages, or whether they represent different activation or differentiation states of cells within a single lineage. In mice, conflicting data exists on the relationship between NKp46⁺ and

NKp46$^-$ ILC. Genetic approaches indicated that these two cell types belong to separate lineages and do not have a precursor-progeny relationship [25]. Conversely, transfer of purified populations of ILC showed that NKp46$^-$ cells could give rise to NKp46$^+$ ILC in-vivo [26]. In humans, these relationships are even less clear. After in-vitro culture, freshly isolated NKp44$^-$ fetal LN-derived ILC acquired NKp30, NKp44 and NKp46 [8]. In-line with this, the adult-lymph node-derived ILC also up regulate NKp44 when cultured (KH and TC, unpublished). Whether the observed IL-22 production by lymph node ILC is functionally linked to this expression of NKp44 is currently under investigation. Our current results indicate that in ex-vivo isolated ILC, NKp44 predicts for IL-22 production whereas in mice NKp46 is a marker for IL-22 producing ILC, again adding to the complexity of comparing data gathered in human and mouse studies.

NKp44$^+$ ILC are preferentially found in mucosal tissues like tonsil and the intestines (this report and [17]). Within this mucosal environment NKp44$^+$ ILC constitutively produce IL-22 that, based on mouse models, acts on intestinal epithelial cells and conditions these cells to cope with the hostile environment to which they are exposed [4,5,32].

Here we show that the appearance of ILC in the human intestine is developmentally programmed and NKp44$^+$ ILC are present from early second trimester. In mice NKp46$^+$ ILC is also a programmed event independent of microflora, yet these cells only appear after birth [25,28]. The observed differences between mouse and man in this respect might be due to the longer pregnancy in humans. NKp44$^+$ ILC in the developing human intestine initiate low level *IL22* transcription during second trimester pregnancy. However, these *IL22* levels are much lower than those found in NKp44$^+$ ILC from pediatric tonsils or adult intestines. Current it is unknown whether this difference reflects a role for microbial colonization of the intestines in inducing a postnatal increase in IL-22 levels. This would be different from the observations done in mice, where microbial colonization was not essential for IL-22 induction but actually induces a decrease in intestinal IL-22 postnatal [23,25,28]. Again, an alternative and perhaps more likely explanation is that due to the longer gestation time in humans compared to mice, the low levels of *IL22* transcription found during the second trimester will steadily increase throughout the third trimester, reaching levels comparable to postnatal ILC just before birth. Even thought the intestines are culture sterile, they are by no means devoid of potential immune stimuli such as TLR ligands and cytokines and these could still be a factor in ILC development and activation [30,31].

NKp44$^-$ ILC reside within peripheral lymph nodes throughout life but in the absence of activating signals do not transcribe detectable levels of *IL17A* or *IL22*. However, upon stimulation NKp44$^-$ ILC can produce at least IL-22, and also initiate low level *IL17A* transcription. This raises the hypothesis that secondary lymphoid organs are a reservoir for ILC that can participate in inflammatory responses in an antigen independent manner. Detailed analysis of human lymph node biopsies from inflammatory diseases or the study of relevant animal models is needed to determine the contribution of LN-derived ILC to systemic immune responses.

MATERIALS AND METHODS

Human tissues

The use of all human tissues was approved by the Medical Ethical Commission of the Erasmus University Medical Center Rotterdam, and use was contingent on informed consent. Fetal tissues were obtained from elective abortions and gestational age was determined by ultrasonic measurement of the diameter of the skull or the femur and ranged from 7 to 22 weeks gestation. Tonsils were obtained from routine pediatric tonsillectomies. Adult hepatic and inguinal lymph nodes were collected post-mortem during multi-organ donation procedures. Adult small intestine, ileum, was collected as residual material after intestinal surgery for colon carcinoma. Healthy ileum was obtained from a clear distance from the tumour. Patients undergoing radiotherapy or chemotherapy were excluded. Informed consent was obtained from all patients, according to the Medical Ethical Commission of the Academic Medical Center, Amsterdam, the Netherlands.

Cell preparation

Fetal lymph nodes were dissected from the mesentery using dissecting microscopes, and cell suspensions were prepared by digestion with 0.5 mg/ml collagenase type IV (Sigma, St. Louis, MO) in PBS for 30 min at 37°C, while stirring continuously, and subsequently filtered through a 70 μm nylon mesh. Tonsils and adult lymph nodes were cut into small pieces and cell suspensions were prepared by disrupting the tissue with a GentleMacs (Miltenyi Biotech) in the presence of 0.5 mg/ml collagenase type IV (Sigma, St. Louis, MO). Mononuclear cells were isolated from ficoll gradients. Tonsils were further enriched for ILC by labeling with CD117 microbeads (Miltenyi Biotech) and positive selection using MidiMacs (Miltenyi Biotech).

Adult intestinal ILC were isolated as described [36] ileum was incubated with HBSS (Gibco) containing DTT (154 μg/mL), 0.1% β-mercaptoethanol and 5mM EDTA at 37°C to eliminate mucus and epithelial cells. Thereafter mucosa was cut into small pieces and digested for 30 minutes at 37°C with Liberase TM (125 μg/mL) and DNaseI (200μg/mL) (Roche). Cell suspensions were filtered through a 70-μm nylon mesh and lamina propria mononuclear cells (LPMC) were isolated by Ficoll-Paque PLUS (GE, Healthcare).

Flow cytometry

The following antibodies were used: CD117-PercP.Cy5.5, NKp44-AlexaFluor647, NKp46-AlexaFluor647, NKp30-Pe (Biolegend, San Diego, CA); CD127-APC-eFluor780, CD45-PeCy7, RORγt-Pe (eBioscience, San Diego, CA); CD56-Pacific Blue, CD69-Pe, CD19-PeDY590, CD3-PeDY590, CD14-PeDY590 and CD34-PeDY590 (Exbio, Praha, CZ). Flow cytometry was performed on a BD LSRII and cell sorting on a BD ARIA (Becton Dickinson). Analysis were performed using FlowJo software (Tree Star Inc., Stanford, CA)

Cell culture

Sorted ILC subsets were cultured overnight in the presence of IL-7 (Peprotech) and SCF R&D Systems) (both at 10 ng/ml) in the presence or absence of PMA (50 ng/ml; Sigma) and Ionomycin (100 ng/ml; Sigma) or for 3 days with IL-7, SCF and either IL-23 or IL-1b. Cells were cultured in 200µl DMEM supplemented with 10% FCS (Hyclone). IL-17a and IL-22 production was measured using the DuoSet ELISA development kit (R&D systems). ELISAs were performed according to the manufacturers' instructions.

PCR

From fetal samples, adult lymph nodes and tonsil, RNA was extracted using the RNA-XS kit (Machery Nagel) followed by reverse-transcription with random hexamer primers. For quantitative PCR, a Neviti Thermal Cycler (Applied Biosystems) and DyNAmo Flash SYBR Green qPCR kit (Finnzymes) were used, with the addition of MgCl2 to a final concentration of 4 mM. All reactions were done in duplicate and are normalized to the expression of GAPDH (glyceraldehyde phosphate dehydrogenase). Relative expression was calculated by the cycling threshold (CT) method as $2^{-\Delta t}$.

Primers used were:

GAPDH (Accession: NG_007073.2) FW GTC GGA GTC AAC GGA TT; Rev AAG CTT CCC GTT CTC AG

IL22 (Accession: NM_020525.4) FW CCC ATC AGC TCC CAC TGC; Rev GGC ACC ACC TCC TGC ATA TA

IL17A (Accession: NM_002190.2) FW GAA GGC AGG AAT CAC AAT C; Rev GCC TCC CAG ATC ACA GA

For cDNA synthesis of the adult ileum samples, the high-capacity cDNA archive kit (Applied Biosystems) was used. PCR was performed using the SYBR Green I master mix (Roche) for LightCycler 480 Instrument II (Roche).

Quantification of expression levels was performed with LinRegPCR software [36]. All reactions were performed in duplicate were normalized using 18S rRNA expression levels and expressed in arbitrary units.

Specific primers were as follows:

18S rRNA FW: AAT CTG GAG CTG GCC TTT CA; Rev CTG GAA GAT CTG CAG CCT TT

Statistical analysis

Samples were analyzed by Mann-Whitney *U* tests in SPSS PASW 17.0.2. P values < 0.05 were considered significant.

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CHAPTER 3

THE DEVELOPMENT OF HUMAN B CELL FOLLICLES AND ECTOPIC GERMINAL CENTER-LIKE STRUCTURES

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ABSTRACT

Upon diagnosis with follicular lymphoma (FL), more than 70 % of patients present with germinal center-like structures in the bone marrow (BM). FL cells are the malignant counterpart of normal germinal center cells and have the capacity to remodel the bone marrow stroma to resemble germinal center stromal cells in order to acquire essential signals for survival in the ectopic microenvironment. The mechanisms driving the development of B cell follicles and ectopic lymphoid follicles are poorly characterized. We set out to study the interactions between FL B cells and BM stromal cells required for ectopic follicle formation. Here, we present an *in vitro* 3D co-culture system and xenograft model in RAG-2$^{-/-}$γc$^{-/-}$ mice as experimental models for studying ectopic FL B cell behavior. Furthermore, to understand the stromal changes induced during B cell follicle formation we analyzed B cell follicles formation ex-vivo in human lymph nodes and in-vivo in a murine model. We show that the development of B cell follicle in human fetal lymph nodes is completed by 18 gestational weeks. At 20 gestational weeks, stromal cells of the developing B cell follicle express high levels of VCAM-1, similar to an activated B cell follicle. Micro array analysis of the non-hematopoietic compartment of lymph nodes of RAG-1$^{-/-}$ mice 7 days after B cell transplantation revealed a yet unknown stromal source for IL-22 production. What the role is of IL-22 in B cell follicle formation remains enigmatic since lymph nodes of IL-22$^{-/-}$ mice do not display abrogated B cell follicles. Together, our data show that B cell follicle development induces an activated phenotype in precursor FDCs and that in adult RAG-1$^{-/-}$ mice IL-22 is produced by an as yet unidentified stromal cell subset.

INTRODUCTION

Follicular lymphoma (FL) is the second most common non-Hodgkin's lymphoma in the western world. The disease is considered to have an indolent course that is characterized by a slow progression and a median survival of 10 years[1]. Treatment of patients can vary from the "watch-and-wait" method to aggressive therapy including high doses of chemotherapy and hematopoietic stem cell transplantation. Most patients experience relapse after treatment and 25-30% progresses towards the aggressive diffuse large B-cell lymphoma (DLBL).

Several classification schemes have been proposed for FL. The World Health Organization adopted the method developed by Bernard and Mann. This scheme grades FL from I to $III_{a/b}$ based on the number of centroblasts in 10 neoplastic follicles per high power field. Grade I and II are considered indolent lymphomas whereas stage III is more advanced. The majority of indolent FL, i.e. grade I and II show a follicular growth pattern. Some FL infiltrates show a partially or even complete diffuse growth pattern as reviewed by E. Leich et al[2]. Even though almost all FL arises in lymph nodes, at time of diagnosis most patients (>75%) present stage III with involvement of the spleen, liver, peripheral blood and the bone marrow.

FL is the neoplastic counterpart of germinal center (GC) B cells. GCs are the main anatomical sites where high-affinity antibody-secreting plasma cells and memory B cells are generated. During this process proliferating GC B cells (centroblasts) undergo somatic hypermutations (SHM) which results in antibody gene diversification. This enables clonal selection based on affinity competition by B cells for antigen presented by FDCs within the center of the follicle. At a certain point, centroblasts become centrocytes and stop proliferating and are selected for their capacity to bind antigen presented by FDCs and for the ability to elicit help from T_{fh}[3,4]. The end stage of this process is that GC B cells emigrate from the B cell follicle and differentiate into long-lived plasma cells and memory B cells. As such, FL partially recapitulates the GC in that tumor cells often grow in close association with follicular helper T (T_{fh}) cells and follicular dendritic cells (FDCs)[5].

FL mimics the GC reaction in a "frozen state", i.e. follicular lymphoma cells are in close association with FDCs but do not differentiate towards long-lived plasma cells or memory cells but do show clonality , exhibit a complete BCR that has undergone SHM and have a follicular growth pattern (low grades)[6]. FL cells are $CD19^+CD20^+CD10^+$ and in addition express *BCL-6*. The latter is essential for GC B cell formation and inhibitis differentiation towards plasma cells by binding to STAT 3[7-10]. In contrast to GC B cells, the majority of the FL cases (80-90%) constitutively express *BCL-2*. This is largely due to translocation t(14;18)(q32; q21) which results in the juxtaposition of the *BCL-2* gene in the immunoglobulin heavy chain locus on chromosome 14, leading to its constitutive transcription. *BCL-2* does not promote cell cycle progression or cell proliferation but rather controls the cellular apoptotic threshold by preventing programmed cell death[11]. Over time, follicular lymphomas tend to convert from grade I to grade III and ultimately progress towards an aggressive diffuse large B cell lymphoma. This coincides with decreased expression

of CD10 and BCL-2[12]. In DLBL, down regulation of CD10 and BCL-2 is often accompanied by mutations of BCL-6 and by the accumulation of p53 mutations and/or inactivation of p16.

The role of the microenvironment as a prognostic factor for FL progression has gained appreciation. Most studies, which conducted gene expression and/or immunohistochemical analysis demonstrated that the non-malignant microenvironment, i.e. T cells, macrophages and (follicular) dendritic cells, can function as a strong predictor for life expectancy of FL patients[13-19]. In contrast to normal GC B cells in LNs where primary follicles are already present prior to a GC reaction, FL remodels its (ectopic) stromal microenvironment in such a way it resembles first a follicle-like structure and secondly mimics a GC-like environment. Invaded lymph nodes exhibit a modulated reticular meshwork and can present with an enormous variety in the differentiation status of follicular dendritic cells[20,21]. Upon progression towards higher grades, FL cells loose the dependency on the local microenvironment, and this correlates with a more diffuse growth pattern. As a result FDCs in infiltrated LNs loose one or more typical FDC markers or FDCs even disappear[21].

FL infiltrates in the BM are in most cases localized at peritrabecular sites[22,23]. The phenotype of FL in the BM is virtually identical to its nodal counterpart and a meshwork of $CD21^{+}CD23^{+}$ FDCs can be demonstrated in larger infiltrates. Similar to FL in lymph nodes, BM infiltrates of FL frequently contain high numbers of intermingled T cells[22-24]. In addition to this, BM biopsies of FL patients can show the up regulated expression of CD35, CD40 and VCAM-1 by adventitial reticulum cells[24]. An elegant *in vitro* study shows that cells with characteristics of fibroblastic reticular cells, a cell type typically present in the T zone of lymphoid organs[25], can be induced from bone marrow derived mesenchymal cells upon interaction with primary FL cells *in vitro*[26].

Even though FL cells express constitutively the anti-apoptotic protein BCL-2, this is not sufficient for FL survival since primary low grade FL cells spontaneously undergo apoptosis *in vitro*. However, co-culturing of FL cells with FDCs and FDC-like feeders rescues the lymphoma cells from apoptosis[27]. Together, these data suggest that FL can remodel the local BM microenvironment in such a way it starts to resemble a GC-like structure that is capable of and necessary for providing the FL cells with essential survival signals.

One can deduct from the previous section that low grade FL cells are dependent on stimuli from the microenvironment for their survival. Upon dissemination to the BM, FL cells remodel the BM stroma into a GC-like phenotype in order to require stimuli essential for survival in the ectopic microenvironment. The early interactions between FL cells and local BM stromal cells prior to the induction of a GC-like phenotype in the bone marrow are poorly understood.

Murine studies demonstrated an important role for the lymphotoxin (LT) and TNFα pathways in normal B cell follicle development and maintenance and this has been extensively reviewed[28,29]. Rennert and colleagues, showed that mice treated in utero on gestational days 14 and 17 with LTβ-R–Ig or TNF-R55–Ig failed to form discrete B cell follicles in mesenteric lymph nodes and that antagonism of TNF-R55 in adult mice disrupts B cell follicles [30,31]. Furthermore, mice deficient for CXCL13 or its receptor CXCR5, have segregated T and B zones but fail to develop B cell follicles[32,33]. Tumanov and colleagues generated mice in which the LTβ-gene is specific

targeted in B cells. Strikingly and in contrast to mice with a systemic LTβ-gene deletion, B cell follicle development in LNs of B-LTβ$^{-/-}$ was not affected. However, the B cell follicles of spleens of B- LTβ$^{-/-}$ mice were affected and had little or no FDCs[34].

To identify and understand the early interactions between FL B cells and BM stromal cells required for ectopic follicle formation, the early mechanisms of B cell follicle formation under normal non-pathologic conditions need to be understood.

RESULTS

FL cell line induces the expression of VCAM-1 and transglutaminase in primary bone marrow

We wanted to assess whether we could study the remodeling of human bone marrow induced by follicular lymphoma cells *in vitro*. Therefore, we set up a 3-D culture system in which reaggregates of primary fetal bone marrow derived stromal cells mixed with the follicular lymphoma derived cell line WSU-FSCCL, were cultured under high oxygen levels for 7 days and analyzed for the induction of a stromal cell-like phenotype by immunofluorescence microscopy. In patients, neoplastic follicular lymphoma LNs can show high levels of transglutaminase (TG)[20]. Upon culturing reaggregates with WSU-FSCCL (WSU), fetal primary BM derived stromal cells started to express transglutaminase (**Figure 1b**) compared to control (**Figure 1a**). In this culture system, also VCAM-1 was induced upon co-culturing of FL cell with fetal BM stromal cells (**Figure 1d**). No other LN stromal cell markers could not be detected by IF. Furthermore, no transcripts for RANKL, CXCL13, CCL21 and CXCL12 in fetal primary BM derived stromal cells after culturing in reaggregates with WSU cells could be detected.

These data suggest that co-culturing the above described reaggregates without any additional supplements was insufficient to induce a "remodeled" BM phenotype.

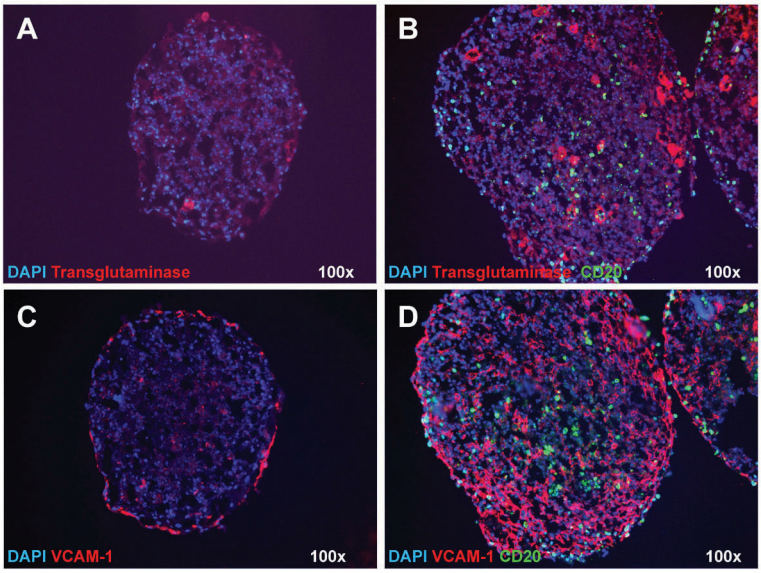

Figure 1: Fetal bone marrow derived (FBMD) stromal cells express Transglutaminase and VCAM-1 after 7 days co-culture with WSU-FSCCL. In order to visualize the remodeling FBMD stromal cells by follicular lymphoma-derived cell line WSU-FSCCL in a 7 days 3-D culture system, frozen sections of reaggregates of FBMD stromal cells with or without $CD20^+$ WSU-FSCCL were stained for CD20, Transglutaminase and VCAM-1. The expression of Transglutaminase and VCAM-1 by FBMD reaggregates (A,C) was induced after 7 days co-culture with WSU-FSCCL (B,D).

FL derived cell line WSU-FSCCL aggregates in clusters in the peri-trabeculae of *RAG-2$^{-/-}$ γc$^{-/-}$ KO femurs*

An *in vivo* model enabling the study of the early interactions between human FL cells and BM was established with the use of RAG-$2^{-/-}\gamma c^{-/-}$ mice. These mice lack B, T and NK cells and are therefore suitable for xenotransplantation studies. Human GFP-transduced WSU-FSCCL cells were xenotransplanted into RAG-$2^{-/-}\gamma c^{-/-}$ mice and four weeks later femurs of the mice were analyzed for the presence of GFP^+-WSU-FSCCL cells in the bone marrow (**Figure 2**). Human derived WSU-FSCCL cells clustered preferentially in the trabeculae of murine femurs (**Figures 2a and 2b**) or at bone-lining niches (**Figures 2c and 2d**). In human patients, disseminated lymphoma cells in the BM also preferentially cluster in the trabeculae or the bone-lining niches of femurs. The transplanted WSU-FSCCL cells were also present in other organs four weeks after transplantation, such as lymph nodes, spleen, liver and kidney (data not shown). Mice sacrificed and analyzed six weeks after transplantation, displayed a vast invasion of GFP^+-WSU-FSCCL cells in the BM as well as in other organs (data not shown).

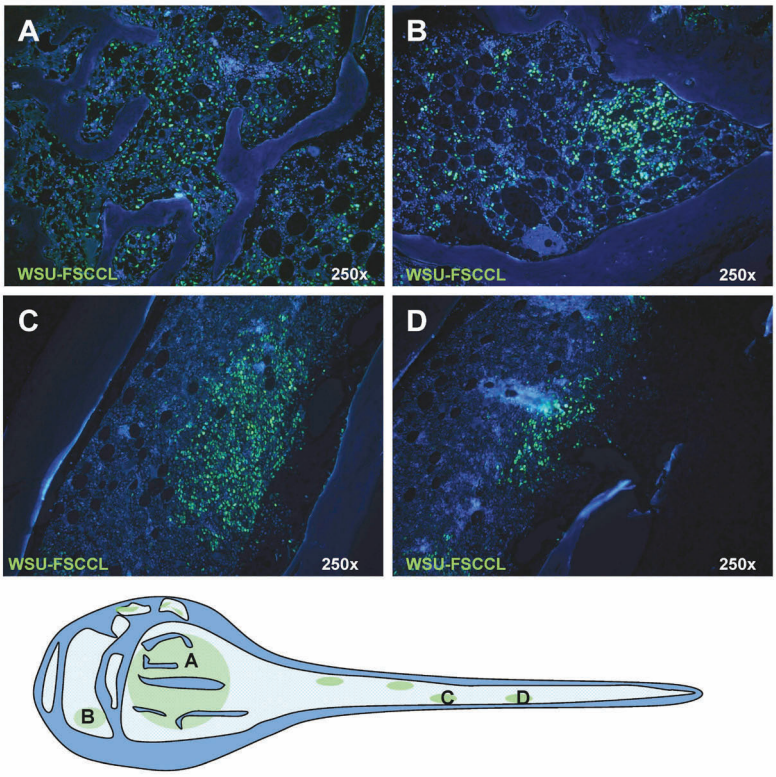

Figure 2. Human WSU-FSCCL cells cluster preferentially in the trabeculae of murine femurs or at bone-lining niches. Parafin-embedded sections of decalcified femurs of RAG-2$^{-/-}\gamma c^{-/-}$ mice were analyzed for the presence and localization of GFP-labeled human WSU-FSCCL cells four weeks after transplantation. Human WSU-FSCCL cell clustered in the trabeculae (A,B) and at bone lining niches (C,D) of murine femurs. Figure 2e is a schematic representation of the murine femur and gives an overview of WSU-FSCCL niches in the murine femur.

These data show that the human follicular lymphoma-derived cell line WSU-FSCCL homes to the BM and organizes in FL-like niches, but that its growth pattern is very progressive and therefore disseminates and spreads out through all organs. This observation shows that WSU-FSCCL does not depend on its local microenvironment for survival and therefore does not mimic the behavior of low grade FL. WSU-FSCCL is not applicable in studies investigating the development of ectopic follicle-like structures..

