CHARACTERIZATION OF LYMPHOID TISSUE INDUCER CELLS AND LYMPHOID TISSUE DEVELOPMENT IN ADULT INTERLEUKIN 7 TRANSGENIC MICE

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ABREVIATIONS

Ab	antibody
Ag	antigen
Bcl	B cell leukemia
BLC	B lymphocyte chemoattractant (CXCL13)
BM	bone marrow
CD	cluster of differentiation
CFSE	carboxyfluoroscein succinimidyl ester
CLP	common lymphoid progenitor
CP	cryptopatch
CIITA	class II transactivator
DC	dendritic cell
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
ELC	EBV-induced molecule-1 ligand chemokine (CCL19)
FAE	follicle-associated epithelium
FCS	fetal calf serum
FDC	follicular dendritic cell
FL	fetal liver
FLT3	fms-like tyrosine kinase 3
FRC	fibroblastic reticular cell
GALT	gut-associated lymphoid tissue
γ_c	common cytokine gamma chain
GC	germinal center
GFP	green fluorescent protein
HEV	high endothelial venule
HRP	horse radish peroxidase
IBALT	inducible bronchus-associated lymphoid tissue
ICAM-1	intercellular adhesion molecule 1
Id2	inhibitory of DNA binding 2
Ig	immunoglobulin
IL	interleukin
ILF	isolated lymphoid folicle
Jak	Janus kinase
KO	knock-out
LCMV	lymphocytic choriomeningitis virus
LN	lymph node
LT$\alpha\beta$	lymphotoxin $\alpha\beta$
LTβR	lymphotoxin β Receptor
LTi	lymphoid tissue inducer
Lyve-1	lymphatic vessel endothelial hyaluronan receptor 1
MAdCAM-1	mucosal addressin cell adhesion molecule 1
MHC	major histocompatibility complex
NALT	nasal-associated lymphoid tissue
NF-κB	nuclear factor-kappa B
NK	natural killer
PBS	phosphate buffer saline

PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PNAd	peripheral node addressin
PP	Peyer's patch
RA	rheumatoid arthritis
RAG	recombination-activating genes
RNA	ribonucleic acid
RORγ	retinoic acid-related orphan receptor γ
RIP	rat insulin promoter
s.c.	sub-cutaneously
SCF	stem cell factor
SLC	secondary lymphoid chemokine (CCL21)
SLO	secondary lymphoid organ
STAT	signal transducer and activator of transcription
TCR	T cell receptor
tg	transgenic
TLO	tertiary lymphoid organ
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF6	TNF receptor associated 6
TRANCE	TNF-related activation-induced chemokine
TRANCER	TNF-related activation-induced chemokine receptor
VCAM-1	vascular cell adhesion molecule 1
WT	wild type

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SUMMARY

During embryogenesis, the development of secondary lymphoid organs (SLOs) such as lymph nodes (LNs) and Peyer's patches (PPs) requires the cellular crosstalk between vascular cell adhesion molecule (VCAM)-1^+ mesenchymal organizer cells and $CD45^+CD4^+lin^-$ lymphoid tissue inducer (LTi) cells. The cascade of events leading to functional SLOs is triggered by the activation of the lymphotoxin β receptor (LTβR) signalling pathway. LTi cells express the corresponding ligand lymphotoxin (LT) αβ, and other tumor necrosis factor super-family members, chemokine receptors, adhesion molecules and Interleukin 7 Receptor alpha (IL-7Rα), which contribute to the formation of SLOs. However, the precise mechanism of surface receptor engagement for lympho-organogenesis and LTi cell function was not fully understood. In addition, it remained unclear if LTi cells could persist in adult mice, and had a function in the adult immune system.

In order to better understand the role of IL-7 in SLO development, we generated a double transgenic mouse model overexpressing IL-7 under the control of an ubiquitous promoter (termed H-IL-7). These mice developed additional ectopic LNs and PPs (1). Ectopic SLO development was strictly dependent on LTi cells. We further showed that the development of ectopic SLOs was mediated by an IL-7-driven increase in the survival of fetal LTi cells and its progenitors.