The stromal cell subsets of the human developing B cell follicle

As discussed in the previous sections, FL is capable of remodeling stroma at ectopic sites in follicle-like structures. Since little is known about the first interactions in human B cell follicle development, we set out to study the stromal cells that co-localize with developing B cell follicles in human fetal lymph nodes. Therefore, fetal human lymph nodes were analyzed by immunofluorescence microscopy.

In human fetal lymph nodes, demarcated B cell follicles were present in the cortex from 17 to 22 gestational weeks (gw.) onward[35]. Figure 3 depicts a mesenteric lymph node (MLN) of 19 gw. The majority of the $CD20^+$ B cells resided in the cortex at the site where a B cell follicle started to develop.(**Figure 3a, white arrow**). In addition, a few cells were positive for CD35, i.e. the complement receptor 1, which is a marker for follicular dendritic cells. Marginal reticular cells in the cortex of the fetal MLN expressed RANKL and co-localized with B cells. However, at sites where B cells clustered and a follicle started to develop, RANKL was absent (**Figure 3c, white arrow**). The bulk of $CD3^+$ T cells present in the fetal MLN resided in the medulla (**Figure 3b**), attracted and retained by the homeostatic chemokine CCL21 that was abundantly expressed by the stromal cells of the T zone (**Figure 1d**). Within the developing B cell follicle both T cells and CCL21 were absent (**Figures 3c and c, white arrow**).

Figure 4 depicts a fetal MLN of 20 gw. with a more matured B cell follicle (white arrow). Here, the absence of RANKL expression by stromal cells in the B cell follicle area was very clear (**Figure 4a**). Marginal reticular cells that surround the B cell follicle were positive for RANKL, VCAM-1 and podoplanin. At this developmental stage, cells with a follicular dendritic cell-like phenotype had developed that resided within the center of the B cell follicle and were $VCAM\text{-}1^{hi}$ and podoplanin positive.

Together, these data show that at early stages in fetal human B cell follicle development, the developing B cell follicle can be identified by the lack of RANKL and CCL21 expression. At later stages in development, local stromal cells in the center of the B cell follicle start to express VCAM-1 and podoplanin, which suggests that these cells might be FDCs.

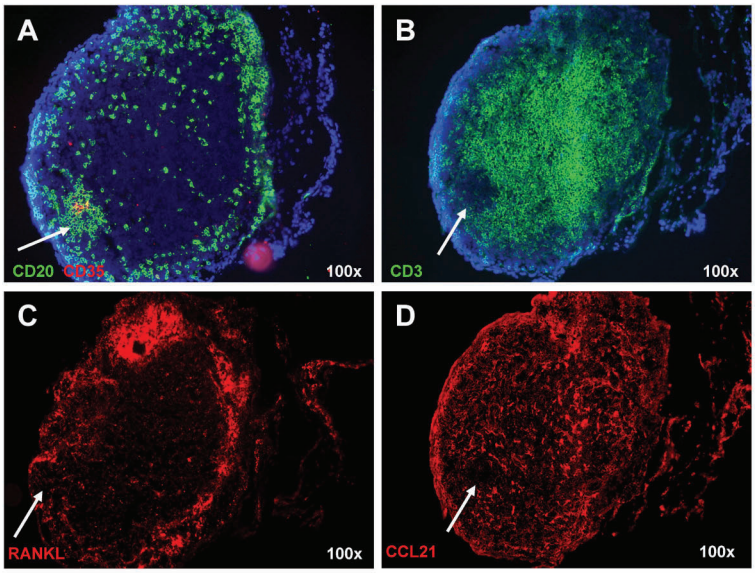

Figure 3. Demarcated B cell follicles are present in human mesenteric fetal lymph nodes of 19 gw. Frozen sections of fetal MLNs of 19 gw. were stained for the indicated markers in order to reveal the presence of an early B cell follicle. (A) At the site where a B cell follicle starts to develop (white arrow) a few $CD35^+$ cells reside. (B) The bulk of $CD3^+$ T cells reside in the medulla of the fetal MLN and are very scarce in the outer cortex and developing B cell follicle. (C) Stromal cells in de cortex express RANKL. and (D) CCL21 is abundantly expressed by stromal cells of the fetal MLN. (C,D) At the site of the developing B cell follicle (white arrow) the expression of both RANKL en CCL21 is absent.

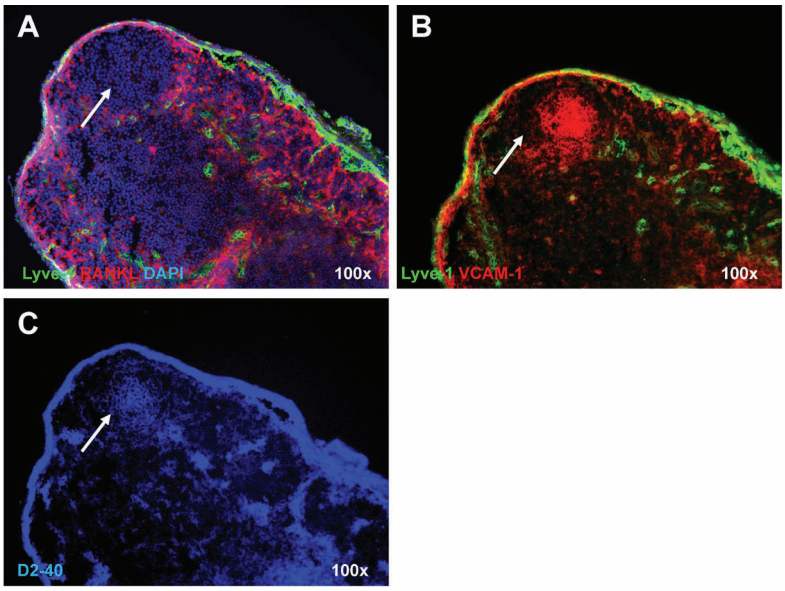

Figure 4. Stromal cells with Marginal Reticular Cell-like and Follicular Dendritic Cell-like phenotypes are present in human fetal mesenteric lymph nodes of 20 gw. Frozen sections of fetal MLNs of 20 gw were stained for Lyve-1, RANKL, VCAM-1 and podoplanin (D2-40) in order to analyze the stromal cells associated with the developing B cell follicle (white arrow). Stromal cells located directly underneath the Lyve-1^{+} subcapsular sinus are positive for RANKL (A), VCAM-1 (A) and podoplanin (C). In addition stromal cells in the center of the B cell follicle (white arrow) express both VCAM-1 (B) and Podoplanin (C).

B cells home to peripheral lymph nodes and organize in follicle-like structures in RAG-1$^{-/-}$ mice and induce IL-22 production by local stromal cells

Next, we established an *in vivo* model in which normal, i.e. non-malignant, B cells were transplanted in RAG-1$^{-/-}$ mice. These mice are deficient for recombination activation gene RAG-1 and display an early arrest of B and T cell differentiation do the inability to perform V(D)J recombination. Lymph nodes and spleen of these animals have no T and B cells and also lack follicular dendritic cells (FDCs). To investigate early molecular changes induced upon interactions with B cells in the non-hematopoietic (CD45^{-}) stromal cell compartment of the lymph node in physiological conditions, we administered spleen derived B cells (donor C57BL/6) to RAG-1$^{-/-}$ mice. To assure that the administered B cells survived after i.v. injection and are capable of homing to secondary lymphoid organs, we analyzed peripheral and mesenteric lymph nodes, and spleen for the presence of adult B cells. As depicted in figure 5, five days post-transplantation clusters of B cells were present in the cortex of the lymph nodes where stromal cells expressed CXCL13 **(Figure 5a).** In the medulla, the T zone, stromal cells expressed CCL21 and the number of B cells was very low with a scattered distribution (**Figure 5b**). At this stage no FDCs were present. Seven days post transplantation FDC-M2 $^{+}$ FDCs could be detected at locations where B cells clustered (**Figure 5c**). To identify genes involved in the early stages of B cell follicle development, a microarray analysis on the CD45^{-} non-hematopoietic stromal cell compartment, including vascular and endothelial cells,

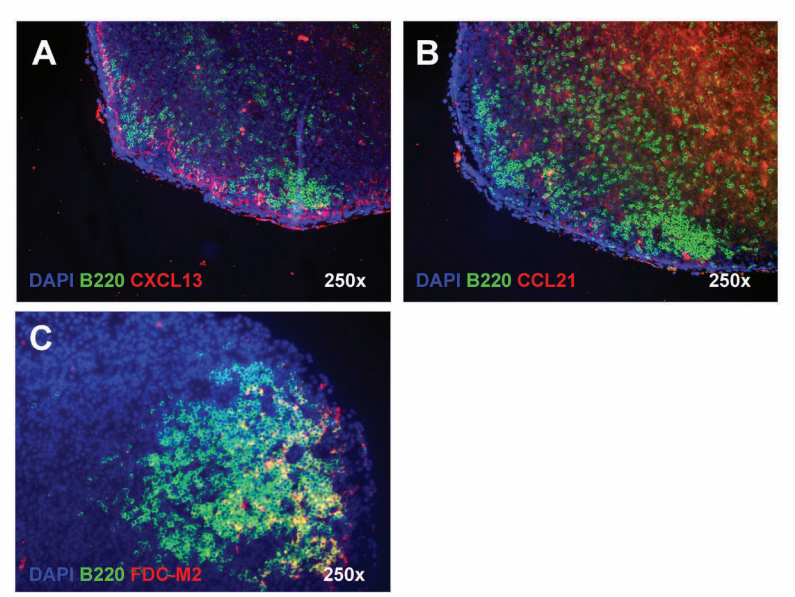

Figure 5. Inoculated B cells home to peripheral lymph nodes (PLNs), cluster in the paracortex and organize in follicle-like structures in RAG-1$^{-/-}$ mice. To visualize the homing of inoculated B cells to PLNs of RAG-1$^{-/-}$ mice by fluorescence microscopy, frozen sections of PLNs of RAG-1$^{-/-}$ mice sacrificed five or seven days after inoculation were stained for B220 , CXCL13, CCL21 and FDM-M2^{+} (A) B220^{+} B cells home 5 days after inoculation to the CXCL13^{+}paracortex. (B) A low number of scattered B220^{+} B cells are present in the medulla where CCL21 is widely expressed. (B) At seven days after inoculation, B cells get organized in follicles and stromal cells start to express the FDC marker FDM-M2 (C).

of lymph nodes of RAG-1$^{-/-}$ mice compared to RAG-1$^{-/-}$ mice transplanted with 10 E^{6} C57/BL6 B cells for 7 days, was performed. Therefore, all CD45- LN derived cells were isolated by flow cytometric cell sorting and analyzed by microarray. **Table 1** represents a condensed list of the 50 most upregulated genes by the total non-hematopoietic compartment (CD45^{-}CD19^{-}CD20^{-}) of lymph nodes of RAG-1$^{-/-}$ mice seven days after inoculation with C57/BL6 B cells compared to control mice. The array was performed with Affymetrix Mouse Genome 430 2.0 chips. In table 1, column 1 (probes) depicts the probe ID, column 2 (gene title) depicts the corresponding gene, column 3 (hits) depicts the levels of expression of the gene (after normalization) in the non-hematopoietic compartment of RAG-1$^{-/-}$ LNs inoculated with B cells and column 4 (fold) depicts the fold increase in expression levels in RAG-1$^{-/-}$ CD45- LN derived cells with B contact compared to negative control. Strikingly the gene Il22 encoding for the IL-22 protein was one of the highest up regulated genes in the total non-hematopoietic compartment after B cell transfer. IL-22 has been known to be produced by RORC^{+} ILC, Tf$_{h}$ cells, T$_{h}$17, T$_{h}$22 and γδ-Tcells. The production of IL-22 by stromal cells has not been reported[36-40]. IL-22 plays a role in tissue immunity by inducing the secretion of anti-microbial peptides and the promotion and differentiation of epithelial cells of mainly the gastrointestinal tract, the urinary tract, the pulmonary tract and the skin that express the IL-22 receptor[40]. A role for IL-22 in the organization of B cell follicles has never been reported in literature. Next to IL-22, CCR7 the receptor for CCL19 and CC21 and the IL-7R were highly up regulated.

Table 1. Fifty most uprelugated genes in RAG-1$^{-/-}$ mice derived CD45^{-} lymp node cells 7 days after B cell transfer

Probe Set ID	Gene Title	hits	fold
1427455_x_at	predicted Igkc	644,2692819	107,2125512
1452557_a_at	---	428,8234527	84,76401382
1425763_x_at	AC160982.1	380,1434176	83,90006365
1427660_x_at	Predicted Igkc	542,0867748	83,30319936
1452417_x_at	2010205A11Rik	517,4443851	71,13758041
1452463_x_at	---	380,080184	61,58597766
1427624_s_at	Il22	390,462449	57,91840629
1428720_s_at	2010309G21Rik	163,7362558	37,54294709
1423466_at	Ccr7	110,5518992	22,35661714
1418937_at	Dio2	147,7659444	22,05081387
1454881_s_at	Upk3b	130,2247629	21,38429885
1454623_at	CPA2	975,9544464	20,19659247
1448186_at	Pnliprp2	140,23377	18,22845017
1449452_a_at	Gp2	133,2165948	17,88341062
1418666_at	Ptx3	724,3684999	16,01375795
1437015_x_at	Pla2g1b	140,9627339	15,35940048
1448281_a_at	Cela2a	1731,011987	15,28442104
1448575_at	Il7r	119,9043343	15,19737239
1423693_at	Cela1	1551,771049	14,32973475
1431763_a_at	Ctrl	742,2081331	14,20394722
1433431_at	Pnlip	3361,524618	11,5882435
1418287_a_at	Dmbt1	604,2492638	11,43385504
1424010_at	Mfap4	100,0751522	11,35361641
1421868_a_at	Pnlip	1363,122969	11,28613808
1415777_at	Pnliprp1	913,6133996	11,27800514
1425152_s_at	Serpini2	120,5485093	11,21501776
1417257_at	Cel	782,4799635	10,53537674
1416523_at	Rnase1	1402,320112	10,36379156
1417601_at	Rgs1	305,4410167	10,2171435
1415805_at	Clps	790,0334894	9,193791828
1437355_at	Zchhc5	156,9422523	8,472219691

Probe Set ID	Gene Title	hits	fold
1455269_a_at	Coro1a	142,7067732	8,362011341
1428358_at	Zg16	172,0554761	8,023323679
1428062_at	Cpa1	1482,791464	7,923676607
1428359_s_at	Zg16	839,9210994	7,648357715
1433573_x_at	Prss2	2114,938505	7,503844856
1417682_a_at	Prss2	6130,546621	7,305028998
1417898_a_at	Gzma	136,4699971	7,126778074
1428102_at	Cpb1	3007,606273	7,003907353
1422789_at	Aldh1a2	159,6147315	6,937423704
1436905_x_at	Laptm5	1869,71155	6,623528604
1426851_a_at	Nov	281,2423414	6,508431938
1435507_x_at	Prss2	940,3604157	6,475582618
1415905_at	Reg1	1041,971426	6,454431366
1436713_s_at	Meg3	140,3504617	6,440041566
1437397_at	Prlr	196,0081728	6,224293109
1417447_at	Tcf21	143,2836317	6,03849908
1433459_x_at	Prss2	1268,3173	5,973721585
1415884_at	Cela3b	1393,21288	5,785934535
1449495_at	Reg3a	237,2772546	5,771554724

Adult IL-22$^{-/-}$ mice do not present an aberrant phenotype in lymph nodes under homeostatic conditions

Next we wanted to analyze adult IL-22$^{-/-}$ mice for abnormalities in B cell follicle architecture. No clear and distinct phenotype in the B cell follicles of IL-22$^{-/-}$ mice could be detected. The B cells were organized in demarcated B cell follicles that appeared normal with FDCs that express CXCL13 (**Figures 6a and 6b**) and VCAM-1 (**Figure 6e**). The Marginal Reticular Cells that reside directly underneath the Lyve-1^{+} subcapsular sinus expressed normal levels of RANKL (**Figure 6c and 6d**) and VCAM-1 (**Figures 6e and 6f**).

These data show the absence of IL-22 does not abrogate B cell follicle development, or B cell follicle integrity under homeostatic conditions.

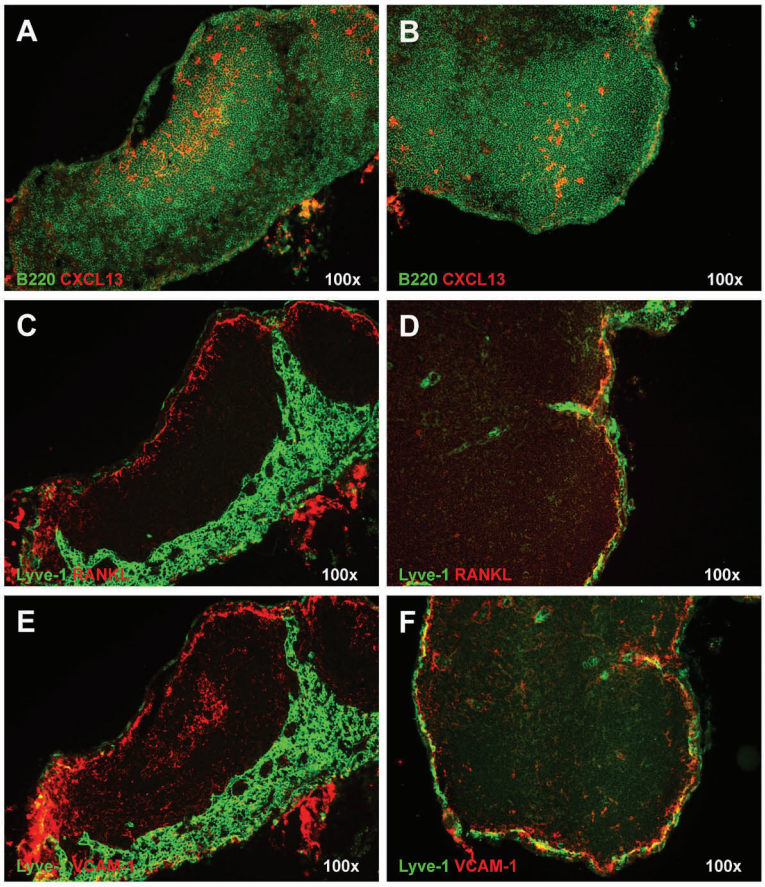

Figure 6. Adult IL-22$^{-/-}$ mice display normal lymph node architecture. Frozen sections of cervical lymph nodes (a,c,e) and inguinal lymph nodes (b,d,f,) of adult IL-22$^{-/-}$ mice were analyzed by fluorescence microscopy . Sections were stained for B220 CXCL13 (a,b), RANKL (c,d) and VCAM-1 (e,f). The localization of B220^{+} B cells in follicles, and the expression patterns of CXCL13, RANKL and VCAM-1 all appear normal compared to wild type (data not shown).

DISCUSSION

In this study we set out to identify genes involved in the early stages of B cell follicle development in order to understand the mechanisms by which disseminated follicular lymphoma is capable of inducing a GC-like structure at extra-nodal sites such as the bone marrow.

Primary low grade follicular lymphoma cells undergo spontaneous apoptosis *in vitro*. Co-culturing these cells with FDCs and/or FDC-like feeders rescues the follicular lymphoma cells from apoptosis[27]. In addition, low grade primary FL cells depend on extracellular stimuli for their survival and are capable of orchestrating a series of events leading to remodeling the ectopic microenvironment[24] in favor of cell survival. In contrast, the FL-derived cell line WSU-FSCCL grows independent from external survival stimuli and and as such it can be cultured in suspension without the addition of supplements. The outcome of the experiments conducted with cell line WSU-FSCCL in both our 3-D *in-vitro* cultures and RAG-2$^{-/-}$γc$^{-/-}$ mice model can be explained by these differences in growth characteristics and grading. It is likely that transplanting primary low grade FL cells in RAG-2$^{-/-}$γc$^{-/-}$ mice is a better model to study FL induced BM remodeling. Repeating both the 3-D cultures and the transplantation experiments with primary low grade FL cells might therefore be more representative with regard to ectopic B cell follicle formation by FL cells.

We observed that lack of expression of CCL21 by stromal cells in the cortex of human fetal LN delineates the early developing B cell follicle. In murine peripheral LN development a role for CCR7 ligands has been reported[41,42]. Mice deficient for CCR7 lacked the characteristic distribution of B cell follicles in peripheral LNs[43]. The role of CCL21 in B cell follicle development is still unknown. Overlapping roles for CXCL13 and CCL21 have been reported with regard to the attraction of LTi cells[41]. Both CXCL13 (data not shown) and CCL21 are abundantly expressed in human fetal LNs, and as such one can envisage that the outer border of the developing B cell follicle has a complex gradient of CXCL13 and CCL21 in which B and T cells are clearly separated and also RORc^{+} ILC reside. In addition, we have observed that in developing human fetal LNs, CD3^{-}CD127^{+}RORc^{+} cells reside in a ring-like pattern surrounding the developing B cell follicle (Hoorweg and Cupedo, unpublished data).

Furthermore, we observed that stromal that co-localize with B cells within the developing B cell follicle do not express RANKL (chapter 4 of this thesis). The importance of RANKL in B cell follicle development has been recently established by Sugiyama et al[44]. In this study, neonatal mice that had been injected with anti-RANKL antibody at 13.5 dpc displayed an absence of B-cell follicle formation and all B cells and FDC-M2^{+} FDCs were localized at the periphery of the LN. In RANKL$^{-/-}$ mice most PLN and all MLN are absent. However, cervical LNs develop occasionally and present with segregated T and B cells, but fail to develop B cell follicles[45]. The mechanisms by which RANKL controls B cell follicle development are not fully understood. Based on our observation, RANKL^{+} MRC play a direct and/or indirect instructive role in the development of RANKL^{-} B cell follicle stroma.

In order to study early events in human B cell follicle development, an *in vivo* B cell transfer model was established using RAG-1$^{-/-}$ mice. This model allowed the monitoring of changes in gene expression of lymph node stromal cells upon contact with introduced B cells during the earliest stages of B cell follicle formation. Surprisingly, IL-22 was found to be highly up regulated by the non-hematopoietic compartment of LNs upon contact with B cells. This result is very interesting since IL-22 is known to be produced by RORC^{+} ILC and subsets of T cells[36-40], but not by stromal cells. The pool of CD45^{-} cells that was analyzed comprises FDC, FRCs, MRCs, lymphatic and vascular endothelial cells and unidentified stromal cells. The next step in this study is identifying an as yet unknown non-hematopoietic cellular source of IL-22.

The role of IL-22 in B cell follicle formation is enigmatic. So far, IL-22 has been described to be involved in tissue immunity and integrity of mainly the gastrointestinal, urinary, and pulmonary tract and the skin. All these tissues also express the IL-22 receptor[46-48]. The observed up regulation of IL-22 might be, for that reason, induced by changes in integrity of the LN structure of RAG-1$^{-/-}$ mice, a result of the entry of large numbers of B cells. To exclude the the possibility that up regulation of IL-22 by the non-hematopoietic compartment of LNs of RAG1$^{-/-}$ mice after transfer of B cells is an artifact, our data should be validated by PCR for IL-22. Furthermore, it should be addressed whether the expression of IL-22 is B cell specific and not a bystander effect.

Our analysis of adult IL-22$^{-/-}$ mice for the presence of aberrant B cell follicles revealed no phenotype suggests that the absence of IL-22 under homeostatic conditions does not hamper B cell follicle formation or maintenance. It remains elusive if lack of IL-22 during development or inflammatory conditions, i.e. GC formation, results in an aberrant phenotype in B cell follicles. The lymphotoxin (LT) pathway is essential for lymph node development and at later stages for lymph node integrity[30,31,49]. B cells express membrane-bound LT and a possible The expression of IL-22 transcripts by stromal cells from RAG-1$^{-/-}$ mice LNs upon B cell contact might be LT dependent. The transfer of LT$^{-/-}$ B cells in RAG-1$^{-/-}$ mice will answer the question whether IL-22 transcripts expressed by the stromal compartment upon B cell contact are B cell derived LT dependent or not. The analysis of bone marrow derived stromal cells that are co-cultured with B cells or WSU-FSCCL cells in three dimensional reaggregates under high oxygen conditions, for differentially expressed IL-22 transcripts might reveal an unknown function for this interleukin in the development of ectopic structures in the BM.