$CD4^+lin^-$ cells were found in significant numbers in all SLOs of adult H-IL-7 mice. This study was performed to characterize $CD4^+lin^-$ cells in adult mice, and to identify their function. Adult $CD4^+lin^-$ cells shared the phenotype with fetal LTi cells, including the expression of retinoic acid-related orphan receptor (ROR) γt. By transferring adult $CD4^+lin^-$ cells into PP-deficient $CXCR5^{-/-}$ mice, we demonstrated their ability to generate lymphoid tissue. Thus, adult $CD4^+lin^-$ cells were *bona fide* LTi cells.

In order to test if adult LTi cells were present in normal wild type (WT) mice, and could respond to IL-7, we treated adult WT mice with IL-7/anti-IL-7 antibody (Ab) complexes. The pool of LTi cells was significantly increased in treated as compared to untreated mice demonstrating that adult LTi cells were responsive to IL-7.

We further investigated the origin of adult LTi cells. We could show that the adult bone marrow (BM) could give rise to LTi cells, which was even more pronounced,

when normal WT mice were treated with IL-7/anti-IL-7 Ab complex. BM cells were, however, far less efficient in generating LTi cells than fetal liver (FL) cells.

It is well established that chronic inflammatory diseases in humans are often associated with a process termed "lymphoid neogenesis". Lymphoid neogenesis leads to the development of tertiary lymphoid organs (TLOs) in non-lymphoid organs. In several autoimmune diseases, a correlation between TLO development and IL-7 production has been reported, but experimental evidence for a causal role of IL-7 in TLO development was lacking. In adult H-IL-7 mice, we observed the development of TLOs in several non-lymphoid organs such as the salivary gland. Moreover, these TLOs were either diffuse or segregated in B and T cell areas, a hallmark of normal SLOs. LTi cells colonized the salivary gland before naive lymphocytes, but were not strictly required for TLO development. In contrast, the expression of LTα was essential for the organization of TLOs into segregated compartments.

To test if local inflammation could trigger the development of TLOs in H-IL-7 mice, we infected mice s.c. with Leishmania major. At sites of infection but not in non-infected H-IL-7 mice, we found additional ectopic LNs. Moreover, the number of LTi cells was significantly increased in the draining LNs of both WT and H-IL-7 infected mice compared to non-infected. Altogether, these data show that the overexpression of IL-7 was able to induce lymphoid neogenesis at ecopic sites.

In summary, this study shows that IL-7 is an important cytokine for the regulation of TLO development in adult mice. We have identified adult LTi cells, which infiltrate TLOs and may have a function in organizing the architecture of TLOs through activation of the LTβR signalling pathway.

1. INTRODUCTION

1.1. Secondary lymphoid organs

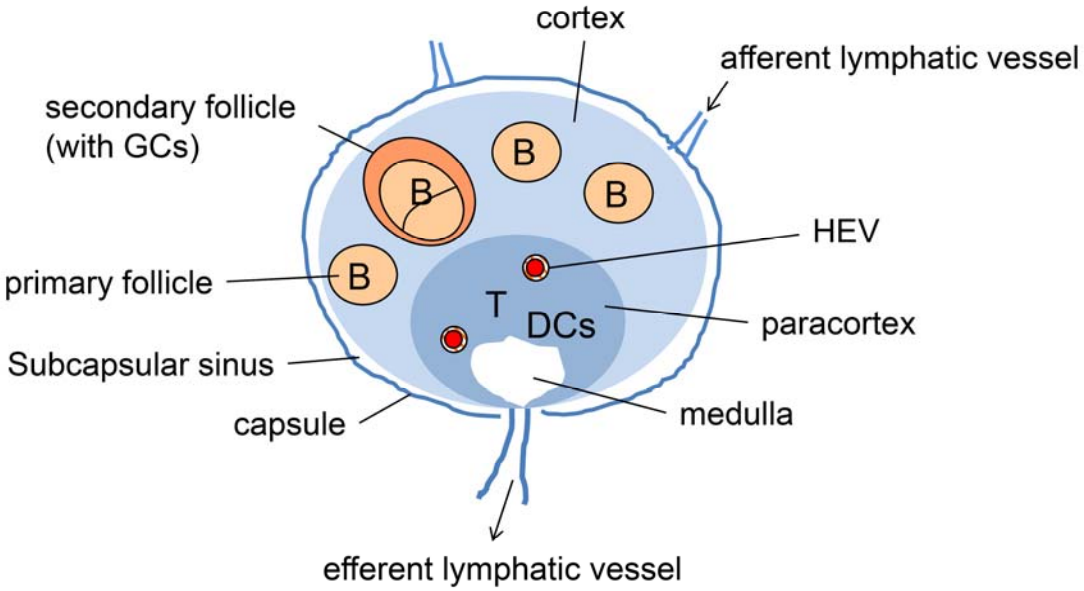
Humans are continuously exposed to various pathogens like viruses, bacteria or worms. The innate and adaptive immune system has evolved as a defence strategy against these pathogens. Adaptive immune responses are initiated in secondary lymphoid organs (SLOs), through the cognate recognition of antigen (Ag) by specific B and T cells, which are the effector cells of the adaptive immune system. SLOs comprise the spleen, lymph nodes (LNs), Peyer's patches (PPs), nasal-associated lymphoid tissue (NALT), tonsils and appendix.

SLOs are found in all jawed vertebrates. They are located at strategic sites of the body allowing a quick and effective activation of Ag-specific B and T cells. The development of SLOs is restricted to embryogenesis (2).

LNs are encapsulated by a lymphatic endothelium and are connected to the lymphatic system. The lymph enters the LN by the afferent lymphatic vessel into the subcapsular sinus. From the subcapsular sinus, trabecular sinuses transport the lymph through the LN, until the efferent lymphatic sinus.

LNs are composed of three compartments, the cortex, the paracortex and the medulla (figure 1). The cortex contains B cell follicles that mature into secondary follicles upon Ag stimulation. Secondary follicles contain germinal centres (GCs), which are the sites for clonal proliferation of B cells inducing the specific expression of immunoglobulins (Igs). Located in B cell follicles, follicular dendritic cells (FDCs) are stromal cells implicated in Ag presentation to B cells. The recruitment and positioning of lymphocytes and dendritic cells (DCs) in LNs are regulated by the localized production of homeostatic chemokines by stromal cells (3, 4). For example, FDCs produce CXCL13, the chemokine responsible for the recruitment of B cells and the formation of B cell follicles. The paracortex is composed of T cells and DCs. Moreover, specific postcapillary high endothelial venules (HEVs) are found in the paracortex. HEVs are a hallmark of LNs and PPs, allowing the entry of naive lymphocytes into PPs and LNs. T and B cells enter the LN by extravasation across HEVs, while soluble Ags and DCs enter via afferent lymphatic vessels. All cells leave the LN via the efferent lymphatic vessel to be delivered to enter the blood circulation.

The medulla contains lymph-draining sinuses, plasma cells, macrophages and memory T cells, but its function is not yet well undestood.



Adapted from Drayton et al. 2006. Nature Immunology 7(4): 344

Figure 1. LN architecture. LNs are surrounded by a subcapsular sinus protected by a capsule. They are composed of the cortex, the paracortex, and the medulla. The cortex is the site where B cell follicles are located. Upon Ag stimulation, primary B cell follicles mature into secondary B cell follicles with GC. The paracortex contains T cells, DCs and HEVs. The expression of various chemokines orchestrates the positioning of the lymphocytes entering the LN. Moreover, HEVs express chemokines that induce the recruitment of naive lymphocytes. All cells exit the LN via the efferent lymphatic vessel.