Together the data described in this chapter show that B cell follicle development induces an activated phenotype in precursor FDCs and that in adult RAG-1$^{-/-}$ mice IL-22 is produced by an as yet unidentified stromal cell subset.

MATERIAL AND METHODS

Human tissue

Human fetal tissues were obtained from elective abortions contingent on the receipt of informed consent. The use of human fetal tissues was approved by the Medical Ethical Commission of the Erasmus University Medical Center of Rotterdam. Gestational age was determined by ultrasonic measurement of the skull or femur and ranged from 18 to 20 weeks.

Mice

RAG2/gc-/- (Balb/c) mice, RAG-1-/- (C57BL/6) mice and IL-22 -/- (C57BL/6) mice were bred and kept at the Erasmus MC animal facility.

Immunofluorescence

Tissue containing GFP+ cells was prior to sectioning fixed overnight in 4% paraformaldehyde with 0.15M EDTA in order to preserve the GFP signal. Cryosections 6 mm in thickness (made on a Cryostat Jung CM3050) were fixed in acetone for 5 min and were air-dried for an additional 10 min. For human tissue: after rehydration, sections of tissue was blocked with 1% (wt/vol) blocking reagent (TSAkits; Molecular Probes) and 10% (vol/vol) normal human and donkey serum, followed by treatment with a streptavidin-biotin blocking kit (Vector Laboratories). Immunofluorescence staining was done with antibodies diluted in 1% blocking reagent. Sections were incubated with primary antibodies for 30 min. at 25˚C, followed by 30 min. of incubation with Alexa Fluor–labeled antibodies (Molecular Probes) and 5% normal human or mouse serum. Biotinylated primary antibodies were incubated overnight at 4˚C and visualized using streptavidin–horseradish peroxidase as the second antibody for 60 min. at 25˚C, followed by treatment with the Tyramide Signal Amplification Kits (Molecular Probes) according to the manufacturer's instructions. Sections were embedded in Vectashield (Vector Labs). Fluorescent images were captures using a Leica DMRXA and processed Adobe Photoshop CS2.

Antibodies

Antibodies used for stainings on human tissue: rat-anti-mouse/human anti-RORg (AFKJ5-9), rabbit-anti-human-CD20 (EP459Y; Epitomics), rabbit-anti-, human-CD3ε (E272; Epitomics), mouse-anti-human-laminin (4c7; Acris), mouse-anti-human-podoplanin (D240; Covance), rabbit-anti-human-Lyve-1 (polyclonal; Abcam), mouse-anti-human-MAdCAM-1 (314G8; HBT), mouse-anti-human-VCAM-1-bio (STA, eBioscience), mouse-anti-human-TRANCE-bio (MIH24; eBioscience), CCL21 (AF336; R&D systems). mouse-anti-human-TG-II-bio (CUB 7402, Thermo Lab Vision).

Antibodies used for stainings on mouse tissue: rat-anti-mouse rat-anti-mouse-CCL21-bio (59106, R&D systems), goat-anti-mouse-CXCL13-bio (polyclonal, R&D systems), biotinylated anti-mouse FDC-M2 (clone FDC-M2; ImmunoKontact), rat-anti-mouse-RANKL-bio (R12-

31,eBioscience), rabbit-anti-mouse-Lyve-1 (polyclonal,Acris), rat-anti-mouse-VCAM-1-bio (429,eBioscience), rat-anti-mouse-B220 (6B2 hybridoma supernatant)

Cell culture

WSU-FSCCL cells (**ACC 612,** Leibniz-Institut DSMZ) and stable GFP-transduced WSU-FSCCL cells (SFFV-GFP) were cultured in RPMI medium 1640 L-glutamine (Gibco) supplemented with 8% FCS (Hyclone).

Reaggregate culture

10^5 fetal bone marrow derived stromal cells were mixed with $4*10^4$ WSU-SF-GFP cells and centrifuged for 5 minutes at 3G in a 96-wells point bottom cell culture plate (Nunc). Subsequently the formed pellet was in submersion culture of 200 µl/well DMEM (Gibco) supplemented with 10% FCS under high oxygen level conditions (1 l. pure O_2 + 50 ml. CO_2 + 300 ml. air) at 4˚ C.

Flow cytometry

Single cell suspensions were prepared from lymph nodes by dicing them into small pieces and then disaggregating with 0.5 mg/ml collagenase type IV (Sigma, St. Louis, MO) DNAse I (Bovine Pancreas, Calbiochem) in PBS for 15 -25 min at 37°C, while stirring continuously. After digestion, the LNs were filtered though a 100µm nylon mesh. Prior to labeling, cells were blocked with 10% (vol/vol) normal human serum.

A FACSAria (Becton Dickinson) was used for cell sorting. Data were analyzed with FlowJo software (TreeStar).

Femur decalicifation

Femurs of mice were prior to paraffin embedding decalcified by the following protocol: 80% EtOH 60 min., 90% EtOH 60 min., 96% EtOH 60 min., 100% EtOH 60 min. , BuOH 12 hrs., paraffine 120 min. and paraffine 120 min.

RAG-2/$yc^{-/-}$ xenograft experiment

RAG-2/$yc^{-/-}$ mice were sublethal irradiated (3 Gy) and subsequently i.v. inoculated with 10^7 GFP^+-WSU-FSCCL cells in 150 µl PBS

Rag-$1^{-/-}$ B cell transfer experiment

Spleens from C57/BL6 mice were isolated, cut into smaller fragments and disrupted with a Gentlemacs (Milteny Biotech) in the presence of 0.5 mg/ml collagenase type IV (Sigma, St. Louis, MO). Subsequently, the single cell suspension was generated by mincing the cell suspension through a 70µm filter (BD). Mononuclear cells were isolated from ficoll gradients. The mononuclear fraction was enriched for $CD19^+$ B cells using a B cell (Milteny) isolation according protocol. B cell purity was (>97%) confirmed by FACS analysis. RAG-$1^{-/-}$ mice were inoculated i.v. the tail with 10^7 B cells (in 200µl PBS).

Microarray

Total RNA of the non-hematopoietic compartment of RAG-1-/- lymph nodes was isolated using the RNA-XS kit (Machery Nagel). RNA quality and quantity was evaluated on a Agilent Bioanalyzer. Antisense biotinylated RNA was prepared and hybridized to Affymetrix Mouse Genome 430 2.0 Gene Chips using the Ovation Pico WTA system (NuGEN cat# 3300) and the Encore Biotin Module (NuGEN, cat#4200) according the manufacturers protocols.

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41

MARGINAL RETICULAR CELLS IN HUMAN FETAL AND ADULT LYMPH NODES

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ABSTRACT

As described in chapter 2 of this thesis, in human fetal and adult lymph nodes different functional subsets of RORC$^+$ ILC reside. In this study, we set out to determine the stoma niches of RORC$^+$ ILC in fetal and adult lymph nodes. We show that both fetal and adult LNs contain populations of marginal reticular cells (MRC). MRC in fetal LNs are located in the outer cortex directly underneath the subcapsular sinus (SCS). In adult lymph nodes MRC are also located directly underneath the SCS but in association with B cell follicles. In both fetal and adult lymph nodes, MRC express RANKL$^+$, MAdCAM-1$^+$ and VCAM-1 and produce CCL21 and CXCL13. This phenotype is reminiscent of murine lymphoid tissue organizer LTo cells that interact with RORC$^+$ ILC and are involved in lymph node development. In fetal LNs, RORC$^+$ ILC colocalize with RANKL$^+$ MRCs, whereas in adult LN RORC$^+$ ILC pre-dominantly reside at the interface of the T/B zone. Collectively these data show that in fetal LNs, RANKL$^+$ MRCs colocalize with RORC$^+$ ILC and in adult LN the niche for RORC$^+$ ILC does not consist of RANKL$^+$ MRCs exclusively.

INTRODUCTION

The family of innate lymphoid cells (ILC) comprises a heterogeneous group of cells that play diverse roles in immunity and maintenance of mainly epithelial homeostasis[1]. In humans, several subsets of ILC have been reported[2-6]. In chapter 2 of this thesis, we described the functional differences, i.e. IL-17 versus IL-22 production, and the distribution of subsets of RORC$^+$ ILC that were characterized by the differential expression of the cell surface markers NKp44 and CD69. We found that in contrast to fetal LNs, NKp44$^-$ CD69$^+$ RORC$^+$ ILC in adult LN do not produce IL-17.

The role of a subset of murine Roryt$^+$ ILC, the lymphoid tissue inducer (LTi) cells, in lymph node development has been well established and involves direct cell-cell contact with stromal cells of mesenchymal origin. Upon stimulation with retinoic acid, stromal cells express CXCL13 which attracts CXCR5$^+$ LTi cells[7]. These cells cluster and start to express surface lymphotoxin induced by signaling through the IL-7Rα and the receptor activator of nuclear factor kappa B (RANK) [7,8]. Subsequently LTi cells ligate the lymphotoxin-β receptor expressed by stromal cells, which are now designated lymphoid tissue organizer (LTo) cells, and this leads to the the expression of RANK-ligand (RANKL)[9] and the production of IL-7, the homeostatic chemokines CCL19/CCL21 and elevated levels of CXCL13 plus the expression of the adhesion molecules VCAM-1, ICAM-1 and MAdCAM-1[8].

The TNF molecule RANKL, also known as TNF-related activation-induced cytokine (TRANCE) or osteoprotegerin ligand (OPGL), has diverse functions but is probably best known for its role in bone homeostasis and is essential for the development and activation of osteoclasts via binding of osteoprotegerin[10]. RANKL has also been described to be involved in the survival of dendritic cells (DCs) and communication between DCs and T cells[10]. Furthermore, as described above, RANKL is essential for lymph node organogenesis in a LT-βR dependent manner[9,11,12].

In 2008, Katakai and colleagues described a novel stromal cell population in murine lymph nodes[13]. These cells are located at the interface between the B cell follicle and the subcapsular sinus and were designated Marginal Reticular Cells (MRCs). Interestingly, these MRCs phenotypically resemble stromal LTo involved in LN development and express RANKL and the adhesion molecules VCAM-1, ICAM-1 and MAdCAM-1. In addition, cell lines generated from MRC produce CXCL13 upon LTβR stimulation. The function and origin of MRC in lymph nodes, remains enigmatic. In human lymph nodes, such stromal cell subset has not been described yet.

In adult lymph nodes, stromal cells provide the optimal microenvironment for activated B cells to undergo affinity maturation and generate high affinity antibodies and for naïve T cells to encounter their cognate antigen presented by antigen presenting cells. In the T cell compartment of the LN, T zone fibroblastic reticular cells (TRCs) reside, that not only provide structural support for the migration of T cells but also deliver survival signals to the migrating T cells in the form of IL-7 and produce CCL21 for the attraction and retention of CCR7$^+$ cells[14-16]. In addition, these TRCs associate with extracellular matrix (ECM) components to form conduits which function as transport channels for low molecular weight antigens through the lymph node cortex[17].

The structural back-bone within B cell follicles is provided by follicular dendritic cells (FDCs), which are essential for generation of high affinity antibodies by presenting immune-complexes to B cells, and by supporting B cell migration and homeostasis[14]. Similar to TRC-conduits, B cell follicles contain a conduit network involved in the transport of small antigens from the subcapsular sinus to FDCs[18] .

In this study, we set out to determine the stomal niches of RORC$^+$ ILC in fetal and adult lymph nodes as knowledge on the role of the diverse stromal cells niches of ILC is currently very poor. Here we show that both fetal and adult LNs contain populations of marginal reticular cells that can be characterized by the expression of RANKL and that specifically colocalize with ILC.

RESULTS

Fetal RORC+ ILC localize in the outer cortex of fetal lymph nodes

Fetal and adult lymph nodes contain RORC$^+$ ILC that are characterized as CD45^{int} lin$^-$RORC$^+$ cells that express CD117 (c-Kit) and CD127 (IL-7Rα). While these cells have been categorized in functional subsets (chapter 2) by the analysis of differences in expression of surface markers NKp44 and CD69 and the differential production of IL-17, little is known about the niches of these cells in vivo. Therefore, we analyzed the location of CD3$^-$ RORC$^+$ ILC in cryosections of fetal lymph nodes varying in age from 17 to 20 gestational weeks. As shown in **Figure 1a,** in fetal lymph nodes CD3$^-$RORC$^+$ cells were present in the outer cortex of fetal lymph nodes. In adult LNs, CD3$^-$RORC$^+$ were localized in the interfollicular region and at the interface of the T/B zone (**Figure 1b**), as has been previously reported by S. Kim et al[19].

Marginal Reticular Cells form a distinct stromal cell subset in human fetal and adult lymph nodes

As described in the previous section, we observed that RORC$^+$ ILC in the fetal LN localized in the outer cortex. Next, we further analyzed the stromal cells that reside in the outer cortex of fetal LNs. In fetal lymph nodes (up to 20 gestational weeks) prior to the presence of B cell follicles (**Figure 2a , right panel**), a population of VCAM-1^{hi}MadCAM-1$^+$ (**Figure 2b, left panel**) and RANKL$^+$ Podoplanin$^+$ (**Figure 2b, right panel**) stromal cells was located in the outer cortex. These VCAM-1^{hi} and RANKL$^+$ stromal cells formed a layer of few cells thick directly underneath the sub- capsular sinus, similar to marginal reticular cells in the murine lymph nodes (**Figure 2c**). Next we wanted to determine, whether a population of MRC-like cells was present in adult lymph nodes. Interestingly, adult lymph nodes contained also MRC-like cells but these were restricted in their localization to the outer border of the B cell follicle directly underneath the sub-capsular sinus and the interfollicular areas **(Figure 2d**). Here, we identified and characterized the RANKL$^+$ stromal cell subset present in human fetal and adult lymph nodes as marginal reticular cell-like cells

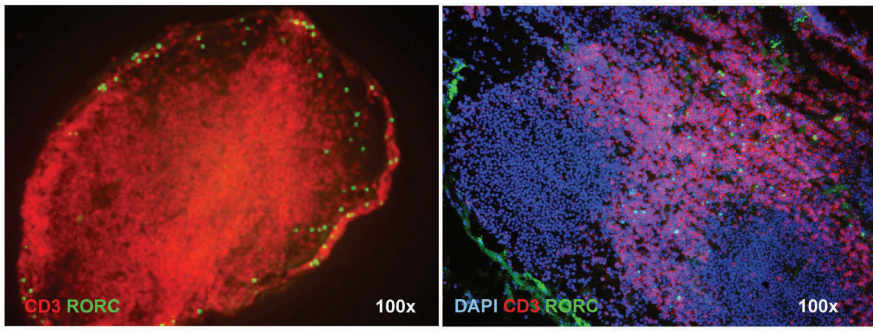

Figure 1. RORc⁺ILC in fetal and adult lymph nodes. Human fetal and adult lymph nodes were analyzed by fluorescence microscopy for the localization of RORC⁺ILCs and stained for RORC and CD3. (A) In fetal mesenteric LNs of 19 gw. CD3⁻RORC⁺ cells are located in the outer cortex.(B) In adult LNs CD3⁻RORC⁺cells reside in the the interfollicular region and at the interface of the T/B zone .

Human MRC-like cells display a gene expression profile similar to LTo

The MRC-like cells present in fetal and adult lymph nodes expressed surface markers that are characteristic for LTo. To determine whether the MRC-like cells also have functional properties of LTo, we set out to analyze the expression of functionally relevant molecules by human MRC. Stromal cells in fetal human lymph nodes were sorted into MRCs and FRCs based on the expression of podoplanin and RANKL (**Figure 3a**). Based on the histology data depicted in **Figure 2b**, podoplanin^{hi} RANKL^{hi} cells were classified as MRCs, while podoplanin^{hi} RANKL^{lo} cells were classified as FRCs. The sorted cells were then analyzed by real time PCR for RANKL, the chemokines CXCL13, CCL19 and CCL21 and IL-7. As shown in Fig 3B, MRCs showed at least two fold higher expression of RANKL. The IL-7 expression in MRCs was found to be lower than in the FRC fraction. CCL19 transcripts were higher in FRCs than in MRCs. CCL21, which has previously been reported to be expressed only in the T cell zone, was found to be expressed at higher levels on MRCs. CXCL13 levels were much higher in human MRC-like cells As shown by real time PCR analysis, we found expression of both CCL21 and CXCL13 by MRC-like cells. MRC-like cells in adult lymph nodes also expressed CXCL13 and CCL21 protein as analyzed by histology on frozen sections (**Figure 3c**).

Together, these data indicate that both fetal and adult lymph nodes contain a population of MRC-like cells that display phenotypic and functional similarities to LTo in the fetal LN anlagen.

RORC⁺ ILC colocalize with RANKL⁺ MRC-like cells in the outer cortex of fetal LNs

In the previous sections we have shown that RORC⁺ ILC in the fetal LN reside in the outer cortex and that the local stromal cell subset is made up of RANKL⁺ MRC-like cells. **Figure 4a** depicts an overview of a fetal LN of 19 gw. The majority of RORC⁺ cells colocalized with RANKL⁺ stromal cells in the outer cortex of the fetal LN, whereas the bulk of CD3⁺ cells resided in the medulla. The RORC⁺ cells in the outer cortex did overall not express CD3 as depicted in **Figure 4b**. Together, these show that in fetal LNs of 15-20 gw MRC-like cells with a LTo phenotype colocalized with RORC⁺ ILC.

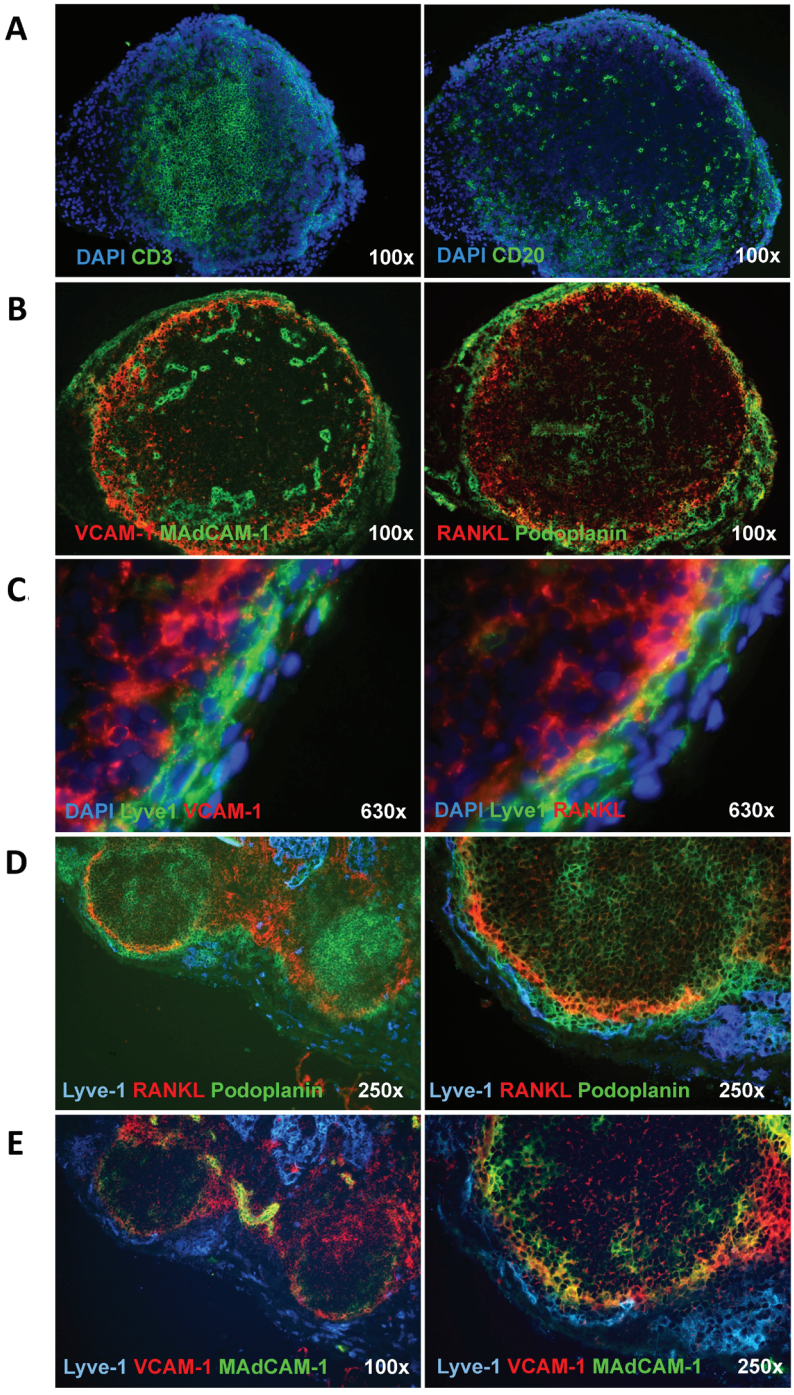
A
DAPI CD3
100x
DAPI CD20
100x
B
VCAM-1 MAdCAM-1
100x
RANKL Podoplanin
100x
C
DAPI Lyve1 VCAM-1
630x
DAPI Lyve1 RANKL
630x
D
Lyve-1 RANKL Podoplanin
250x
Lyve-1 RANKL Podoplanin
250x
E
Lyve-1 VCAM-1 MAdCAM-1
100x
Lyve-1 VCAM-1 MAdCAM-1
250x

Figure 2. MRCs are a distinct subset of stromal fibroblasts in human lymph nodes. In order to analyze fetal and adult lymph nodes for the presence of MRCs, frozen sections of human fetal mesenteric and adult lymph nodes were analyzed by fluorescence microscopy for the distribution of lymphocytes and for the expression of the indicated MRC related markers. (A) $CD3^+$ T cells are located in the paracortex and developing medulla of the lymph node, while $CD20^+$ B cells are located in the cortex. (B) A distinct population of stromal cells in the outer cortex is positive for the adhesion molecules VCAM-1 and MAdCAM-1 (left panel) and in addition positive for RANKL and Podoplanin (right panel). (C) A closer examination at higher magnification shows that the VCAM-1 (left panel) and RANKL (right panel) expressing stromal cells are located directly underneath the Lyve-1 expressing subcapsular sinus. To determine if stromal cells with a MRC-like phenotype are present in adult LNs, non-inflamed hepatic LNs were stained for VCAM-1 and MAdCAM-1 (D) and Podoplanin and RANKL (E). Stromal cells positive for the MRC-like markers are located at the border of B cell follicles in association with lymphatic endothelial cells.