PPs are the most prominent component of the gut-associated lymphoid tissue (GALT) in intestinal mucosa. PPs are located on the anti-mesenteric wall of the small intestine below the follicle-associated epithelium (FAE), containing M cells. These cells transport luminal Ags to lymphoid follicles (5). The number and position of PPs are constant, but depends on the genetic background of the mouse strain. The microarchitecture is composed of B cell follicles and interfollicular T cell zones containing T lymphocytes, DCs, macrophages and HEVs. In addition, DCs are localized in close contact to M cells within the FAE and in the subepithelial dome.

PPs are the sites where primary immune responses against intestinal mucosal pathogens are generated. In PP-deficient mice, however, mucosal immune

responses are still observed. It has been shown that mesenteric LNs may serve as an alternative site for the induction of immune responses (6).

In addition to PPs, the GALT comprises cryptopatches (CPs) and isolated lymphoid follicles (ILFs), present on the anti-mesenteric wall of the intestine. Unlike PPs, CPs and ILFs appear after birth. CPs are lymphoid aggregates mainly composed of lineage negative cells expressing c-Kit (7). ILFs are inducible lymphoid tissues that develop after colonization of the intestine with bacteria (8). Thus, ILFs may replace conventional SLOs during adaptive immune response. ILFs are composed of B cells, DCs, T cells and RORγt$^+$c-Kit$^+$IL-7Rα^+ cells. Apart from cell composition, CPs are similar to ILFs (9, 10). Morphological studies suggest that CPs can differentiate into ILFs upon infection (10).

Colonic lymphoid patches and ILFs in the large intestine are also parts of the GALT (11), and display an architecture similar to PPs and ILFs in the small intestine (11).

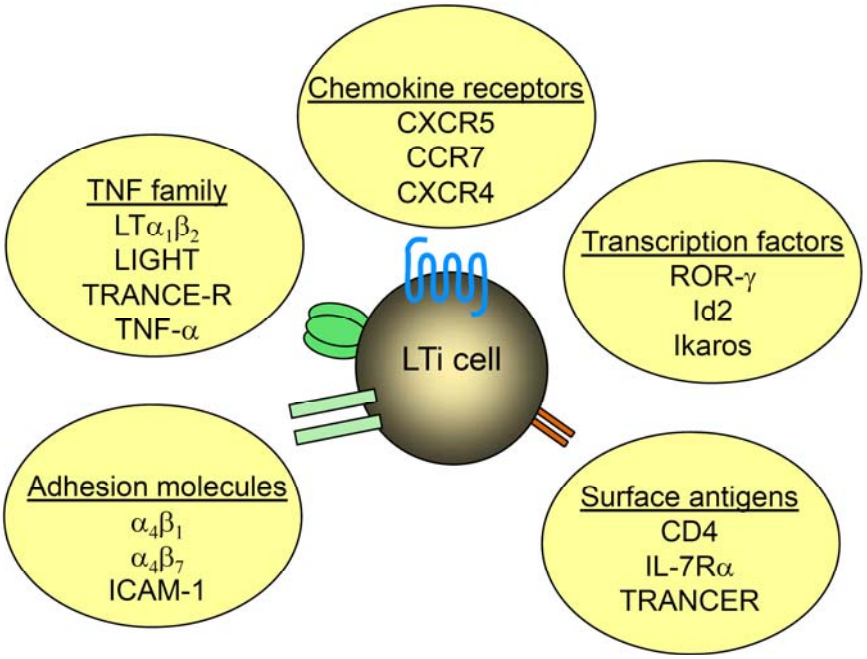
Both, LN and PP development requires the cellular crosstalk between lymphotoxin (LT) $\alpha\beta^+$ hematopoietic and LTβ receptor (LTβR)$^+$ mesenchymal cells (2) (see also 1.1.3 and 1.1.4). Similar to LN and PP development, the splenic white pulp develop during embryogenesis as a result of crosstalk between hematopoietic and mesenchymal cells. Studies in mutant mice, however, have shown that cytokines required for lympho-organogenesis may differ between LNs, PPs and NALT. In many knock-out (KO) mice, the number of peripheral LNs is reduced, whereas mesenteric LNs are present, indicating a site-specific regulation of peripheral versus mesenteric LN development. Similarly, there are some differences in the cellular regulation of LN and PP development. For example, additional cell subsets such as RET$^+$CD11c$^+$ cells have been shown to contribute to the formation of PP anlagen, but not of LNs (12).