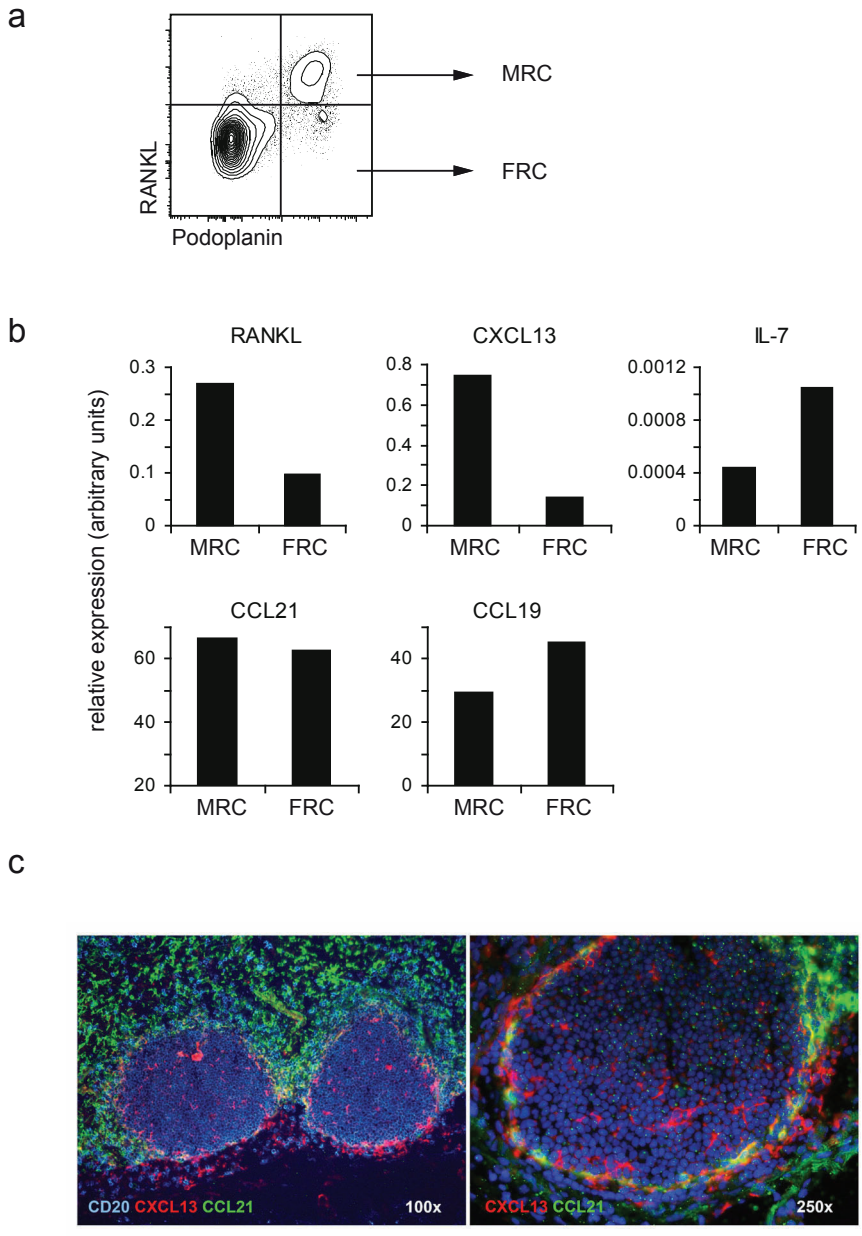

Figure 3. Human MRCs present with a LTo gene expression profile.
(A). The CD45^{-}CD31^{-} fraction of fetal human lymph nodes was sorted into MRCs and FRCs based on RANKL and Podoplanin staining. (B) RNA was extracted from these cell populations and real time PCR was performed for *il-7*, *cxcl13*, *rankl*, *ccl19* and *ccl21*. Gene expression was normalized to *GAPDH*. (C) Frozen sections of human adult non-inflamed LNs were stained for CD20, CCL21 and CXCL13. CCL21 is abundantly expressed by T zone fibroblasts and CXCL13 by follicular dendritic cells within the B cell follicle. Stromal cells located at the border of the B cell follicle express CXCL13, CCL21 or both (right panel).

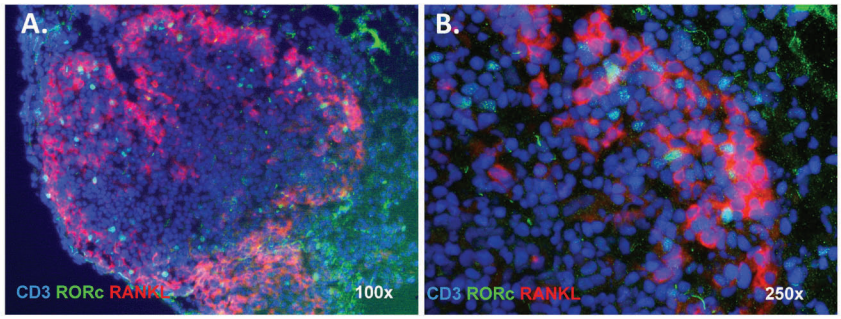

Figure 4. RANKL$^+$ MRCs are associated with CD3$^-$ RORC$^+$ ILC. Human fetal MLNs were analyzed for the localization of RORC$^+$ ILC and MRCs by fluorescence microscopy. Therefore, frozen sections of fetal MLNs of 19 gw. were stained for CD3, RORgt and RANKL. Cells negative for CD3 but positive for RORC are closely associated with RANKL$^+$ stromal cells in the outer cortex of fetal MLNs.

DISCUSSION

The importance of the family of RORC$^+$ ILC in the early defense against mucosal pathogens and in maintaining tissue integrity during homeostasis and disease is gaining appreciation[2,20-22]. While many studies have addressed the characteristics, function and distribution of various subsets of ILC in health and disease, little is known about the niches that these cells occupy in the human system. In this study we identified the niche for RORC$^+$ ILC in human fetal lymph nodes.

We found that RORC$^+$ ILC in fetal lymph nodes of 17-20 gestational weeks co-localized with RANKL$^+$ stromal cells in the outer cortex. The stromal location of RORC$^+$ ILC in adult lymph nodes has been described in a previous report[19] and we could confirm that RORC$^+$ ILC in adult LN reside at the T/B interface.

The RANKL$^+$ stromal cells in present fetal LNs were also observed in adult LN. Further analysis of this stromal population indicated these cells as the human equivalents of the murine MRC that Katakai and colleagues reported in 2008[13]. These human MRC-like cells not only displayed a phenotype that is reminiscent of LTo cells, but also expressed transcripts for the B cell chemokine CXCL13 and the T cell and dendritic cell chemokines CCL19/21. The expression of these homeostatic chemokines suggests that these MRC-like cells might facilitate encounters with RORC$^+$ ILC, with B and T cells and DCs. As shown in figure 1A, we observed that B cells reside in the outer cortex of the fetal LN and co-localized with RORC$^+$ ILCs. Furthermore, we observed DC-sign positive cells co-localizing with RANKL$^+$ MRC-like cells (data not observed). This is in accordance with the role of RANKL for survival signals for DCs.

The function of MRC-like cells in fetal and adult lymph nodes, remains enigmatic. These cells might be direct descendants of LTo cells that are found in the LN anlagen and this could mean that these cells are very plastic since it has been hypothesized that LTo can give rise to all LN stromal cell subsets[8]. MRC produce CXCL13, CCL19/CCL21 and might therefore facilitate encounters of B cells, T cells and DC. Furthermore, RANKL expressed by MRCs was shown to be essential for murine LN development[12,23,24] and more recently, a specific role for RANKL in follicle development was established[25]. Based on these murine studies and the localization of RANKL$^+$ MRC in fetal and adult human lymph nodes, MRCs might play a role in the maintenance of B cell follicle integrity upon infection and damage.

Furthermore, Katakai and colleagues reported that MRCs are developmentally programmed since they could not be observed in induced ectopic gastric lymphoid structures[13]. However, whether MRCs are present in human ectopic lymphoid structures is not known. The analysis of biopsies of patients with rheumatoid arthritis for the presence of MRCs is currently subject of research.

As described in chapter two of this thesis, CD69$^+$NKp44$^-$RORC$^+$ ILC in fetal LN are positive for IL-17a transcripts while this cell subset in adult lymph nodes is negative for IL-17a transcript. It has been documented that IL-17A increases the production of RANKL by osteoclasts[26,27] and a similar function for IL-17A produced by CD69$^+$ LTi cells in the cortex of fetal LNs can be envisaged. It needs to be determined, whether the CD69$^+$NKp44$^-$ RORC$^+$ ILC in fetal and adult

lymph nodes are developmentally related or that they belong to another branch of the RORC$^+$ ILC family.

Together the data described in this chapter show that fetal and adult LNs contain RANKL$^+$ MRCs. In fetal LNs RANKL$^+$ colocalize with RORc$^+$ ILC, in adult LN the niche for RORC$^+$ ILC does not consist of RANKL$^+$ MRCs exclusively.

MATERIAL AND METHODS

Human tissue

Human fetal tissues were obtained from elective abortions contingent on the receipt of informed consent. The use of human fetal tissues was approved by the Medical Ethical Commission of the Erasmus University Medical Center of Rotterdam. Gestational age was determined by ultrasonic measurement of the skull or femur and ranged from 18 to 20 weeks. Lymph nodes were dissected from the mesentery with dissecting microscopes. Adult hepatic lymph nodes were collected post-mortem during multi-organ donation procedures.

Flow cytometry

Single cell suspensions were prepared from lymph nodes by dicing them into small pieces and then disaggregating with 0.5 mg/ml collagenase type IV (Sigma, St. Louis, MO) DNAse I (Bovine Pancreas, Calbiochem) in PBS for 15 -25 min at 37°C, while stirring continuously. After digestion, the LNs were filtered though a 100µm nylon mesh. Prior to labeling, cells were blocked with 10% (vol/vol) normal human serum. The following antibodies were used: Alexa Fluor-488-conjugated mouse-anti-podoplanin (D240; Biolegend), phycoerythrin-conjugated mouse-anti-human RANKL (MIH24; eBioscience), peridinin chlorophyll protein–cyanine 5.5–conjugated mouse-anti-CD45 (2D1; eBioscience), phycoerythrin –cyanine 7- conjugated mouse-anti-human CD34 (8G12; BD Biosciences) and phycoerythrin -TexasRed-conjugated mouse-anti-human CD31 (MEM-05; ImmunoTools). A FACSAria (Becton Dickinson) was used for cell sorting. Data were analyzed with FlowJo software (TreeStar).

PCR

RNA was isolated using the RNA-XS kit (Machary-Nagel), followed by reverse-transcription with random hexamer primers. A Neviti Thermal Cycler (Applied Biosystems) and DyNAmo Flash SYBR Green qPCR kit (Finnzymes) were used for quantitative PCR, with the addition of MgCl2 to a final concentration of 4 mM. All reactions were done in duplicate and are normalized to the expression of GAPDH (glyceraldehyde phosphate dehydrogenase). Relative expression was calculated by the cycling threshold (CT) method as $2^{-\Delta t}$.

Table 1. Primer sequences (5' → 3')

Gene	Forward Primer	Reverse Primer
GAPDH	GTC GGA GTC AAC GGA TT	AAG CTT CCC GTT CTC AG
RANKL	GTG CAA AAG GAA TTA CAA CA	CGG TGG CAT TAA TAG TGA G
CXCL13	CCT CCA GAC AGA ATG AAG TT	AGG GTC CAC ACA CAC AAT
CCL21	GTA CAG CCA AAG GAA GAT TC	GGG GAT GGT GTC TTG TC
CCL19	AGC CTG CTG GTT CTC TG	TGC AGC CAT CCT TGA T
IL7	CCT CCC CTG ATC CTT GTT CT	CGA GCA GCA CGG AAT AAA AA

Immunofluorescence

Cryosections 6 mm in thickness were fixed in acetone for 5 min and were air-dried for an additional 10 min. For intracellular straining, cryosections were fixed in acetone for 20 min at 4°C and air-dried for an additional 10 min. After rehydration, sections of human tissue were blocked with 1% (wt/vol) blocking reagent (TSAkits; Molecular Probes) and 10% (vol/vol) normal human and donkey serum, followed by treatment with a streptavidin-biotin blocking kit (Vector Laboratories). Immunofluorescence staining was done with antibodies diluted in 1% blocking reagent. Sections were incubated with primary antibodies for 30 min. at 25˚C, followed by 30 min. of incubation with Alexa Fluor–labeled antibodies (Molecular Probes) and 5% normal human serum. The Rorγt antibody was incubated for 120 min. at room temperature. Biotinylated primary antibodies were incubated overnight at 4˚C and visualized using streptavidin–horseradish peroxidase as the second antibody for 60 min. at 25˚C, followed by treatment with the Tyramide Signal Amplification Kits (Molecular Probes) according to the manufacturer's instructions. Sections were embedded in Vectashield (Vector Labs). Antibodies used, were as follows: rat-anti-mouse/human anti-RORg(AFKJ5-9)rabbit-anti-human-CD20 (EP459Y; Epitomics), rabbit-anti-, human-CD3ε (E272; Epitomics), mouse-anti-human-laminin (4c7; Acris), mouse-anti-human-podoplanin (D240; Covance), rabbit-anti-human-Lyve-1 (polyclonal; Abcam), mouse-anti-human-MAdCAM-1 (314G8; HBT), mouse-anti-human-VCAM-1-bio (STA, eBioscience), mouse-anti-human-TRANCE-bio (MIH24; eBioscience), goat-anti-human-CXCL13-bio (AF3844; R&D systems), and goat-anti-human-CCL21 (AF336; R&D systems).

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MARGINAL RETICULAR CELLS IN THE HUMAN FETAL SPLEEN

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Submitted

ABSTRACT

The spleen is the largest secondary lymphoid organ and is formed during fetal development. As described in previous chapters of this thesis, the development of LN involves RORC$^+$ ILC and lymphoid tissue organizer (LTo) cells. Although murine spleen development has many overlaps with LN development, Rory$^+$ ILC play no role in the development of the white pulp in mice. Murine studies indicated that lymphotoxin$^+$ B cells are essential for the development of the white pulp of the murine spleen. A stromal organizer-like cell subset involved in fetal spleen development has not been described. In this study we analyzed human fetal spleens varying from 15-20 gestational weeks (gw) for stromal cell subsets. We describe in fetal spleens of 18 gw onwards at sites where B cells cluster, a stromal cell subset that is MAdCAM-1 and VCAM-1 positive. These cells are positive for transcripts of RANKL, CCL21 and CXCL13. RORC$^+$ ILC present in fetal spleens of 18-20 gw displayed random distribution and a phenotype similar to RORC $^+$ILC in fetal LNs. Based on these findings we propose that MAdCAM-1 marks a putative stromal organizer of the T cell zone in human fetal spleen.

INTRODUCTION

The spleen is the largest secondary lymphoid organ (SLO) of the human body. It is the site where immune responses are induced against blood-borne pathogens, where effete erythrocytes are removed from the blood circulation and the spleen functions as a storage compartment for plasma cells [1]. These diverse functions are made possible by the unique structure of the spleen that can be divided into two compartments: the red pulp (RP) with its open sinusoidal system, and the white pulp (WP) that is made up of sheats of lymphoid tissue.

The structure of the adult spleen has been described in several studies[2-7]. The bulk of T and B lymphocytes that reside in the WP are compartmentalized in the periarteriolar lymphoid sheath (PALS) and B cell follicles, respectively. This cellular organization resembles lymph nodes and enhances the chance of efficient encounters of naïve T cells with antigen presenting dendritic cells (DCs) as well as encounters of T cells with B cells. The distinct T/B compartments are supported by specialized stromal cell subsets that also interact with the residing hematopoietic cells, not only to enhance survival but also to optimize proper immune responses. The stromal compartment of the T zone (the PALS) is mainly made up of fibroblasts that correspond to fibroblastic reticular cells (FRCs) that form a network of conduits to which $CCR7^+$ T cells and DCs adhere via the expression of the chemokines CCL19 and CCL21[8,9]. In literature the reticular cells of the PALS are also designated interdigitating cells (IDCs)[10,11]. The reticular cells of the PALS wind around collagen fibers that function as anchors along which T cells travel and dendritic cells adhere[12,13]. The stromal cells of the B cell area are the follicular dendritic cells (FDCs), the reticular cells of the marginal zone and the marginal reticular cells (MRCs). FDCs are the best characterized stromal cell subset of the B cell area and express high levels of CXCL13 thereby attracting $CXCR5^+$ B and T_{fh} cells. FDCs express Fc receptors and complement receptors which are involved in the presentation of unprocessed antigen in the form of immune-complexes to B cells. FDCs play an important role in the development of germinal center reactions and isotype switching and somatic hypermutations of the B cell receptor [14]. The marginal zone (MZ) is a structure exclusively found in the spleen and acts as a boundary between the WP and the RP. The MZ is important in the early defense against encapsulated bacteria in the blood. The MZ harbors a unique set of B cells, the MZ B cells, that can respond very quickly to encapsulated bacteria by the production of IgM antibodies in a T cell independent manner. Compared to rodents, the human spleen has a larger MZ and is surrounded by a perifollicular zone that separates the WP from the RP. The unique MZ is made up of stromal reticular cells that are a scaffold for the MZ B cells as well as DCs and macrophages. In rodents, there is a population of macrophages that is exclusively found in the MZ. In humans no MZ specific macrophage population has been described. In 2008 Katakai and colleagues described a novel stromal cell subset that presents with characteristics of stromal lymphoid tissue organizer (LTo) cells and are common to murine secondary lymphoid organs, the marginal reticular cells (MRCs)[15]. In the murine spleen, MRCs are located in the inner ring of the marginal zone in close contact to the B cell follicle and express

CXCL13, VCAM-1 and MAdCAM-1 and RANKL. Currently, no data exist on the presence of MRCs in the human fetal and adult spleen.

The development of SLOs such as lymph nodes depends on signals downstream of the LTβ-R. In mice deficient for LTα or the LTβ-receptor, lymph nodes and PPs are absent and T/B cell compartmentalization of the white pulp in the spleen is lost[16,17]. Mice deficient for Rorγt and/or the inhibitor of DNA binding protein Id2, lack LTi cells and no LNs and PPs develop. However, the spleens of these gene-deficient mice do have segregated B and T cell compartments[18,19]. Tumanov and colleagues generated mice with the LT-β gene specifically deleted in B cells (B-LTβ-KO)[20]. In contrast to the systemic deletion of LT-β, in B-LTβ-KO mice only the splenic microarchitecture was affected with only a few FDCs and not able to induce a proper immunoglobulin response. In these mice, LN and PPs developed normally, albeit that the latter was reduced in size. Together these data indicate that LT^+ B cells are involved in the development of the WP of the murine spleen. The specific role of LTi cells in WP development is still not fully elucidated. This is partly due to the fact that while LTi cells are already present in the developing murine spleen from embryonic day (E) 13 onwards, most data about the requirement of lymphotoxin (signaling) for proper development of the WP is obtained from spleens from newborn mice from day 4 and onwards. These spleens contain LT^+ B cells that colonize the spleen and T cell influx has begun[21-23].

The human spleen develops *in utero*. The developmental stages and cells involved in early spleen development are therefore not well defined. Several studies have characterized the development of the human fetal spleen by performing electron microscopy and immunohistochemistry on paraffin embedded fetal spleens. Together, these studies give an overview of the cellular hematopoietic and non-hematopoietic organization in fetal spleens varying from the 11th gestational week (gw.) until neonatal children[10,11,24,25]. However, no study has touched upon the presence of stromal LTo-like cells in WP development and the interaction of this stromal cell subsets with B, T cells and LTi cells. The presence of stromal LTo(-like) cells in the developing human spleen has never been evaluated.

In this study we analyzed human fetal spleens varying from 15-20 gestational weeks for the presence of LTo-like cells and (precursors) of the major stromal cell subsets of the WP by immunofluoresence which allows the combination of multiple markers. The presence of a LTo-like stromal cell subset was analyzed in correlation with B and T cell organization and the presence of LTi cells in the developing human fetal spleen[26].

RESULTS

Identification of distinct stromal cell subsets in the adult human white pulp

The bulk of T and B lymphocytes that resided within the white pulp of the adult spleen were compartmentalized in distinct B and T zones as depicted in **Figure 1a**. T cells were clustered around central arterioles (white arrows), hence the name periarteriolar lymphoid sheeth for the T zone of the adult spleen and B cells were organized into B cell follicles. The red pulp of the adult spleen contained an open sinusoidal system that was made up of $CD31^+$ (PECAM-1) vascular tissue (**Figure 1b**). In the white pulp, CD31 staining was absent except for the central arteriole (**Figures 1b and 1c**). Although the expression of CD31 is not restricted to vascular endothelial cells and is also present on the surface of macrophages, neutrophils monocytes and platelets, CD31 staining as a marker for vascular endothelium of the spleen has been established[27]. The stromal cell subsets that make up the WP present with a differential expression of homeostatic chemokines, adhesion molecules and extracellular matrix proteins. As such, these markers could be used to identify the different stromal cell subsets in the WP of the adult spleen. The B cell attracting chemokine CXCL13 was expressed by $CD31^-$ stromal cells that were associated with the B cell follicle and by the $CD31^+$ central arteriole that in the WP. The marginal reticular cells that surrounded the B cell follicle and the follicular dendritic cells that resided within the B cell follicle also both expressed CXCL13 (**Figure 1c**). MRCs and activated FDCs also expressed the adhesion molecule VCAM-1 (**Figure 1c**). Reticular cells delineating the B cell follicle were positive for MAdCAM-1 adjacent to the follicular side and positive for smooth muscle actin (SMA) adjacent to red pulpa, but negative for VCAM-1. These cells were characterized as the reticular cells of the marginal zone (MZ) (**Figures 1d and 1e**). The PALS was made up of mainly fibroblastic reticular cells that expressed MAdCAM-1, VCAM-1 (**Figure 1d, white arrow**), smooth-muscle actin (SMA) (**Figure 1e, white arrow**). Furthermore, this T zone area was rich in collagen I and IV fibers (**Figure 1f, white arrow**). Based on the expression of collagen IV one can easily distinguish the white pulp from the red pulp (**Figure 1f**). In the red pulp, collagen IV was abundant while the presence of collagen IV in the white pulp was very limited.

These data suggest that with the application of the above described markers, determination of the major stromal cell subsets in the developing fetal spleen of 15-20 gw can be performed.

B and T cells cluster around CXCL13-expressing arterioles and are not segregated into distinct B and T cell compartments at 15-18 gestational weeks

B cells present in the developing fetal spleen of 15 gw. clustered around a CXCL13 expressing arteriole, while the few T cells had a more scattered distribution throughout the developing spleen. (**Figures 2a and c**). At 18 gw. B cell clusters had increased in number and size and were interspersed with T cells (**Figures 2b and d**). At 15-18 gw. B and T cell compartmentalization was not observed. This finding is in accordance with previous reports that described that splenic B cell follicle development occurs from 24 gw. onwards.

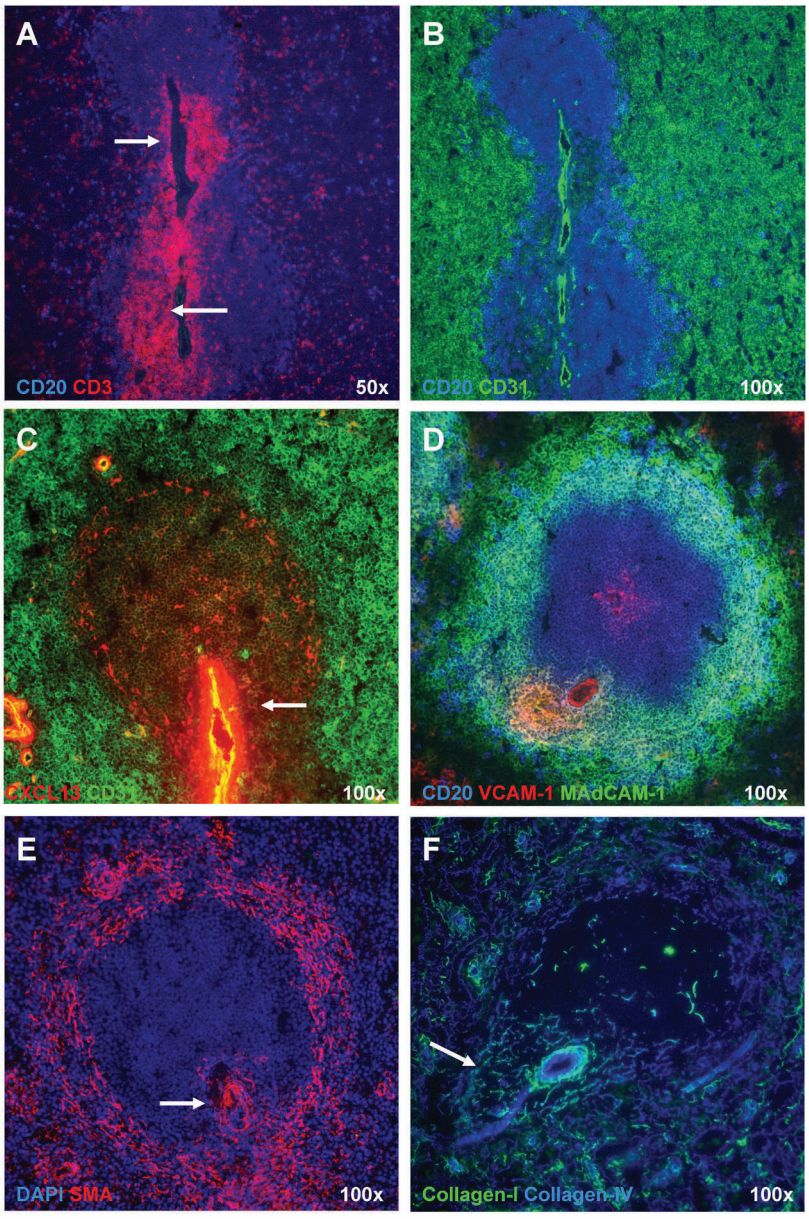

Figure 1. **Stromal cell subsets of the human adult white pulp (WP).** To visualize the lymphocyte distribution and the stromal cell subsets of the WP of the human adult spleen, frozen sections were stained for the indicated markers and analyzed by fluorescence microscopy.(A) In the WP of the human adult spleen the bulk of $CD20^+$ B and $CD3^+$ T lymphocytes are compartmentalized in follicles and the PALS (white arrows), respectively. (B) CD31 (PECAM-1) is expressed by the endothelium of the red pulp and by the central arteriole, but not by stromal cells of the WP. The different stromal cell subsets present in the WP can be identified by the differential expression of the applied markers. (C) CXCL13 (red) and CD31 (green), (D) CD20 (blue), VCAM-1 (red), MAdCAM-1 (green), (E) DAPI (blue), smooth muscle actin (SMA) (red), (F) Collagen-I (green), Collagen-IV (blue).

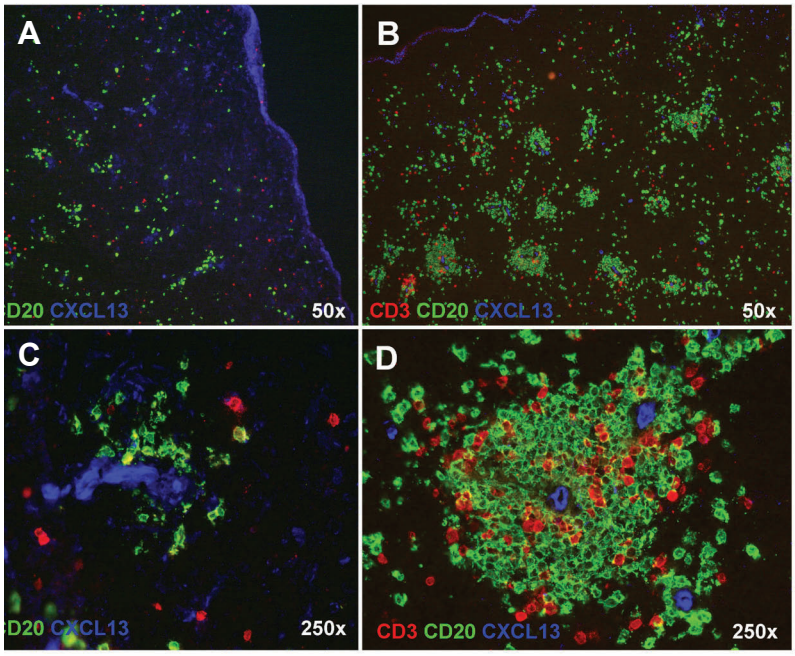

Figure 2. Lymphocyte clusters around arterioles in developing human fetal spleens of 15-18 gw. Human fetal spleens were stained for CD3, CD20 and CXCL13 and analyzed by fluorescence microscopy for the distribution of and clustering of B and T cells. At 15 gw., B lymphocytes were higher in numbers than the few scattered T cells and were the first lymphocytes to cluster around CXCL13^{+} arteries (A,D). At 18 gw, the number and size of lymphocyte clusters was increased and consisted of B lymphocytes interspersed with T cells (B,D).

Putative stromal organizer-like cells express MAdCAM-1 and VCAM-1

The adhesion molecule MAdCAM-1 was widely expressed by endothelial cells in the developing fetal spleen of 15 gw. and 18 gw. (**Figures 3a and b**). As described in the preceding section, between 15 and 18 gw, the number and size of B cell clusters interspersed with T cells increased. From 18 gw. onwards a subset of non-hematopoietic cells co-localizing with B and T lymphocyte clusters started to express MAdCAM-1 (**Figure 3b, white arrows**). This was not observed in smaller B cell clusters at earlier time-points during development (**Figure 3c**). These MAdCAM-1^{+} cells were not of vascular or lymphatic endothelium origin since they did not express the lymphatic endothelium marker Lyve-1, nor the vascular endothelium marker CD31 (**Figures 3e,f and g**). The MAdCAM-1^{+} stromal cells were found to be aso positive for the adhesion molecule VCAM-1 (**Figures 3f and g**).

The expression of both MAdCAM-1 and VCAM-1 by non-vascular and non-lymphatic stromal cells at sites of periarteriolar B cells and T cell cluster, suggests that these cells might be stromal organizer-like cells involved in the development of the white pulp. The expression of MAdCAM-1 by CD31^{-} stromal cells may therefore be the earliest marker for WP stroma.

MAdCAM-1$^+$CD31$^-$ stromal cells in fetal spleens express transcripts with a LTo signature

The MAdCAM-1$^+$ VCAM-1$^+$ stromal cells we observed in spleens from 18-20 gw were negative for vascular and lymphatic endothelial markers, and were solely observed at sites where large B cells clusters were found. We wanted to confirm the presence of this stroma cell subset by flow cytometry and analyzed fetal spleens from 14-15 gw and fetal spleens from 18-20 gw onwards for the presence of CD45$^-$CD31$^-$MAdCAM-1$^+$ stromal cells (**Figure 4a**).

In concordance with our earlier observations on cryosections, CD31$^-$MAdCAM-1$^+$ stromal cells were virtually absent in fetal spleens from 14 gw but were present in fetal spleens of 18-20 gw. We hypothesized that theseCD31$^-$ MAdCAM-1$^+$ stromal cells might represent a LTo-like cell subset and could be involved in the organization of the white pulp of the human fetal spleen. To test this hypothesis we analyzed purified CD31$^-$MAdCAM-1$^-$ and CD31$^-$MAdCAM-1$^+$ stromal cells from fetal spleens from 18-20 gw for the relative expression of transcripts of LTo signature genes, i.e. LTβ-R, RANKL and the homeostatic chemokines CXCL13 and CCL21. **Figure 4b** depicts the results obtained from four different donors. Even though there was heterogeneity amongst the four samples, a clear difference in the expression of LTo related gene transcripts between the MAdCAM-1 positive and negative stromal cell subset could be observed. The mean of relative expression levels for transcripts for LTβ-R, RANKL and the homeostatic B cell chemokine CXCL13 was respectively 19.6, 5.37 and 1.83-fold higher in the MAdCAM-1$^+$ stromal cell subset compared to the MAdCAM-1 negative population. The most striking difference in levels of relative gene transcripts expression between the two stromal cell subsets, was observed for CCL21. Transcripts for this homeostatic chemokine were exclusively and highly expressed in the MAdCAM-1$^+$ stromal cells.

Together, these data suggest that in human fetal spleens from 18-20 gw MAdCAM-1$^+$VCAM-1$^+$ LTo-like stromal cells reside that co-localize with large B cell clusters and might be involved in the development of the WP.

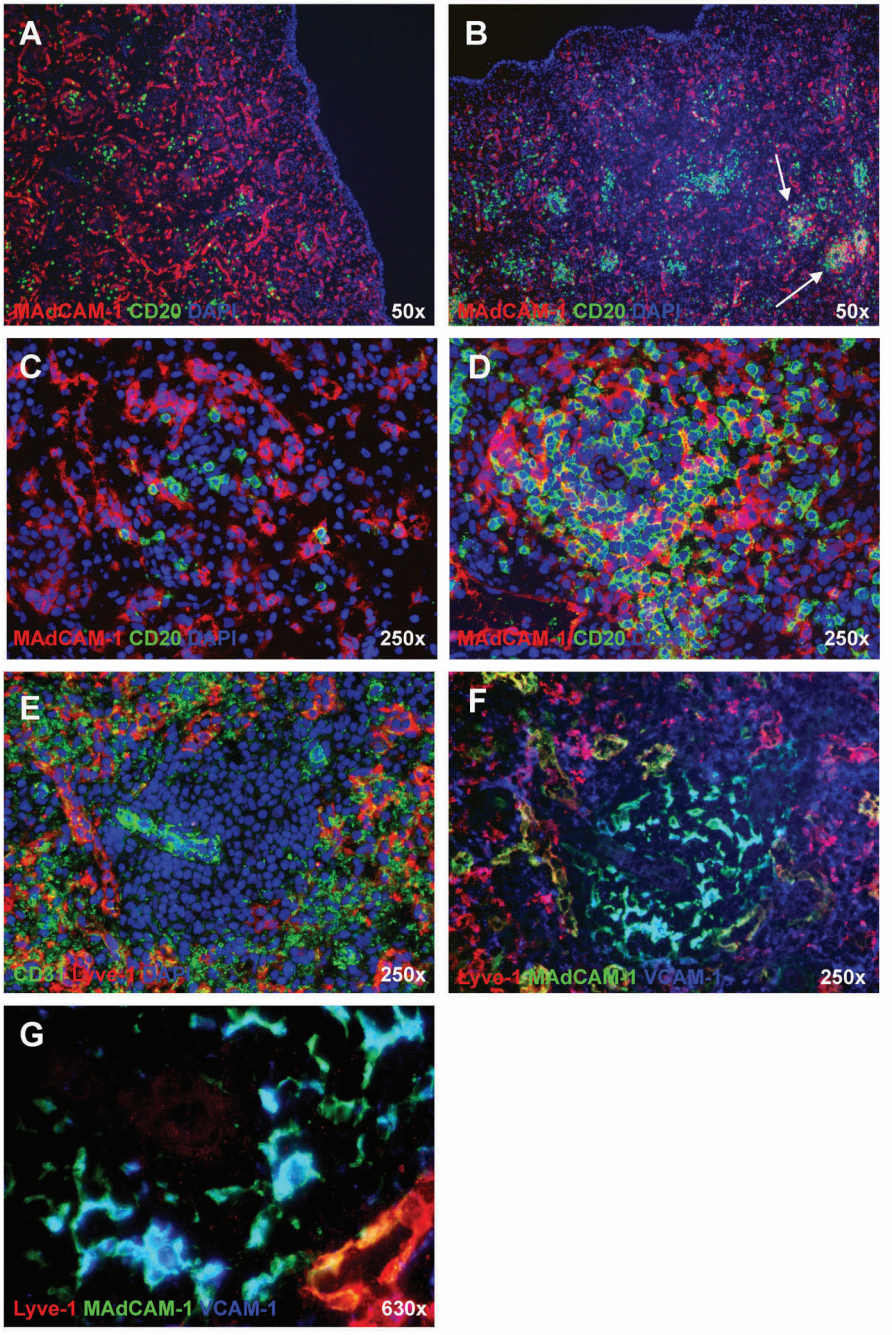

Figure 3. Fetal MAdCAM-1 and VCAM-1 expressing stromal cells co-localize with large B cell clusters. To visualize the stromal cell subset that supports the large B cell clusters in human fetal spleens of 15 and 18 gw., frozen sections were stained for the indicated markers and analyzed by fluorescence microscopy. In human fetal spleens of 15 gw weeks $CD20^+$ B cells are low in number (A,C). From 18 gw onwards $CD20^+$ B cell numbers increase and larger B cells clusters develop (B, white arrow, and D). Stromal cells that co-localize with large B cell clusters do not express Lyve-1 and CD31 (E) but do express both MAdCAM-1 and VCAM-1 (F and G).

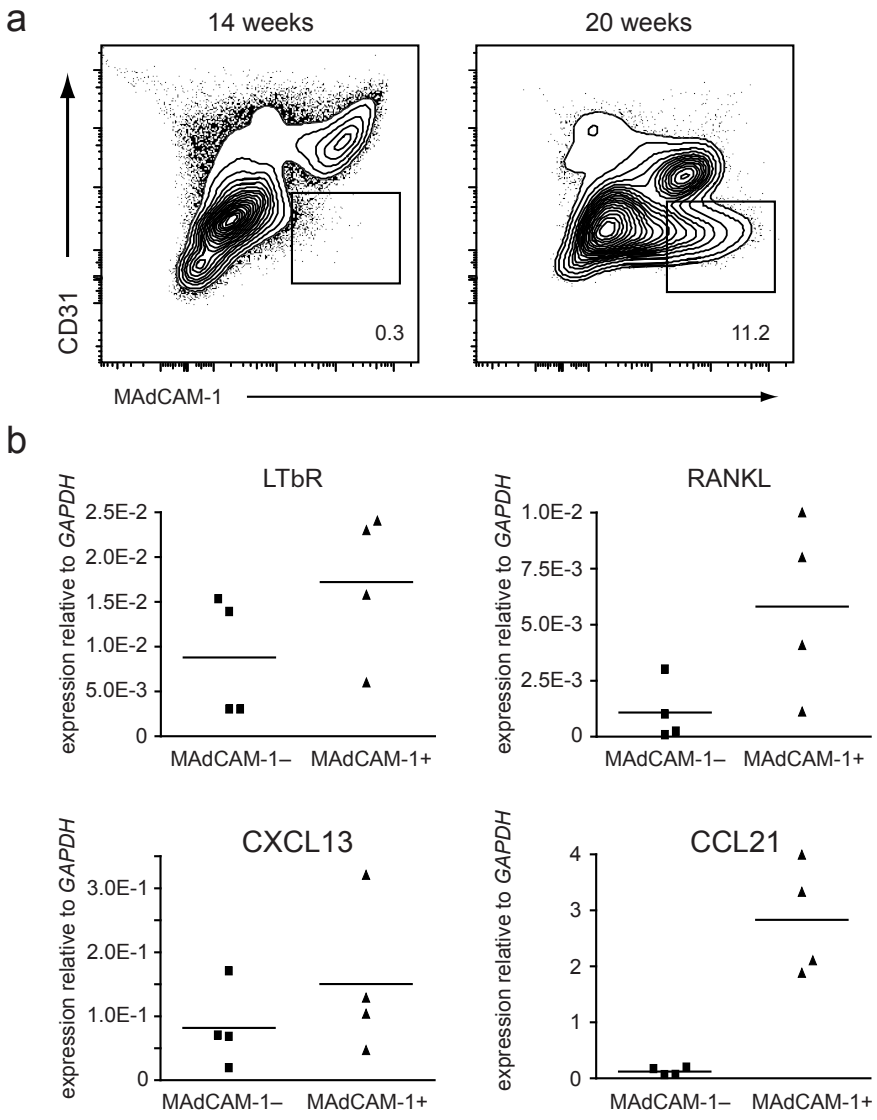

Figure 4. Non-vascular MAdCAM-1$^+$ stromal cells in human fetal spleens of 18-20 gw, display a LTo gene expression profile. (A). Flow cytometric analysis of human fetal spleens of 14 gw. and 20 gw. for the presence of CD45$^-$CD31$^-$ MAdCAM-1$^+$ cells . Cell suspensions were depleted from B cells (B) MAdCAM-1$^+$ and MAdCAM-1$^-$ non vascular stromal cells isolated from spleen from 18-20 gw were sorted and analyzed for the expression of gene transcripts of LTβR, RANKL, CXCL13 and CCL21 relative to GAPDH.

Precursors of marginal zone reticular cells of the white pulp are not present in the human fetal spleen of 19 gestational weeks

As discussed previous, the reticular cells of the marginal zone (MZ) of the human adult spleen were positive for both MAdCAM-1 and smooth muscle antigen (SMA) (**Figures 1d and e**). We also observed that the fetal spleen of 18-19 gw. of age had no compartmentalization of B and T lymphocytes. In previous reports it has been described that from 24 gw. onwards the T zone starts to develop[24] and after the formation of the PALS from 26 gw onward, B cells get organized into a B follicle and the marginal zone develops. Currently, there is controversy about the initiation of MZ development in the human fetal spleen. At 19 gw, SMA was expressed by vessels and at a very low level by reticular cells colocalizing with the large B lymphocyte clusters (Figure 5a). At this stage of development, the expression of MAdCAM-1 (**Figure 5b**) and SMA by stromal cells of the early white pulp appeared to be mutually exclusive .

Together these data show that MZ reticular cell-like cells are absent at 19 gw.

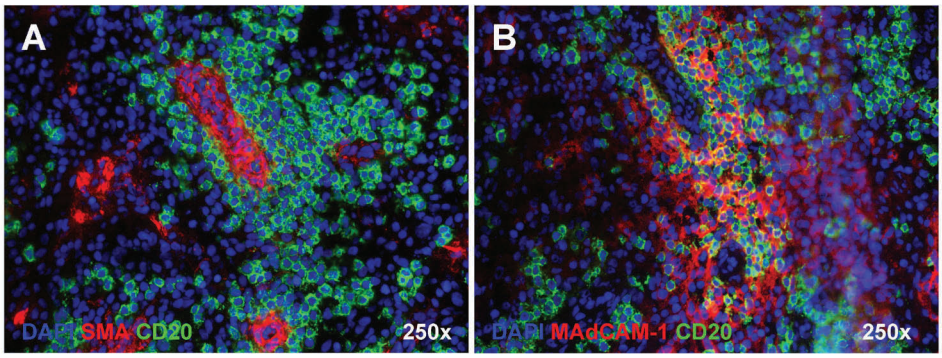

Figure 5. Stromal cells with a marginal zone reticular-like phenotype are absent in developing fetal spleens of 19 gw. The WP of fetal spleens of 19 gw. was analyzed by fluorescence microscopy for the presence of smooth muscle actin (SMA) and MAdCAM-1 expressing stromal cells. (B) MAdCAM-1 is expressed by stromal cells that colocalize with large B cell clusters in contrast to (A) SMA expressed by vessels at this stage.

LTi cells in the human fetal spleen have a phenotype similar to LTi cells in fetal mesenteric lymph nodes

Human LTi cells have been described in fetal MLNs and LTi-like cells have been described in postnatal tonsils and adult intestines. To determine the presence of LTi cells in human fetal spleens, we analyzed lineage$^-$ (lin$^-$; CD3$^-$CD20$^-$CD14$^-$CD34$^-$) cells that express intermediate levels of CD45 and high levels of CD117 (c-Kit) and CD127$^+$(IL-7Rα) for the expression of intracellular RORc protein. Of the lineage$^-$CD45^{int} cells, only 1.9% was CD117^{hi}CD127^{hi} LTi cell (**Figure 6a**). As depicted in **Figure 6b**, all cells in this gate expressed the RORc protein. To determine whether expression of NCRs by LTi cells from fetal spleens was a fetal lymph node phenotype or a mucosal/ postnatal phenotype, we analyzed the expression of NKp46, NKp44 and NKp30 on LTi cells derived from fetal spleen. The expression of NCRs by LTi cells from fetal spleens was similar to LTi cells derived from fetal MLN. About 52% of the LTi cells expressed NKp30 while expression of NKp46, and NKp44 was lacking (**Figures 6c and d**). The expression of CD69 and

CD25, both markers of T cell activation was restricted to a minority of the fetal splenic LTi cells (**Figures 6e and f**). In the fetal MLN, CD69 expression is correlated to IL-17a production by LTi cells. Whether CD69$^+$ LTi cells in the fetal spleen produce IL-17 is subject of further research as is the observed CD25 expression by fetal spleen derived LTi cells. Together these data show that LTi cells in the fetal spleen have a phenotype similar to LTi cells in fetal MLN. The cytokine profile of LTi cells in fetal spleen is subject of further investigation.

RORC$^+$ lymphoid tissue inducer cells do not co-localize specific with MAdCAM-1 expressing stromal cells of the early white pulp

Recent murine data indicate that lymphoid tissue inducer (LTi) cells do not play a role in the development of the white pulp into lymphoid tissue with segregated B and T zones. So far, we showed the presence of LTi cells in the human fetal spleen by flow- cytometry. To determine the localization of LTi cells *in situ* we double-stained frozen sections of fetal spleen for LTi cells with MAdCAM-1. As expected, the number of LTi cells was very low. The spatial distribution of the LTi cells showed no specific localization and was not associated with MAdCAM-1$^+$ stromal cells at sites of large B and T cell clusters (**Figures 7a,c and e**).

The random distribution of LTi cells in the fetal spleen of 19 gw. suggests that these cells are not directly involved in the induction of white pulp stroma. A role for LTi cells in imprinting of the stroma, as has been suggested for mice, or if they play a role in later stages of development remains to be determined.

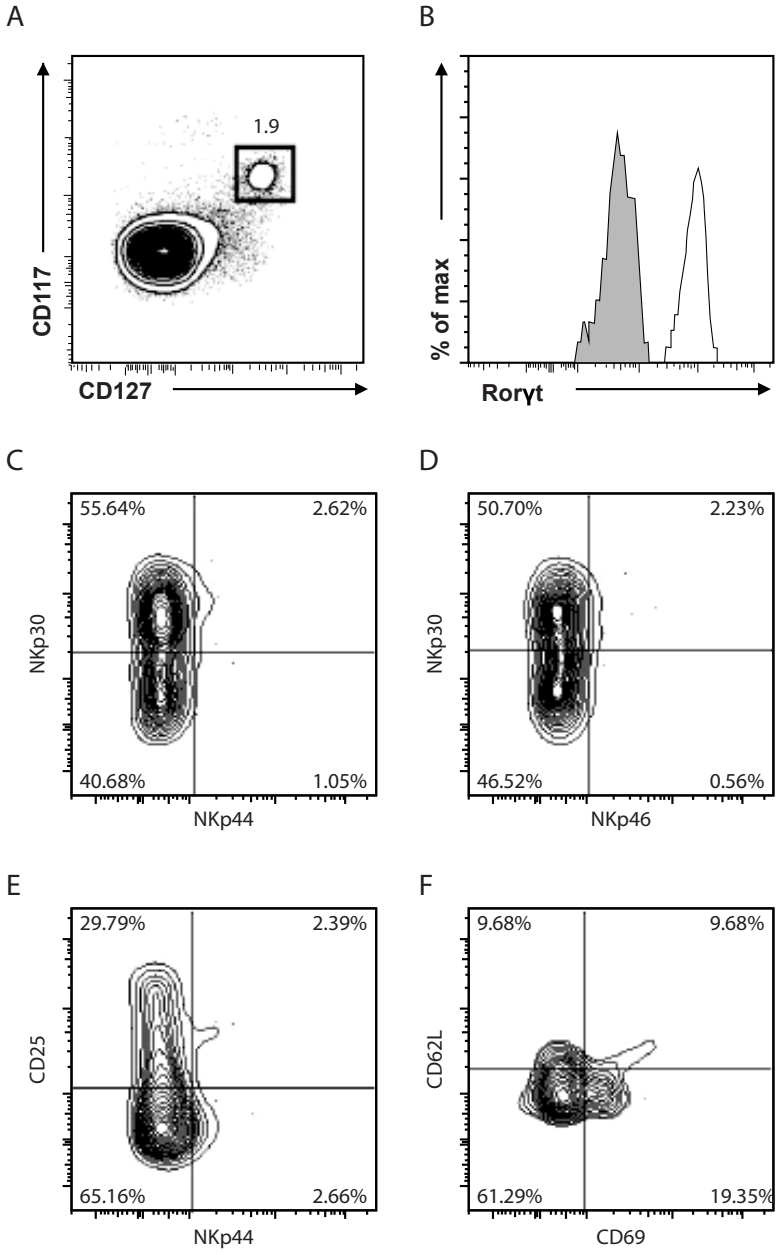

Figure 6. Human fetal spleen derived RORC⁺ LTic display a phenotype similar to RORC⁺ LTi in fetal MLN. LTic were characterized as lineage negative CD127⁺CD117⁺ cells (A) and analyzed for the expression of the intracellular Rorc protein by flow cytometry (B). (C,D) Flow cytometric analysis of ILC from fetal spleens for NKp44, NKp30 and NKp46 (representative example of 2 independent experiments). (E). Flow cytometric analysis of CD25and NKp44 and (F) CD62L and CD69 expression on LTic from fetal spleens (representative of three independent experiments).

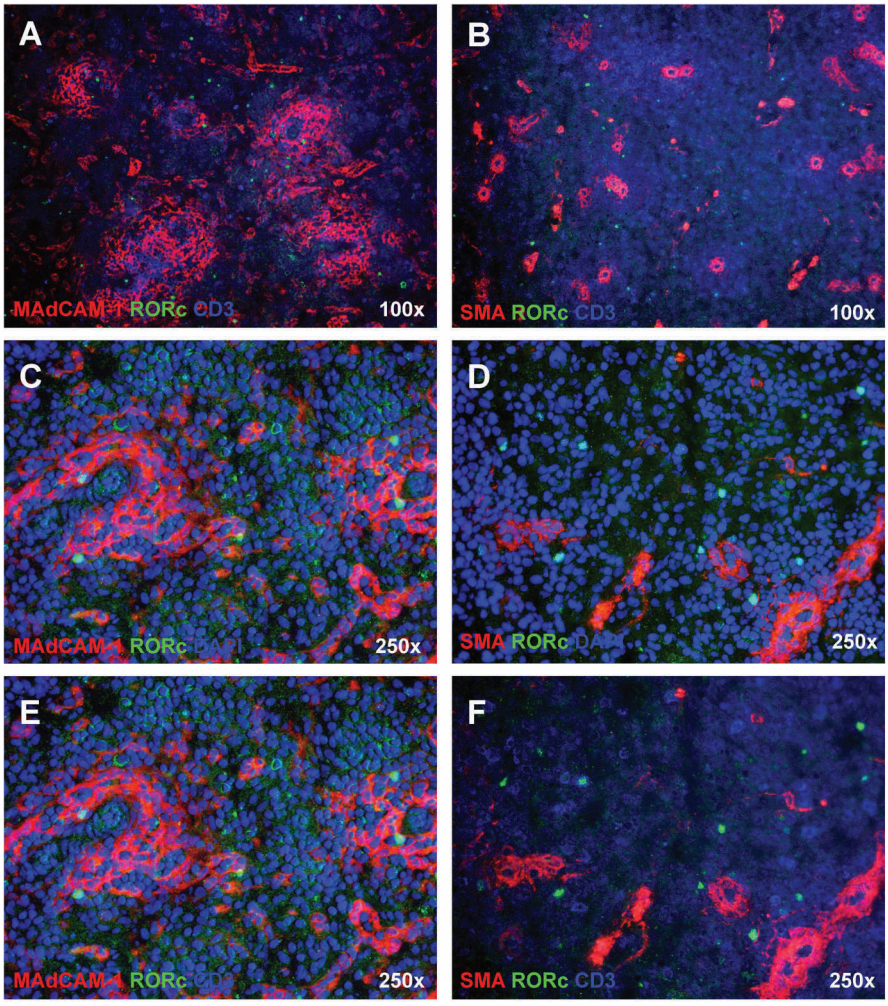

Figure 7 CD3$^-$RORC$^+$ LTic do not exclusively colocalize with MADCAM-1$^+$ LTo-like cells. The role of LTi cells in the development of the human WP is uncertain. In order to visualize the localization of RORc$^+$ LTic in the fetal spleen, frozen section of fetal spleens of 19 gw were stained for RORC, CD3, MAdCAM-1 and smooth muscle actin (SMA). RORc$^+$CD3$^-$ ILC are present but few in numbers and do not preferentially co-localize with MAdCAM-1$^+$ (A, C and E) or SMA$^+$ (B, D and F) stromal cells.

DISCUSSION

The spleen has a unique anatomy that supports its diverse functions and functions as a storage site for several populations of immune cells, iron and erythrocytes, is involved in the removal of effete erythrocytes and is important in the defense against blood-borne pathogens. Patients after splenectomy or with functional asplenia are at risk of developing overwhelming post-splenectomy infections (OPSI)[28].

Knowledge on the development of the human fetal spleen is, for obvious reasons, restricted to data obtained from descriptive microscopy studies performed on tissue sections[2-5,7,10,11,24,29]. Functional data on the development of the spleen comes mostly from murine studies in which signaling pathways known to be involved in the development of SLOs are affected or ablated, as extensively reviewed by Mebius and Kraal in 2003[1]. A good understanding of the development of the human spleen is currently lacking. The murine spleen develops with overlapping but slightly different rules and players when compared to lymph node development. In addition to this, the human spleen differs anatomically from its murine counterpart.

In this study we identified a stromal cell subset within the human fetal spleen of 18-20 gestational weeks that is equivalent to the Marginal Reticular Cells present in the murine spleen [15] as well as the Marginal Reticular Cell–like cells present in human fetal and adult lymph nodes (chapter 4 of this thesis). Furthermore, we showed that fetal spleen derived LTi cells have a similar phenotype as RORC$^+$ LTi cells present in fetal lymph nodes (chapter 2) but that these cells do not co-localize with MRC-like stromal cells in the fetal spleen of 18-20 gw.

Both murine MRCs and human MRC-like cells express RANKL at the protein level as can be visualized by immunofluorescence microscopy[15 and chapter 5]. In human fetal and adult spleens, we could not detect RANKL protein expressing cells . However, gene transcripts for RANKL were present in the isolated MRC-like population from fetal spleen of 18-20 gw. Whether the MRC-like cells do not translate RANKL transcript in protein is currently not known. Another possible explanation for our inability to identify RANKL at the protein level, might that RANKL proteolytically processed into its soluble form (sRANKL) which would be washed off during our protocols for protein staining on cryosections. It has been described in literature that MMP14 is capable of cleaving RANKL into sRANKL and can be produced by many cell types such as endothelial cells and macrophages[30]. In fetal spleens from 14-20 gw DC-Sign positive cells were observed in large numbers (unpublished data). In addition, Steiniger and colleagues observed CD4$^+$CD68$^+$ macrophages[24]. In the human fetal spleen, MRC-like cells develop at sites where large clusters of B cells intermingled with T cells reside adjacent to open endings of arteries. These stromal cells are VCAM-1$^+$MAdCAM-1$^+$ and express transcripts for RANKL, LTβ-R, CXCL13 and CCL21. This gene expression profile corresponds with a MRC-like phenotype and is similar to that of lymphoid tissue stromal organizer (LTo) cells present in embryonic lymph node anlagen[31and chapter 5 of this thesis]. The high levels CCL21 transcript expression observed in the MRC-like cells are in conclusion with data of previous reports that describe that the human PALS is formed from the 17th gw onward prior to the development of the B cell follicle[24] which

develops from 24 gw onward[24,25,32]. In addition to this, in mice CCL21 expression was found to be obligatory for the segregation of B and T cells and was found to be induced in a LT dependent manner by both B cells and to a lesser extend LTi cells[33].

Similar to our observations in the human fetal lymph node, B cells in the fetal spleen co-localized with MRC-like cells. However, in contrast to fetal LNs of 15-22 gw., RORC$^+$ ILC in fetal spleens of the same gestational age did not co-localize with MRC-like cells. This finding suggests that at this stage in development, RORC$^+$ ILC do not play a direct role in the development of the human WP. This observation is in conclusion with a study performed by Vondenhoff et al., which shows that LTi cell in neonatal mice do not express membrane bound lymphotoxin and are therefore suggested to play no further role in the development of the WP[23]. In addition to this, it has been described that mice with a specific deletion of the LTβ gene in B cells show aberrant development of the white pulp indicating that B cells are required for the proper LT signal to the LT-βR expressed by the local stromal cells[20]. Together these data suggest that B cells in the fetal spleen of 18-19 gw, might signal via the LT-LTβ-R pathway to induce further development of the white pulp.

In conclusion to previous reports, we could not observe an developing MZ at this stage in fetal spleen development [24,25]. Our data complement the findings of Steiniger that B cells in fetal spleens of approximately 15-18 gw reside around SMA$^+$ reticular cells located adjacent to large arteries[24]. We observed that SMA$^+$ reticular cells also resided within the MAdCAM-1$^+$ MRC-like area and could not detect any overlap of these two subsets of reticular cells in the developing fetal WP.

In this study we identified MRC-like cells with a LTo-like phenotype that are putatively involved in the development of the human WP in the fetal spleen of 18-20 gw. The observation that these MRCs do not co-localize with LTi cells but do appear at sites where large B cell clusters intermingled with T cells reside, indicates that B cells rather than LTi cells interact with MRCs thereby inducing the development of the WP.

MATERIAL AND METHODS

Human tissue

The use of all human tissues was approved by the Medical Ethical Commission of the Erasmus University Medical Center of Rotterdam and contingent on informed consent. Fetal tissue was obtained from elective abortions. Gestational age was determined by ultrasonic measurement of the skull or femur and ranged from 15to 20 weeks. Adult spleens were obtained post-mortem during multi organ donation procedures.

Immunofluorescence

Cryosections 5-6 mm in thickness were fixed in acetone for 5 min and were air-dried for an additional 10 min. When stained for RORγt, sections were fixed in acetone for 20 minutes by a temperature of -20 °C and air-dried. After rehydration, sections of human tissue were blocked with 1% (wt/vol) blocking reagent (TSAkits; Molecular Probes) and 10% (vol/vol) normal human and donkey serum, followed by treatment with a streptavidin-biotin blocking kit (Vector Laboratories). Immunofluorescence staining was done with antibodies diluted in 1% blocking reagent. Sections were incubated with primary antibodies for 30 min. at 25˚C, followed by 30 min. of incubation with Alexa Fluor–labeled antibodies (Molecular Probes) and 5% normal human serum. Biotinylated primary antibodies were incubated overnight at 4˚C and visualized using streptavidin–horseradish peroxidase as the second antibody for 60 min. at 25˚C, followed by treatment with the Tyramide Signal Amplification Kits (Molecular Probes) according to the manufacturer's instructions. Sections were embedded in Vectashield (Vector Labs). Antibodies used, were as follows: rabbit-anti-human-CD20 (EP459Y; Epitomics), rabbit-anti-human-CD3ε (E272; Epitomics), mouse-anti-human-laminin (4c7; Acris), mouse-anti-human-MAdCAM-1 (314G8; HBT), mouse-anti-human-VCAM-1-bio (STA; eBioscience), goat-anti-human-CXCL13-bio (BAF801; R&D systems), and goat-anti-human-CCL21 (AF336; R&D systems), mouse-anti-human collagen I (COL-1; Abcam), rabbit-anti-human collagen IV (polyclonal; Abcam), mouse-anti-human SMA (1A4; Sigma), rat-anti-mouse/human ROR(g)t (AFKJS-9; eBioscience).

Cell preparation

Fetal spleens were dissected from the mesentery with dissecting microscopes. Single cell suspensions were prepared by dicing spleens into small pieces and then disaggregating with collagenase type IV (0.5 mg/ml; Sigma, St. Louis, MO) and DNAse I (Bovine Pancreas, Calbiochem) in PBS for 15 -20 min at 37°C. After digestion spleens were filtered (70 μm for lymphocytes and 100μm for stromal cells) in DMEM (Invitrogen) supplemented with 8% (vol/vol) FCS (Hyclone) and DNAse I. Erythrocytes were lysed using Lysing Solution (Stem-Kit Reagents, Beckman Coulter). Prior to labeling for stromal cell sort, cells were further enriched for obtaining a CD45- population using the DynaMag-50 in combination with Dynabeads Biotin Binder (both Invitrogen, Dynal AS, Oslo, Norway) .

Flow cytometry and cell sorting

The following antibodies were used: Alexa Fluor 647–conjugated anti-NKp46 (9E2) and anti-NKp44 (P44-8), peridinin chlorophyll protein-anti-CD117(A3C6E2), phycoerythrin-anti-NKp30(P30-15), biotinylated-anti-CD117(104D2), allophycocyanin-anti-VCAM-1 (STA), pacific blue-conjugated-anti-CD31 (WM59) (all from Biolegend), allophycocyanin –eFluor 780–anti-CD127 (eBioRDR5), phycoerythrin Cy7-anti-CD45(HI30)(all from eBioscience), phycoerythrin -anti-CD69(FN50), phycoerythrin-DY-590–conjugated anti-CD3(MEM-57), phycoerythrin-DY-590–conjugated anti-CD19(LT19), phycoerythrin-DY-590–conjugated anti-CD14(MEM-15), phycoerythrin-DY-590–conjugated -CD34(4H11) (all from ExBio), phycoerythrin -anti-ROR(g)t (AFKJ5-9; Ebioscience), biotin-conjugated anti-CD45 (eBioscience), phycoerythrin-anti-CD62L (SK11; BD), MAdCAM-1 (314G8; HBT), FITC-conjugated anti-CD25 (Immunotools). For cell sorting and FACS analysis, a FACS Aria (Becton Dickinson) was used. Data were analyzed with FlowJo software (TreeStar).

Quantitative RT-PCR Analysis

RNA from human tissues was extracted using the RNA-XS kit (Machery Nagel) followed by reverse-transcription with random hexamer primers. A Neviti Thermal Cycler (Applied Biosystems) and DyNAmo Flash SYBR Green qPCR kit (Finnzymes) were used for quantitative PCR, with the addition of $MgCl_2$ to a final concentration of 4 mM. All reactions were done in duplicate and are normalized to the expression of GAPDH (glyceraldehyde phosphate dehydrogenase). Relative expression was calculated by the cycling threshold (CT) method as $2^{-\Delta t}$.

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GENERAL DISCUSSION

INTRODUCTION

The human body contains approximately 600-700 lymph nodes (LNs)[1]. These secondary lymphoid organs (SLO) function as central players in our immune system by facilitating encounters of antigen presenting cells with lymphocytes and enhancing the chance of activation of antigen specific lymphocytes. The development of human lymph nodes occurs during fetal life and starts approximately at 8-11 gestational weeks[2,3]. The number and type of studies on human LN development are limited and functional studies on LN development are obtained predominantly from mouse studies. Murine lymph node development starts between 12-14 days of gestation and involves direct cell-cell contact of lymphoid tissue inducer (LTi) cells with lymphoid tissue organizer (LTo) cells. Upon retinoid acid induced CXCL13 expression by stromal cells, $CXCR5^+$ LTi cells cluster and start to express surface lymphotoxin induced by signaling through the IL-7Rα and the receptor activator of nuclear factor kappa B (RANK) [4,5]. The mature LTi cells ligate the lymphotoxin-β receptor expressed by LTo cells and this event leads to the expression of RANK-ligand (RANKL)[6] and the production of IL-7, the homeostatic chemokines CCL19/CCL21, elevated levels of CXCL13 plus the expression of the adhesion molecules VCAM-1, ICAM-1 and MAdCAM-1 all involved in the attraction and retention of LTi cells and circulating lymphocytes[5]. Mice deficient for Rorγt and/or the inhibitor of DNA binding protein Id2 lack LTi cells and hence no LNs and Peyer's patches (PPs) develop. However, spleens develop normal in these gene-deficient mice and contain segregated B and T cell compartments[7,8]. In addition, in mice deficient for LTα or the LTβ-receptor, LNs and PPs are absent and T/B cell compartmentalization of the white pulp in the spleen is lost[9,10]. Together these data indicate that related yet different mechanisms can give rise to lymphoid structures with segregated B and T cell compartments.

Human $RORC^+$ILC

In 2009, IL-17a producing cells resembling murine LTi cells were described in fetal lymph nodes[11]. At the same time studies reported a IL-22 producing NK/LTi-like cell population present in human tonsils and adult intestines[11,12]. The discovery of these cytokine producing cells led to a gain of interest in innate immunity[11-21]. Crellin et al. showed in an elegant study, that human IL-22 producing NK/LTi-like cells are a stable $RORC^+$ expressing line and therefore are not related to NK cells[22]. In a proposal for uniform nomenclature, IL-17a and IL-22 producing $RORC^+$ LTi-like cells were categorized in the expanding family of innate lymphoid cells (ILC)[23]. ILC make up a family of lymphocytes that lack a specific antigen receptor and are capable of initiating rapid responses to pathogens[24]. From mouse studies we know that all ILC derive from a common $Id2^+$ progenitor cell in the fetal liver and bone marrow. The subsequent differentiation into a mature ILC is mediated by specific transcription factors[25]. Based on the cytokine production profiles and the expression of lineage specific transcription factors, ILC have been classified into three distinct groups resembling the various T helper cell subgroups[23].

Group 1 comprises the ILC1. This group of ILC expresses the transcription factor T-bet and produces "type 1" cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α) in order to induce immune responses to intracellular pathogens[24]. The expression of the lineage transcription factor T-bet is not required for the development of ILC1 since NK cells, the best studied member of this group, develop in the absence of the T-box transcription factor[26-29].

Group 2, the ILC2, depend for lineage specification on transcription factor GATA-3 [30]. ILC2 are widely expressed and secrete IL-5 and IL-13 in order to mediate immune responses during helminth infections and allergies[24,31-34]. Human ILC2 are defined by the expression of CRTH2[35].

Lineage specification into the ILC3 lineage requires the stable expression of Roryt[36]. LTi cells involved in the development of LNs and PPs and postnatal IL-17 and IL-22 producing RORC$^+$ ILC belong to the ILC3 family. Cytokine producing RORC$^+$ ILC are recognized as important mediators of early innate immunological defenses against mucosal pathogens and as essential players in tissue integrity during infections. Human ILC3 can produce a variety of effector molecules involved as reviewed by Cornelissen et al[37].

Functional distinct subsets of human RORC$^+$ILC

Human RORC$^+$ ILC can produce IL-17 and IL-22. In mice, Rorc$^+$ ILC derived IL-22 was shown to play an important role in the early defense against intestinal bacterial infection with *Citrobacter rodentium*[15] while Rorc$^+$ ILC-derived IL-17a was shown to be pathogenic in T-cell independent intestinal inflammation[38].

A detailed analysis of the distribution and role of human RORC$^+$ ILC in homeostasis and pathological conditions was up to now hampered due to the absence of markers that distinguish IL-17a$^+$ RORC$^+$ ILC from IL-22$^+$ RORC$^+$ ILC. In chapter 4 we show that expression of NKp44 is a good predictor for RORC$^+$ ILC that are actively transcribing IL-22 and are mainly found in mucosal tissues. In addition, our data suggest that non-inflamed lymph nodes may function as a reservoir of resting, RORC$^+$ non-cytokine secreting ILC that lack NKp44 expression, yet retain the capacity to secrete IL-22 after appropriate cytokine activation. Furthermore, RORC$^+$ ILC already present in the intestine at early second trimester start to express NKp44 but display low levels of IL-22 transcription which gradually increases in levels comparable to postnatal. The role of bacterial colonization in inducing higher levels of IL-22 is still under debate[39-41].

IL-17a has been associated with several diseases and is associated with an inflammatory response against mucosal infections as well as several autoimmune diseases[42-44]. Freshly isolated CD69$^+$NKp44$^-$ RORC$^+$ ILC from fetal LNs are positive for transcripts of *IL-17a.* The biological function of IL17a produced by RORC$^+$ ILC in fetal LNs is still not unraveled. Even though LN development resembles a controlled inflammation in many aspects, IL-17a is unlikely to have an essential function during organogenesis as IL-17Rα deficient mice display no gross abnormalities in LN development (KH and TC, unpublished observations)[45,46]. However, the development of inducible bronchus associated tissue (iBALT) in mice was shown to depend on CD4+ T cell derived IL-17[47]. The production of IL-17a by T cells is stringent controlled in order to avoid

unwanted or excessive inflammation [48]. Freshly isolated CD69$^+$NKp44$^-$ ILC in non-inflamed lymph nodes lack *IL17A* transcripts suggesting that similar levels of restraint are operational for ILC. Unraveling the exact signals that can either induce or inhibit ILC activation is needed to understand regulation and control of innate IL-17 secretion.

RORC$^+$ ILC colocalize with RANKL$^+$ in fetal LNs

In murine LN anlagen LTi cells interact with LTo cells. In 2008, Katakai et al. described Marginal Reticular Cells in adult lymphoid organs. In fetal LNs of 15-20 gw, prior to B cell follicle formation, RORC$^+$ ILC are located at the outer cortex (chapter 4) while in adult LNs, RORC$^+$ ILC reside at the interface of the T/B zone which was in concordance with an earlier finding by Kim et al.[49], and at the apical site of B cell follicles. Stromal cells that reside directly underneath the subcapsular sinus in the cortex of fetal LNs of 17-20 gw and thus co-localize with RORC$^+$ ILC display a phenotype reminiscent of LTo cells, and MRC cells, i.e. RANKL$^+$VCAM-1$^+$MAdCAM-1$^+$ and positive for transcripts of the homeostatic chemokines CXCL13 and CCL21. In adult LN MRC-like cells co-localize with RORC$^+$ ILC. The function of MRC-like cells in fetal and adult lymph nodes remains enigmatic. The expression of the homeostatic chemokines suggests that MRC-like cells might facilitate encounters with RORC$^+$ ILC, with B and T cells and DCs. We observed in fetal LNs, the co-localization of RORC$^+$ ILC and RANKL$^+$ MRCs suggesting a direct or indirect interaction between these two cell types. Furthermore, unpublished data (TC and KH) suggests that RORC$^+$ ILC co-localize with DC-Sign$^+$ cells in the RANKL$^+$ outer cortex of fetal LNs.

RORC$^+$ ILCs and MRCs in the human fetal and adult spleen

Spleen development in the mouse has overlapping but slightly different mechanisms compared to lymph node development. In addition, the human spleen differs anatomically from its murine counterpart[50]. In chapter five of this thesis we identified a MAdCAM-1$^+$ stromal cell subset in the developing white pulp of the human fetal spleen from 18-20 gestational weeks onwards that has LTo characteristics. MAdCAM-1$^+$ MRC-like cells in the human fetal spleen expressed VCAM-1 and transcripts for RANKL, LTβ-R, CXCL13 and CCL21. This gene expression profile corresponds with a MRC-like phenotype and with LTo cells present in embryonic lymph node anlagen[5] and chapter 5 of this thesis. While murine and human MRC in fetal and adult LNs express the RANKL protein[51] and chapter 5, no RANKL protein was observed in human fetal and adult spleens. Whether the MAdCAM-1$^+$MRC-like cells of the fetal spleen do not translate RANKL transcript in protein is currently not known. In contrast to RORC$^+$ ILC in fetal LN, we did not observe a specific co-localization of RORC$^+$ ILC with MAdCAM-1$^+$ MRC-like stromal cells in the fetal spleen of 18-20 gw. This finding suggests that at this stage in development, RORC$^+$ ILC do not play a direct role in the development of the human WP. This observation is in line with a study performed by Vondenhoff et al., which shows that splenic LTi cell in neonatal mice do not express membrane bound lymphotoxin and are therefore suggested to play no further role in the development of the WP[52]. In addition, it has been described that mice with a specific deletion of the LTβ gene in B cells show aberrant development of the white pulp

indicating that B cells are required for the proper LT signal to the LT-βR expressed by the local stromal cells[53]. Together these data suggest that B cells in the fetal spleen of 18-19 gw might signal via the LT-LTβ-R pathway to induce further development of the white pulp.

In line with previous reports, we could not observe MZ-like structure this stage of spleen development[54,55]. Our data complement the findings of Steiniger that B cells in fetal spleens of approximately 15-18 gw reside in proximity of SMA^+ reticular cells located adjacent to large arteries[55]. We observed that SMA^+ reticular cells also reside within the $MAdCAM\text{-}1^+$ MRC-like area and could not detect any overlap of these two subsets of reticular cells in the developing fetal WP.

B cell follicle development and GC-like structures

In human adults, tertiary lymphoid organs (TLO) can develop at ectopic sites under persistent inflammatory conditions or by low grade malignancies such as follicular lymphoma in the bone marrow (BM)[56-58]. The mechanisms and cellular components required for secondary lymphoid organ (SLO) development, might remain functional throughout adulthood and contribute to the formation of TLO[59]. In order to understand the mechanisms by which disseminated follicular lymphoma is capable of inducing a GC-like structure at extra-nodal sites such as the bone marrow we set out to study B cell follicle formation *in vitro* and *in vivo*.

Low grade FL cells depend on the local microenvironment for survival. Due to the lack of a cell line reflecting an indolent B cell lymphoma, we performed 3-D *in-vitro* cultures and xenograft experiments in $RAG\text{-}2^{-/-}\gamma c^{-/-}$ mice using cell line WSU-FSCCL. This cell line grows independent of external stimuli and might therefore not be representative for primary low grade FL. However, in our 3D co-culture with WSU-FSCCL we observed the induction of transglutaminase (TG) and VCAM-1 on fetal BM derived stromal cells. Interestingly, Vega et al. describe the upregulation of TG by the FRC network in FL infiltrated LN[60]. In addition the upregulation of VCAM-1 during FL B cell induced lymphoid stroma differentiation has also been described[61]. Together these data suggest that remodeling of fetal BM derived stromal cells in our 3-D *in vitro* model could be used with primary low grade FL cells. The results from the $RAG\text{-}2^{-/-}\gamma c^{-/-}$ xenograft model shows that human malignant B cells disseminate in the bone marrow preferentially to bone lining niches and the trabeculae suggesting $RAG\text{-}2^{-/-}\gamma c^{-/-}$ are a good model to study *in vivo* behavior of FL B cells at ectopic sites.

Stromal cells of early developing B cell follicles lack expression of CCL21 and RANKL (chapter 4 of this thesis). Mice deficient for CCR7 lack the characteristic distribution of B cell follicles[62]. In murine peripheral LN development, a role for CCR7 ligands has been reported[63,64]. We observed that absence CCL21, the ligand for CCR7, in cortical stromal cells in human fetal LN delineates the early developing B cell follicle from the residing MRC. A putative role for CCL21 in B cell follicle development has not been put forward yet. However, overlapping roles for CXCL13 and CCL21 have been reported in regard to the attraction of LTi cells[63]. In addition we have observed in developing human fetal LNs, $CD3^-CD127^+$ cells forming a rim around the developing B cell follicle (Hoorweg and Cupedo, unpublished data). The importance of RANKL in follicle devel-

opment has been recently established by Sugiyama et al[65]. In this study, neonates injected with anti-RANKL antibody at 13.5 dpc displayed an absence of B-cell follicle formation. Furthermore, all B cells and FDC-M2$^+$ FDCs were localized at the periphery of the LN. RANKL$^{-/-}$ mice lack most PLN and all MLN. Cervical LNs do develop occasionally and present with segregated T and B cells, but fail to develop B cell follicles[66]. The mechanisms through which RANKL is involved in B cell follicle development are not fully understood. The study of Sugiyama and our observation, suggest that RANKL$^+$ MRC have an instructive role in the development B cell follicle stroma. However, the answer to this question remains enigmatic.

Current knowledge on the genetic changes of stromal cells during B cell follicle formation is poor. FL cells can remodel ectopic BM stroma in such a way that a follicle-like structure is formed. To gain insight in the changes in gene expression of lymph node stromal cells during the earliest stages of B cell follicle formation we used an *in vivo* B cell transfer model in RAG-1$^{-/-}$ mice. Seven days post inoculation we harvested lymph nodes and analyzed the CD45$^-$ cells including FDC, FRCs, MRCs, lymphatic and vascular endothelial cells and un-identified stromal cells. We found the induced expression of IL-22 by the non-hematopoietic compartment of LNs upon contact with B cells. Literature does not report the production of IL-22 by CD45- cells. It is known to be produced by RORC$^+$ ILC and subsets of T cells[67-71]. IL-22 has been described to play a role in tissue immunity and integrity of mainly the gastrointestinal, urinary, and pulmonary tract and the skin that express the IL-22 receptor[12,72,73]. The observed up regulation of IL-22 might be, for that reason, induced upon loss of integrity of the LN structure of RAG-1$^{-/-}$ mice upon the entry of large numbers of B cells. To exclude that up regulation of IL-22 by the non-hematopoietic compartment of LNs of RAG1$^{-/-}$ mice after transfer of B cells is an artefact, our data should be validated by PCR for IL-22 on the same samples used for the microarray analysis. Furthermore, it should be addressed whether the expression of IL-22 is B cell specific and not a by-stander effect. The analysis of adult IL-22$^{-/-}$ mice for the presence of aberrant B cell follicles revealed no phenotype suggesting that absence of IL-22 does not hamper B cell follicle formation or B cell follicle maintenance in homeostatic conditions. A role for IL-22 in the maintenance of B cell follicle integrity during development and under inflammatory condition can be studied in IL-22$^{-/-}$ by analyzing LN isolated during different developmental stages and under inflammatory conditions.

The lymphotoxin pathway is essential for lymph node development and at later stages for B cell follicle development and integrity[74-76]. B cells express membrane-bound LT and IL-22 could be involved in B cell follicle formation. The upregulation of IL-22 by CD45$^-$ lymph node derived cells upon contact with B cells might be LT mediated. The transfer of LT$^{-/-}$ B cells in RAG-1$^{-/-}$ mice can give a conclusive result on this question. In addition, the analysis of gene transcripts of BM stromal co-cultured in 3-D reaggregates with B cells or WSU-FSCCL cells for IL-22 transcripts might reveal a role for this interleukin in the development of ectopic structures in the BM.

Future directions for research on MRC & IL17$^+$ and IL-22$^+$ RORC$^+$ ILC

Tertiary lymphoid organs (TLO) develop at sites of pathogenic infection in order to facilitate the production of antibodies specific for the pathogen. However, TLO develop also at numerous types of chronic inflammatory lesions leading to excessive inflammation and as a consequence tissue destruction. Several chronic autoimmune disorders such as rheumatoid arthritis (RA), Sjögren's syndrome (SS) and Hasimoto's thyroiditis (HT),low grade follicular lymphoma (FL) and chronic graft rejection can present with TLO which are associated with a poor outcome[77,78]. Therefore, it has been suggested that blocking the formation of TLO could be a strategy in the treatment of the excessive inflammations. In RA and Sjögren's syndrome patients and during the experimental formation of inducible bronchus-associated lymphoid tissue (iBALT), the degree of cellular organization of TLO has been associated with the expression of the homeostatic chemokines CCL21 and CXCL13 and molecules involved in SLO development by local stromal cells[47,79-83]. From mouse studies we know that TLO development shares many similarities with SLO development[77]. While prenatal SLO development requires the LTi-LTo cell interaction, a similar cell-cell interaction for TLO formation has not been identified yet. The role of Rorc$^+$ ILC in the formation of TLO remains to be addressed since in mouse models in the absence of these cells iBALT and TLO in inflammatory bowel disease develop[84,85]. The identification of human MRC in adults (chapters 4 and 5) shows that cells reminiscent of LTo cells reside throughout adulthood in man. Currently it is unknown whether human MRC are restricted to developmentally programmed lymphoid tissues as Katakai suggested for murine MRCs or that these cells also reside and / or can be induced in other non-lymphoid organs[51]. The presence of MRC cells in ectopic lymphoid structures has never been reported to be extensively evaluated.

Targeting the microenvironment of ectopic lymphoid structures

Follicular lymphoma is an indolent B cell lymphoma that depends for survival on its specialized microenvironment. Microarray analyses showed that the clinical outcome of FL patients is primarily predicted by molecular features of non-malignant cells[86].$^+$ In addition, a large panel of predictive markers reflecting the number, activation, and spatial distribution of infiltrating immune non-B cell subsets in FL infiltrates has been identified[87]. FL B cells interact with stromal cells in order to create the optimal FL niche that upon activation by FL B cells provide pro-survival signals and are involved in the organization of a tumor promoting GC-like niche[88]. However, the number and spatial distribution of the stromal cell subsets of infiltrated follicular lymphoma LN has been poorly characterized. The presence of MRC in FL niches has never been investigated. An accurate functional characterization of the major stromal cell subsets, including MRCs, of FL infiltrated LN in various stages of grading might lead to the determination of stromal cell markers that contribute to the prediction of the clinical outcome of patients. In addition the course of stromal cell differentiation in the follicular lymphoma microenvironment can be characterized.

Upon diagnosis, up to 70% of the FL patients display bone marrow involvement. FL cells are capable of remodeling the BM stroma in a lymphoid like structure in order to create an optimal local niche[60,89-91]. It has been shown that stromal cells of infiltrated LN and in the BM provide

essential pro-survival factors such as hedgehog (Hh) ligands that in addition indirectly could be involved in stroma-mediated chemotolerance[92-94]. Stromal cells of the ectopic FL microenvironment are because of the aforementioned reasons emerging as therapeutic targets. However, mesenchymal stromal cells in the BM difficult to target and are also radio resistant[95].

Ibrutinib is a Bruton tyrosin kinase (Btk) inhibitor which in B cell chronic lymphocytic leukemia (CLL) reduces the migration towards CXCL12 and CXCL13 and delibitates the adhesion of primary CLL B cells in the lymph node and bone marrow microenvironment[96]. In addition to this study, an *in vitro* study determining the effects of Ibrutinib on multiple myeloma and octeoclasts and BM stromal cells, shows that Ibrutinib acts on the bone resorption activity of osteoclasts (OC), to inhibit tumor growth factors from OC and BM stromal cells and to reduce the secretion of MIP1-α, MIP1-β, SDF-1, TGF-β, activin A, APRIL, BAFF, and IL-8. [97]. Together these studies show that inhibiting B cell receptor signaling via inhibiting of Bruton tyrosine kinase results in interfering with the migration and adhesion of the malignant B cells towards the ectopic microenvironment results and in loss of contact with the beneficial microenvironment. This strategy could sort a similar effect in follicular lymphoma B cells in their local microenvironment. Moreover, a recent study suggests that another tyrosine kinase pathway, the Syk-mTor pathway, is involved in FL cell invasion through the regulation of metalloproteinase-9 and angiogenesis as an upstream regulator of vascular endothelial growth factor[98]. Currently clinical trials evaluating the Syk inhibitor in various relapsed or refractory hematologic malignancies including FL might yield promising therapeutic targets.

RORC⁺ ILC derived cytokines in health and disease

Postnatal, human RORC⁺ innate lymphoid cells reside predominantly in the skin and mucosal tissues. RORC⁺ ILC produce besides IL-17 and IL-22 the cytokines IL-2, IL-5, IL-13 and BAFF and this is reviewed by Cornelissen et al[37]. Here, I will focus on IL-17 and IL-22 producing ILC.

From mouse studies we know that Rorc⁺ ILC play important roles in innate immune responses at mucosal surface to pathogens. ILC derived IL-17 acts within hours upon epithelial injury or activation by pattern recognition receptors (PPRs) and induces inflammatory cytokines such as IL-6, G-CSF and SCF and the recruitment of neutrophils[(reviewed in 99)]. In mouse models IL-17 producing Rorc⁺ ILC were shown to drive intestinal inflammation upon *H. hepaticus* infections[38,100] and to be protective against mucosal *Candida* infections[101]. In addition, a colitis model in mice demonstrated that ILC derived IL-17 can be pathogenic[38]. IL-22 can serve both as a protective and inflammatory mediator depending on several factors. Mouse models demonstrated a protective role for IL-22 in de the early defense against *Citrobacter rodentium*-induced colitis[15,102], systemic infection with *Salmonella enterica*[103] and gastrointestinal *Candidasis* in the absence of IL-17R[104]. In contrast, IL-22 is required for the development of *Toxoplasma gondii*-induced immunopathology in the small intestine[105].

However, emerging data indicate a prominent role for RORC⁺ ILC in chronic inflammatory diseases. In humans, IL-17 has been associated with several disease processes including IBD, rheumatoid arthritis, and psoriasis[106]. In Crohn's disease (CD) patients, IL-17 producing ILC

were present and suggested to contribute to the disease[38,107]. In addition, patients suffering from CD display high levels of IL-22, which could be (in part) NKp44$^+$ RORC$^+$ ILC mediated[108]. Also in synovial fluids and sera of RA patients, increased levels of IL-17 and IL-22 have been described[109,110].

Currently, the role of RORC$^+$ ILC derived cytokines in autoimmune diseases has been poorly evaluated. The distribution and conceivably shift in balance of RORC$^+$ ILC subsets in tissues from patients compared to healthy donors should be analyzed in detail. When a significant shift in the composition of IL-17$^+$ vs IL-22$^+$ RORC$^+$ ILC subsets is observed, it needs to be addressed how this phenomenon and the cytokine production of these cells is regulated in vivo.

The high levels of IL-17A in rheumatoid synovial tissue, causes cartilage and bone degradation. In vitro experiments suggested that IL-1β, an endogenous damage associated molecular pattern (DAMP), is involved in the regulation of IL-17 secretion by ILC[20,111]. In CD and joints of RA patients, levels of IL-1β are relatively high. However, since T_h17 cells require two signals in order to secrete IL-17 it is likely that a second activation signal is required. Currently, Canakinumab, an antibody directly against IL-1β is studied in Phase III trials. Whether the production of IL-17 by RORC$^+$ ILC (if present in synovial tissue and fluid) is abrogated has not been evaluated yet[112]. Furthermore, in literature it has been proposed that IL-17 controls the pro-inflammatory versus tissue-protective properties of IL-22[113]. In this model, blocking IL-17 will affect the function of IL-22. Presently, IL-17 is recognized as an attractive therapeutic target in RA. The anti-IL-17A antibody secukinumab is very promising with phase III trials ongoing in patients on background methotrexate who had inadequate responses to previous tumor necrosis factor blocker therapy[114].

Furthermore, a recent report decribes that chondrocytes express NKp44 ligand.[115] . Nkp44 signaling in RORC$^+$ ILC activates a pro-inflammatory program resulting in the production of TNF which is involved in chondrocyte destruction[116]. Blocking NKp44 in RA may therefore be a promising therapeutic target.

In sum, the work described in this thesis is of basic biomedical nature and can facilitate translational research for identifying new therapeutic targets for the management of diseases suffering from ectopic TLOs and RORC$^+$ ILC derived cytokines.

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SUMMARY

Human secondary lymphoid organs (SLOs) develop during embryogenesis. As described in **chapter 1** of this thesis, knowledge on human SLO development is very limited and most data on SLO development are obtained from murine studies. In relation to various chronic inflammatory diseases, there is a gaining interest in tertiary lymphoid structures (TLS). These TLS can at various degrees recapitulate the complex and highly organized structure of lymph nodes with segregated T and B cell zones, germinal centers (GCs), high endothelial venules (HEVs), and follicular dendritic cells (FDCs). While TLS are formed during inflammatory responses in order to create an optimal environment for the eradication of infections, evidence is culminating that during chronic or autoimmune inflammation TLS contribute to sustaining the aberrant inflammation and serve as a site for activation of auto reactive lymphocytes[1]. The mechanisms that drive the development of human ectopic lymphoid structures are not well characterized. From murine models, we know that many pathways and players in the development of TLS are similar to those involved in the development of SLOs. Since relatively little is known about SLO development in human, we set out to identify and characterize the cellular components involved in the development of human SLOs.

Lymph node development in mice starts between days 11 and 14 gestation. Upon CXCL13 dependent clustering of specialized circulating lymphoid tissue inducer cells (LTic) at prospective lymph node locations, stromal lymphoid tissue organizer (LTo) cells start to express the lymphotoxin-β-receptor (LTβR) which is ligated by surface-bound lymphotoxin (LTα1β2). Ligation of this receptors triggers the expression of the adhesion molecules ICAM-1, VCAM-1 and MAdCAM-1 and homeostatic chemokines such as CXCL13, CCL19 and CCL21 that attract and retain more LTic. The clustering of more LTic induces a local self-sustaining primordium in which, over time, high endothelial venules will be induced and postnatally T and B cell areas will be shaped and germinal centers can form.

In 2009, Cupedo at al. identified the human LTi cells in fetal LNs. These lineage negative RORC$^+$ innate lymphoid cells were shown to interact with mesenchymal cells through lymphotoxin and tumour necrosis factor and to produce IL-17. In adult man and mice, RORC$^+$ ILC were shown to be present in SLO, skin and in the intestine. In mice, RORC$^+$ ILC are essential in the early defense against attaching and effacing pathogens like *Citrobacter rodentium* via the production of IL-22. In human, IL-22 producing ILC were described in mucosal tissue such as tonsils, Peyer's patches and the intestine. In **chapter 2 of** this thesis we show that functionally distinct subsets of human RORC$^+$ innate lymphoid cells are enriched for secretion of IL-17a or IL-22. While both subsets have an activated phenotype they can be distinguished based on the presence or absence of the natural cytotoxicity receptor NKp44. We found that NKp44$^+$ IL-22 producing cells are present in tonsils and adult intestines while NKp44$^-$ IL-17a producing cells are present in fetal developing lymph nodes. We also show that the development of human intestinal NKp44$^+$ ILC is

a programmed event independent of bacterial colonization. Furthermore, NKp44^{-} ILC remain present throughout adulthood in peripheral non-inflamed lymph nodes as resting, non-cytokine producing cells. However, upon stimulation lymph node ILC can swiftly initiate cytokine transcription suggesting that secondary human lymphoid organs may function as a reservoir for innate lymphoid cells capable of participating in inflammatory responses.

As mentioned in the previous section, the development and cellular organization of lymph nodes is guided by subsets of stromal reticular cells. Fibroblastic Reticular Cells (FRCs) and FDCs are the major stromal constituents of the adult T cell zone and B cell follicles respectively. In 2008 Katakai et al. described a novel stromal cell subsets in adult murine lymphoid organs that are phenotypically equivalent to fetal stromal organizer cells. These RANKL^{+} Marginal Reticular Cells are located in the outer follicular region immediately underlying the subcapsular sinus in LNs and in the follicular dome area of Peyer's patches. As described in chapter 2 of this thesis, in human fetal and adult lymph nodes different functional subsets of RORC $^{+}$ILC reside. In **chapter 4**, we set out to determine the stromal niches of RORC^{+} ILC in fetal and adult lymph nodes. We showed that both fetal and adult LNs contain populations of MRCs which resemble murine lymphoid tissue organizer cells. Human MRCs can be characterized by the expression of RANKL and are positive for CXCL13 and CCL21. In fetal LNs, RORC^{+} ILC co-localize with RANKL^{+} MRCs whereas in adult LNs RORC^{+} ILC reside at the interface of the T/B zone. Collectively these data show that in fetal and adult lymph node cells RANKL^{+} stromal cells reside which are remiscent of murine LTo cells found in LN anlagen.

Studies with genetically altered mice indicated an essential role for signals downstream of the lymphotoxin-beta receptor in lymph node and Peyer's patches development. While mice deficient for LTα or the LT receptor, lack LNs and Peyer's patches, compartmentalization of the white pulp (WP) of the spleen was lost. Murine studies indicated that LT^{+} B cells are involved in the development of the while a specific role of LTi cells in white pulp development could not be addressed. In addition, the presence of stromal LTo-like cells in the fetal spleen involved in WP development have not been described. In **chapter 5** of this thesis we analyzed human fetal spleens varying from 15-20 gestational weeks (gw) for a stromal organizer cell. We show in fetal spleens of 18 gw. a MAdCAM-1^{+} VCAM-1^{+}stromal cell subset that is positive for MRC-related gene transcripts: RANKL, CCL21 and CXCL13. RORC^{+} ILC in fetal spleens of 18-20 gw displayed a random distribution and a phenotype similar to RORC^{+} ILC in fetal LNs. These data suggest that in fetal spleens MRC-like cells are involved in WP development.

In **chapter 3** we set out to study the interactions between follicular lymphoma (FL) B cells and BM stromal cells required for ectopic follicle formation. Our results derived from *in vitro* 3D co-culture experiments and a xenograft model using RAG-2$^{-/-}$γc$^{-/-}$ mice were obtained with the progressive FL derived cell line WSU-FSCCL. In contrast to primary FL cells, WSU-FSCCL cells grows independent of external signals for its survival and therefore does not need to induce

changes to its microenvironment. Nevertheless, results show that WSU-FSCCL induces the expression of VCAM-1 by human primary bone marrow stromal cells *in vitro*. Furthermore, the RAG-2$^{-/-}\gamma c^{-/-}$ xenograft model showed that WSU-FSCCL disseminated to peritrabeculaer sides. Together, these data hold the promise that with primary follicular lymphoma B cells interesting result can be obtained. Furthermore, we show that human fetal B cell follicle development starts at 18 gw and induces an activated phenotype in pre-cursor FDCs by the high expression of VCAM-1. To identify differentially expressed genes by LN stromal cells upon B cell, we conducted a B cell transfer experiment in adult RAG-1$^{-/-}$ mice. This revealed the production of IL-22 by a yet unidentified stromal cell subset in murine LNs.

In this thesis we describe the identification and characterization of RORC^{+} ILC and MRC in lymphoid tissues. We found these cells, which are phenotypically and functionally reminiscent of LTi and LTo cells, to be present in adult tissues. Currently, knowledge on the presence and role of RORC^{+} ILC in homeostatic and pathological conditions is poor and for human MRC lacking. The cytokines and chemokines produced by these cell types suggest roles in immune responses. In addition one could envision a role for RORC^{+} ILC and MRC in the development of ectopic tertiary lymphoid structures.

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NEDERLANDSE SAMENVATTING

Cellen betrokken bij de immuunrespons zijn georganiseerd in gespecialiseerde lymfoïde organen om zo optimaal mogelijk te kunnen functioneren. De belangrijkste lymfoïde organen kunnen worden onderverdeeld in primair en secundair. De primaire lymfoïde organen, het beenmerg en de thymus, zijn de locaties voor respectievelijk B en T lymfocyt ontwikkeling. De secundaire lymfoïde organen bieden een omgeving waarin afweer reacties worden gestart en waar de kans op interacties tussen lymfocyten en antigen presenterende cellen verhoogd is en omvatten o.a. de lymfeklieren, de milt en de platen van Peyer.

Secundaire lymfoïde organen (SLO) ontwikkelen zich tijdens de embryogenese. Het bestuderen van SLO ontwikkeling in de mens kent hierdoor grote beperkingen en derhalve is de wetenschappelijke kennis over dit onderwerp voornamelijk verkregen middels muis studies. Lymfeklierontwikkeling in de muis begint tussen dag 11 en 14 van de embryogenese wanneer circulerende lymfeweefsel inducerende cellen (engelse afkorting: LTic) clusteren en lokale stromale cellen induceren tot de expressie van de lymphotoxine bèta (LTβ) receptor. Activatie van de LTβ-receptor door LTic-membraan gebonden lymfotoxine, induceert de expressie van homeostatische chemokines (CXCL13, CCL19 en CCL21) en adhesiemoleculen (ICAM-1, VCAM-1 en MAdCAM-1) door de stromale lymfoïde organiserende (engelse afkorting: LTo) cellen. Dit resulteert in het aantrekken en behouden van meer en meer LTic waardoor er een positieve feedback loop ontstaat. Verder in de ontwikkeling worden de typische hoog endotheliale venules geïnduceerd en postnataal de T en B cel gebieden gevormd.

Vanuit de medische wetenschap is er een grote interesse in tertiaire lymfoïde organen (TLS). Deze ectopische lymfoïde structuren worden gevormd tijdens ontstekingreacties om zo een optimaal lokaal milieu te genereren van waaruit de infectie bestreden kan worden. TLS zijn er qua structuur en complexiteit in verschillende gradaties en lijken in meer of mindere mate op lymfeklieren met T en B cel gebieden, hoog endotheliale venules, folliculair dendritische cellen en secundaire B cel follikels. Echter, TLS kunnen ook een negatief effect sorteren wanneer ze ontwikkelen tijdens chronische ontstekingen of auto immuunziekten en dienen als een plaats voor auto-reactieve lymfocyt activatie. De mechanismen die ten grondslag liggen aan de ontwikkeling van humane ectopische lymfoïde structuren zijn niet goed gekarakteriseerd. Studies met muismodellen hebben laten zien TLO ontwikkeling veel overeenkomstigheden heeft met SLO ontwikkeling. Zoals hierboven besproken is de wetenschappelijke kennis over humane SLO ontwikkeling zeer beperkt. Daarom hebben wij in deze studie de cellulaire componenten betrokken bij de ontwikkeling van humane SLO getracht te identificeren en te karakteriseren.

De humane LTic werd in 2009 geïdentificeerd in foetale lymfeklieren door Cupedo et al. Deze LTi cellen die behoren tot de familie van de RORC$^+$ aangeboren lymfoïde cellen (engelse afkorting: RORC$^+$ ILC), bleken te communiceren met mesenchymale cellen via lymfotoxine en tumor

necrosis factor en produceerden IL-17a. In volwassen mensen evenals in volwassen muizen zijn RORC$^+$ ILC aanwezig en wel in tonsillen, lymfeklieren, de huid en in de darm. In volwassen muizen is aangetoond dat RORC$^+$ ILC in de darm essentieel zijn in de eerste verdediging tegen *Citrobacter rodentium* via de productie van IL-22. In de mens zijn IL-22 producerende ILC aangetoond in volwassen mucosaal weefsel zoals de tonsillen, de platen van Peyer en de darm. Samengevat: in foetale lymfeklieren resideren IL-17a producerende ROR$^+$ILC en in tonsillen en de adulte darm resideren IL-22 producerende RORC$^+$ ILC. De vraag die wij ons stelde was of de IL-17a en IL-22 producerende RORC$^+$ ILC konden worden onderscheiden van elkaar op basis van fenotypische kenmerken.

In **hoofdstuk 2** van dit proefschrift laten we zien dat functioneel verschillende subsets van humane RORC$^+$ ILC verrijkt zijn voor de secretie voor IL-17a of IL-22. Beide subsets hebben een geactiveerd fenotype maar kunnen onderscheiden worden op basis van de aanwezigheid of afwezigheid van de natuurlijke cytotoxiciteits receptor NKp44. We vonden dat NKp44$^+$ IL-22 producerende cellen aanwezig zijn in tonsillen en adulte darm en dat NKp44$^-$ IL-17a producerende cellen aanwezig zijn in de foetale lymfeklieren. Verder tonen we aan dat de ontwikkeling van NKp44$^+$ ILC in de humane darm is aangeboren, wat betekent dat ontwikkeling van deze cellen onafhankelijk is van bacteriële kolonisatie. Ook in perifere niet-ontstoken lymfeklieren resideren NKp44$^-$ ILC maar deze produceren geen IL-17a noch IL-22. Echter, stimulatie van adulte lymfeklier RORC$^+$ ILC induceert de transcriptie van cytokine genen. Dit suggereert dat secundaire humane lymfoïde organen kunnen fungeren als een reservoir voor RORC$^+$ ILC die indien nodig in staat zijn te participeren in ontstekingsreacties.

In adulte SLO zijn fibroblastische reticulaire cellen (FRCs) en folliculair dendritische cellen (FDCs) de belangrijkste stromale cellen van respectievelijk de volwassen T en B cel gebieden. In 2008 beschreef Katakai et al. een tot dan toe nog onbekende stromale cel subset in de lymfoïde organen van adulte muizen. Deze RANKL$^+$ Marginal Reticular cellen (MRC) doen fenotypisch denken aan de stromale lymfoïde organizer cellen en zijn gelegen in het buitenste folliculaire gebied direct onder de subcapsulaire sinus van de lymfeklier en in de folliculaire dome van platen van Peyer. In **hoofdstuk 4** tonen we aan dat zowel foetale als volwassen humane lymfeklieren populaties van MRCs bevatten die MAdCAM-1 en VCAM-1 positief zijn en worden gekenmerkt door de expressie van RANKL.Verdere analyse toonde aan dat humane MRCs positief zijn voor de homeostatische B cell en T cell chemokines, CXCL13 en CCL21. RORC$^+$ILC in foetale LK co-lokaliseren specifiek met RANKL$^+$ MRCs. In adulte lymfeklieren bevinden de RORC$^+$ ILC zich in het gebied tussen de T en B cel gebieden en de follikels waar zich ook RANKL$^+$ MRCs kunnen bevinden. Tezamen tonen onze data aan dat humane foetale en adulte lymfklieren RANKL$^+$ MRCs bevatten die fenotypisch en functioneel lijken op de stromal lymfoïde weefsel organiserende cellen zoals in LN anlagen van de muis.

Nu we in foetale en adulte humane lymfeklieren de aanwezigheid van RANKL$^+$ MRC hadden aangetoond, wilden we foetale en volwassen milten ook analyseren voor de aanwezigheid van deze stromale cel subset. In **hoofdstuk 5** tonen we aan dat de foetale milt van 18 gw een MAdCAM-1$^+$VCAM-1$^+$ stromale cel bevat die positief is voor RANKL, CCL21 en CXCL13. RORC$^+$ ILC aanwezig in de foetale milt van 18-20 gw. hebben een fenotype overeenkomstig met RORC$^+$ ILC in foetale lymfeklieren maar clusteren niet specifiek met de MAdCAM-1$^+$VCAM-1$^+$ stromale cellen. Deze data suggereren dat in de foetale milt MRC-achtige cellen aanwezig zijn die mogelijk betrokken zijn bij de ontwikkeling van de witte pulpa.

In **hoofdstuk 3** bestudeerden we de interacties tussen folliculair lymfoom (FL) B-cellen en stromale beenmerg cellen (BM) nodig voor ectopische B cel follikel vorming. De *in vitro* 3D co-cultuur experimenten en het xenograftmodel in RAG-2$^{-/-}\gamma c^{-/-}$ muizen werden uitgevoerd met de progressieve FL afgeleide cellijn WSU-FSCCL in plaats van met primaire folliculair lymfoom cellen. In tegenstelling tot primaire FL cellen is WSU-FSCCL niet afhankelijk van externe signalen voor survival en is de nood voor het veranderen van het lokale micromilieu om zo de overlevingskans te verhogen gering dan wel afwezig. Ondanks dit gegeven laten onze resultaten zien dat WSU-FSCCL de expressie van VCAM-1 door humane primaire beenmerg stromale cellen induceerde *in vitro*. Het RAG-2$^{-/-}\gamma c^{-/-}$ xenotransplantatie model leerde ons dat WSU-FSCCL evenals primaire FL cellen, preferentieel clusterde in de peritrabeculae van het BM. Om de eerste differentieel tot expressie gebrachte genen in de niet hematopoeietische cellen van de lymfeklier na het eerste B cel contact te analyseren, hebben we adulte RAG-1$^{-/-}$ muizen geïnoculeerd met B cellen en na 7 dagen geanalyseerd met microarray analyse. Met dit experiment vonden we heel verrassend een nog niet eerder beschreven stromale bron van IL-22 productie. Welke cellen dit zijn en wat de functie is van IL-22 in deze, is onderwerp van verder onderzoek.

Gezamenlijk, dragen de data in dit proefschrift bij aan een beter begrip van de ontwikkeling van humane secundaire lymfoïde organen.. De identificatie en karakterisering van "inducers" (RORC$^+$ ILC) en de "organizers" (RANKL$^+$ MRCs) in foetaal en adult lymfeweefsel kan gebruikt in studies naar de ontwikkeling van ectopische tertiaire lymfoïde structuren en de rol van deze structuren tijdens ontstekingsziekten.

ABBREVIATIONS

AMP	adenosine monophosphate
Bcl	B cell lymphoma
BCR	B cell receptor
BM	bone marrow
CCL19	c-c chemokine motif ligand 19
CCL21	c-c chemokine motif ligand 21
CXCL13	c-x-c chemokine motif ligand 13
CXCR5	c-x-c chemokine motif receptor type 5
CXCR7	c-x-c chemokine motif receptor type 7
CD	cluster of differentiation
cKIT	proto-oncogene KIT, CD117
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cells
DLBL	diffuse large B cell lymhoma
DNA	desoxyribonucleic acid
ELISA	enzyme-linked immuno sorbent assay
FDC	follicular dendritic cells
FRC	fibroblastic reticular cells
FL	follicular lymphoma
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
gw	gestational weeks
GC	germinal center
HEV	high endothelial venule
IDC	interdigitating cells
ICAM-1	intercellular adhesion molecule 1
Id2	inhibitor of DNA binding protein 2
IFN-y	interferon gamma
IL-17	interleukin 17
IL-22	interleukin 22
IL-7	interleukin 7
IL-7Rα	interleuking 7 receptor alpha
ILC	innate lymphocyte cell

JAK	Janus kinase
LTic	lymphoid tissue inducer cell
LT	lymphotoxin
LTβ-R	lymphotoxin-beta receptor
LToc	lympoid tissue organizer cell
MAdCAM-1	mucosal addressin cell adhesion molecule 1
MRC	marginal reticular cell
MZ	marginal zone
NCR	natural cytotoxicity receptor
NK	natural killer
NKp	natural killer cell related protein
PALS	periarteriolar lymphoid sheath
PECAM-1	platelet endothelial cell adhesion molecule
PNAd	peripheral node addressin 1
PPs	Peyer's patches
RAG	recombination activating gene
RALDH2	retinaldehyde dehydrogenase 2
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
RET	rearranged during transfection proto-oncogene
Roryt	RAR-related orphan receptor yt
RORc	RAR-related orphan receptor C
SCID	severe combined immune disease
SHM	somatic hypermutation
SLO	secondary lymphoid organ
SMA	smooth muscle actin
TLO	tertiary lymphoid organs
TLS	tertiary lymphoid structure
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule 1
WP	white pulp
WSU-FSCCL	follicular small cleaved cell lymphoma

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alleen maar een beeld van de kritische professor te schetsen, want naast je strenge doch recht -vaardige kant heb ik je leren kennen als een hele prettige, sociale en benaderbare man.

En dan is het nu tijd voor Natalie. Lieve Natalie, heel erg bedankt voor al het werk dat je hebt verricht. Zonder de door jou uitgevoerde experimenten zou dit boekje een stuk dunner zijn geworden. Ik weet nog goed dat je in het begin wel even moest wennen aan zo'n halve zool naast je in het lab. En dan deed hij ook nog altijd de radio aan. Maar al redelijk snel konden we het zeer goed met elkaar vinden en spraken we over de meest uiteenlopende onderwerpen. Wel zo prettig als je samen de hele dag experimenten aan het uitvoeren was. Dat je geen 9.00-17.00 uur mentaliteit had, was wel een zegen. De beruchte dinsdagen konden weleens laat worden…Zeker in de tijd van vóór de positieve CD117 selectie. Zo aan het einde van die lange dagen, kwamen de flauwe grappen op tafel. Gek genoeg begonnen ze vaak op precies dezelfde manier. Vanaf 20.00 uur ging ik poppetjes tekenen bij de ARIA en jij moest raden wie het was: van zo'n beetje de hele afdeling zijn er karikaturen getekend. Het avondje stappen in Birmingham is vereeuwigd middels een foto camera. Die grote fles cava/crémant de bourgogne in de taxi op de heenweg….. schitterend! Ik hoop bij deze dat jij de foto's niet meer hebt. Ik heb ze overigens nog wel. Je was een hele fijne collega en wat dat betreft heb ik erg veel geluk gehad om samen met je te mogen werken. Heel erg bedankt voor al je inspanningen ten bate van mijn promotie!

Beste Eric, als collega promovendi zaten we in het zelfde schuitje en deelden we gelijke ervaringen. Die bespraken we dan onder het genot van een lekkere koffie. Hoe vergaat het je overzees? Verkopen ze daar ook Snelle Jelle? En zo niet, met wat vervang je dan dit essentiële stukje koek? Ik hoop vurig dat jouw bijzondere dromen stuk voor stuk uitgebracht gaan worden als stripboek. Deel 1 zal dan moeten gaan over je ontsnapping, samen met De Burgemeester, aan een reuze octopus die de stad komt terroriseren. Mocht je geen geschikte tekenaar vinden, bij deze bied ik mij aan als vertolker van het werk. Ik ben ervan overtuigd dat als je net zulke bijzondere vragen blijft stellen in wetenschappelijke context als dat je hebt laten zien in sociale context (bv. "Als je een voertuig kon zijn, wat zou je dan kiezen?"), je een mooie carrière voor je hebt. Heel veel succes! Uiteraard moet ik me ook even richten tot Elwin. Ontelbaar vaak heb ik iets aan je gevraagd, heb je geholpen met de meest uiteenlopende zaken en was het soms behelpen als je er eens een keer niet was. Dank voor al die momenten. Rogier, we hebben in feite maar kort samengewerkt maar lang genoeg om te kunnen zeggen dat je een fijne collega was. Ik heb altijd fijn met je kunnen praten en lachen. Ambitie en talent zijn goed vertegenwoordigd in je persoonlijkheid en ik wens je het beste toe in je verdere carrière als onderzoeker. Ferry, ik hoop dat na alles wat je hebt meegemaakt het vuur dat in je huisde weer is gaan branden. Want jouw gedrevenheid, heeft een diepe indruk op me gemaakt. Patricia, ik hoop dat je onderhand meer Nederlands spreekt dan ik Spaans en dat je een prettige loopbaan zult hebben in de wetenschap. Eric Braakman, jij bedankt voor alle suggesties tijdens werkbesprekingen

Ik wil bij deze de leescommissie bestaande uit Prof. dr. Reina Mebius, Prof. dr. Rudi Hendriks en Prof. dr. Steven Pals bedanken voor het beoordelen van mijn manuscript. Natuurlijk bedankt ik ook de overige leden van de promotiecommissie, Prof. dr. Ivo Touw, dr. Eric Lubberts en dr. Marc Raaijmakers voor het zitting nemen.

Beste King Lam en Jeanette Doorduijn, jullie wil ik bedanken voor alle inspanningen ten bate van het folliculair lymfoom project. Aan alle medewerking die ik van jullie heb mogen ervaren heeft het niet gelegen! Ook wil ik dr. Hans Hoeve van de KNO afdeling van het Sofia Kinderziekenhuis bedanken dat we op zijn afdeling toestemming voor het gebruik van tonsillen mochten vragen. In het verlengde hiervan bedank ik alle chirurgen, OK personeel, verpleegkundigen en dames van de OK planning voor hun medewerking. Jaap Kwekkeboom en Geert Kazemier wil ik bedanken voor het adult lymfeklier en milt weesfel dat zeer belangrijk voor mijn ondezoek en dit proefschrift is geweest. Natuurlijk wil ik ook Dicky Lindenberg bedanken voor al haar hulp en geduld. Charlotte Peters, Jenny Mjösberg en prof. dr. Hergen Spits wil ik graag bedanken voor de vruchtbare samenwerking. En natuurlijk wil ik de medewerkers van het EDC bedanken voor de prettige samenwerking.

I want to thank Marc Coles and Priyanka Narang for the ongoing collaboration. I believe that combining your mouse data with our human data will result in a beautiful publication.

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Lotte en Marleen, laatst dacht ik “mijn” aantekeningen van de bachelor BMW en de master in Oncology op te ruimen die ik in al die studiejaren had verzameld. Gek genoeg was er maar heeeeeeeeel weinig te vinden en was datgene dat ik vond met een ander handschrift geschreven: wel te lezen en met ronde letters. Misschien dat jullie de handschriften herkennen? De leuke tijd op de VU die ik met jullie en Lot in het bijzonder had, is de opmaat geweest voor het willen

promoveren. Een retrospectieve beschouwing leert mij dat ik naar een manier zocht om die leuke tijd te verlengen.

Het nadeel van het soort werk zoals beschreven in dit proefschrift, is dat vrienden zoals Maikel of de mannen van iSoulation een afwezige blik in hun ogen krijgen wanneer ik zo simpel als mogelijk tracht te vertellen wat ik al die jaren heb gedaan. Nu is het allemaal gebundeld in dit boekje mannen! Ik weet 't..... dat verandert niks.

Timothy, Swalter, Scherrie, Schalie en in principe ook Sjako: het oorspronkelijke idee voor het schrijven van een proefschrift, alleen konden we dat toen als zodanig niet herkennen, werd geboren op een camping in Biscarosse in het jaar dat Cassius met het album 1999 de hitlijsten aanvoerde. Dit proefschrift is nu het tweede exemplaar van de hand van een van ons en ik denk dat we een klein onderzoekje moeten opzetten waarin het proefschrift van Wouter getest wordt tegen dit proefschrift. In deze studie zullen de volgende handelingen geïncludeerd moeten worden: 1. Het voorlezen aan kinderen voor het naar bed gaan: hoe snel vallen ze in slaap? 2. Vijf mannelijke proefpersonen (genoeg power! muscles….) moeten na het drinken van teveel lauwe rode wijn uit een jerrycan een passage uit beide boekjes navertellen: hoe ver komt het proefpersoon? De winnaar zal nipt winnen, immers beide boekjes hebben genoeg potentie om boven de ander te eindigen. Jongens, het is een rijk gevoel om zulke goede vrienden als jullie te hebben.

Tot slot wil ik mijn familieleden bedanken voor hun onvoorwaardelijke steun. Nelly, en in het bijzonder, Johan wil ik extra bedanken voor hun betrokkenheid bij het afronden van dit proefschrift en de daarmee gepaard gaande interesse en bezorgdheid. En tot slot natuurlijk mijn Liesje, mijn alles, mijn liefje. Jij bent samen met Lucas mijn alles. Als er geen gekke dingen gebeuren, hebben we binnenkort gezinsuitbreiding. Jullie zijn het allermooiste in mijn leven, daar kan geen titel of diploma tegenop!

CURRICULUM VITAE

Kerim Hoorweg werd geboren in Willemstad (Curaçao) op 30 juni 1978. Exact één jaar later zat hij in het vliegtuig naar Nederland alwaar hij opgroeide in de gemeente Renkum. In 1997 behaalde Kerim het VWO diploma aan het Dorenweerd College en aansluitend studeerde hij tot 1998 Biologie aan de Katholieke Universiteit Nijmegen. In de zomer van 1998 werd het testimonium Natuurkunde aan het James Boswell Institute behaald. Na een periode van werken en twee jaar Technische Bedrijfskunde aan de Universiteit Twente ging hij in 2002 Biomedische Wetenschappen (BMW) studeren aan de Vrije Universiteit te Amsterdam. Na de bachelor BMW volgde Kerim tot 2007 met succes de Master in Oncology aan het VU medisch centrum (VUmc). Als student aan de VU heeft Kerim verscheidene onderzoeken gedaan. Tijdens de bachelor BMW onderzocht hij "TRANCE expression in lymph nodes at E16.5 in wild type and lymphotoxin deficiënt mice" op de afdeling Moleculaire Cel Biologie and Immunologie van het VU medisch centrum (VUmc) onder de supervisie Prof. Dr. R. Mebius. Tijdens de Master in Oncology deed Kerim onderzoek naar "Mechanisms of glucocorticoid resistance in childhood acute leukemia" op de afdeling Hematologie van het VUmc onder de supervisie van dr. Jacqueline Cloos en onderzoek naar "Inducing enteroendocrine differentiation in colorectal cancer cell line LS174 by ways of evading NOTCH signaling en over expressing atonal bHLH transcription factors" in het Clevers-lab van het Hubrecht Instituut onder de supervisie van dr. Wim de Lau. In 2007 begon Kerim als promovendus in de groep van dr. Tom Cupedo met als promotor Prof. dr. Jan Cornelissen op de afdeling Hematologie van het Erasmus Medisch Centrum. Dat onderzoek staat beschreven in dit proefschrift. Op dit moment is Kerim werkzoekende en in afwachting van een mooie baan is hij met veel plezier fulltime huisvader.

PUBLICATIONS

Onder L., Narang P., Scandella E., Chai Q., Iolyeva M., Hoorweg K., Halin C., Richie E., Kaye P., Westermann J., Cupedo T., Coles M. and Ludewig B.,IL-7 producing stromal cells are critical for lymph node remodeling, *Blood*, 2012 Sep 6

Kerim Hoorweg, Charlotte P. Peters, Ferry Cornelissen, Patricia-Aparicio Domingo, Natalie Papazian, Geert Kazemier, Jenny M. Mjosberg, Hergen Spits and Tom Cupedo, Functional differences between human NKp44^{-} and NKp44^{+}RORC^{+} innate lymphoid cells, *Frontiers in Immunology*, 2012 April, 3(72): 1-10

Vondenhoff M.F., Greuter M., Goverse G., Elewaut D., Dewint P., Ware C.F., Hoorweg K., Kraal G., Mebius R.E., LTbetaR signaling induces cytokine expression and up-regulates lymphoangiogenic factors in lymph hode anlagen, *Journal of Immunology,* 2009 May; 182(9):5439-45

Hoorweg K., Cupedo T., Development of human lymph nodes and Peyer's patches, *Seminars in Immunology,* 2008 Jun; 20(3):164-70

PHD PORTFOLIO

Name PhD student: Kerim Hoorweg
Erasmus MC department: Hematology
Research School: Molecular Medicin

PhD period: 01-10-2007 - 31-12-2011
Promotor: Prof. Dr. J.J. Cornelissen
Co-promotor: Dr. T. Cupedo

PHD TRAINING	Year	ECTS
Courses		
• Animal experimentation Article 9 WOD	2007	4.2
• Animal imaging by AIME	2008	1.4
• The Course Molecular Medicin (MM)	2008	1.8
• Advanced Immunology (ALIFI)	2009	4.2
Workshops & Seminars		
• 3rd symposium & masterclasses on Mucosal Immunology	2008	0.5
• Browsing genes and genomes w Ensemble IV (MM)	2009	0.6
• Basic data analysis on gene expression arrays (MM)	2009	0.5
• Erasmus Hematology Lectures	2007-2011	2.0
Presentations		
Oral		
• Hematology presentations	2007-2011	3.5
• AIO/Postdoc presentations	2007-2011	1.5
• Literature discussions	2009-2011	1.5
• Workdiscussions	2007-2011	6.0
(Inter)national conferences		
• Molecular Medicin day, poster	2008-2009	1.0
• The Dutch Hematology Congress, oral	2008-2009	2.0
• Annual meeting of the dutch society of immunolog poster	2008	1.0
• Microanatomy of Immune responses in health and Disease, Birmingham, poster	2009	2.0
• Annual meeting of the dutch society of immunology, oral	2009	1.0
Teaching activities		
• Supervising Master Student	2009-2010	7
Organizing invited speaker lunch	2009-2010	1.0
• Organizing GOL-day	2008-2009	2
Total ECTS		**44.7**

STELLINGEN

1. De lokalisatie in perifere lymfeklieren van aangeboren lymfoïde cellen die de celkern-hormoon receptor RORC tot expressie brengen, suggereert dat deze geactiveerd kunnen worden door via lymfe aangevoerde antigenen (*dit proefschrift*).

2. De karakterisatie van interleukine (IL)-17A en IL-22 producerende aangeboren lymfoïde cellen biedt de mogelijkheid om een verschuiving in cel populaties en daarmee cytokine balans in ziektes waaraan deze cellen mogelijk bijdragen, waaronder inflammatoire darm-ziekten, te monitoren *(dit proefschrift).*

3. De Marginale Reticulaire Cel (MRC) is een tussen muis en mens evolutionair geconserveerde stromale cel die op basis van fenotype en functionaliteit direct verwant lijkt te zijn aan de stromale voorlopercel betrokken bij lymfeklier ontwikkeling (*dit proefschrift*).

4. De productie van interleukine 22 tijdens de ontwikkeling van B cel follikels in de lymfeklier suggereert dat de vorming van het stromale netwerk van de lymfeklier overeenkomsten vertoont met wondheling (*dit proefschrift*).

5. Het chemokine profiel van stromale cellen die de adhesie factor MAdCAM-1^{+} tot expressie brengen in de foetale milt suggereert dat dit de stromale voorlopercellen zijn van het T cel gebied van de witte pulpa (*dit proefschrift*).

6. De Marginale Reticulaire Cellen in de platen van Peyer lijken essentieel te zijn voor de differentiatie van M (micro-plooi) cellen uit epitheliale stamcellen die de G-proteïne-gekoppelde receptor Lgr5 tot expressie brengen *(The Journal of Immunology, 2008, 181: 6189–6200. J Immunol* 2009; 183:5738-5747*, Mol Cell Biol. 2012 September; 32(18): 3639–3647).*

7. De factor die wordt aangemaakt bij een te lage zuurstofspanning "Hypoxia-Inducable-Factor-1" (HIF-1) alpha speelt mogelijk een rol in de ontwikkeling en functie van het aangeboren lymfoïde systeem en verdient om die reden nader wetenschappelijk onderzoek (*Nature Reviews Immunology 5, 712-721; Cell 2011 Sep 2;146(5):772-84; Eur. J. Immunol. 2012. 42: 1226–1236).*

8. Men zou zich meer dik moeten maken over de overwichtsproblematiek in onze samenleving (*Draft action plan for the prevention and control of noncommunicable diseases 2013–2020, WHO, 17 jan 2013*).

9. Muziekles zou tot de verplichte lesstof moeten behoren op alle basisscholen omdat het de cognitieve vermogens van kinderen significant verbeterd (*Psychol Sci. 2004 Aug;15(8):511-4*).

10. De kans dat een wetenschappelijk onderzoek naar koffieverslaving door een niet koffieverslaafde vakgenoot wordt beoordeeld is zeer klein.

11. De weg naar een academische promotie kent een duidelijk vertrek- en eindpunt. De route naar het eindpunt is voor elke promovendus anders in tegenstelling tot de drijvende kracht die nodig is om van vertrek- tot eindpunt te geraken: ambitie.