1.1.1. Lymphoid tissue inducer cells

In the early 1990's, Kelly and Scollay have identified a new population of cells expressing CD4 but not CD3 (13). These cells are present in the neonatal LN at a high proportion and they are Thy-1^{low}CD44$^+$. They have a lymphoid morphology, but their origin and function have not been determined at that time. Some years later, Mebius and colleagues have shown that CD4$^+$CD3$^-$ cells express LT$\alpha\beta$ but, except

for CD4, are negative for all other lymphoid, myeloid and erythroid lineage markers. Moreover, these cells are amongst the earliest hematopoietic cells colonizing the fetal intestine as well as LN anlagen and spleen (14-16), suggesting a role in the development of lymphoid tissue architecture. Yoshida et al. have shown that blocking IL-7Rα function prevents the development of PPs. Since $CD4^+CD3^-$ cells express IL-7Rα, they have proposed that these cells are required for PP development (16). Two experimental approaches have demonstrated that $CD4^+CD3^-$ cells are lymphoid tissue inducer (LTi) cells. Firstly, the adoptive transfer of the cells could restore the formation of PP and NALT anlagen in $CXCR5^{-/-}$ or $Id2^{-/-}$ mice, respectively (17, 18). Secondly, mice lacking $CD4^+CD3^-$ cells have been shown to completely lack LNs and PPs (19, 20).

The phenotype of IL-$7R\alpha^+CD4^+CD3^-$ cells has been carefully described (15, 16). IL-$7R\alpha^+CD4^+CD3^-$ cells express LTαβ, TRANCE, LIGHT, TNFα, which are ligands of the tumor necrosis factor (TNF) family, the chemokine receptors CXCR5 and CCR7, the adhesion molecules α4β1, α4β7 and ICAM-1. Moreover these cells are positive for CD25, CD44, CD90, CD122, CD117, CD132 (15, 21) (figure 2). Amongst IL-$7R\alpha^+CD3^-$ cells, both $CD4^+$ and $CD4^-$ subsets exist that have been shown to share the same surface marker expression (16). Different proportions of $CD4^+$ and $CD4^-$ were found in various LNs (22). Functional experiments with $CD4^-$ cells have not been done and it remains to be tested if they belong to the LTi cell population.



Other surface markers expressed by LTi cells:

CD45, CD25 (IL-2Rα), CD132 (γ_c), CD44, CD90.2 (Thy1.2), CD117 (c-Kit), CD122, MHC I, MHC II.

Surface markers not expressed by LTi cells:

CD3, CD8, TCRαβ, TCRγδ, CD40, CD80, CD86, B220, CD19, CD5, CD11c, NK1.1, Ter119, Gr-1, F4/80, CD62L, Sca-1,

Figure 2. Phenotype of LTi cells (15, 21). LTi cells express CD4 and IL-7Rα, as well as TNF family members, chemokine receptors and adhesion molecules. Moreover, LTi cells are dependent on the RORγ, Id2 and Ikaros transcription factors for their differentiation.

Two IL-7Rα^+ LTi cell progenitor populations have been described in the fetal liver (FL) (23, 24). Firstly, Mebius and co-workers have characterized IL-7Rα^+Sca-1^{low}c-Kit^{low} cells in the FL (23). These FL cells were able to differentiate *in vivo* and *in vitro* into CD45$^+$CD4$^+$CD3$^-$ LTi cells, T cells, B cells, natural killer (NK) cells and DCs. In addition, *in vitro* these progenitors were able to differentiate into macrophages. Nishikawa and colleagues have described a lin$^-$IL-7R$\alpha^+\alpha_4\beta_7^+$ cell population that could differentiate into CD45$^+$CD4$^+$CD3$^-$ LTi cells, T cells, NK cells and DCs. No differentiation of these progenitors into B cells could be observed (24).

The differentiation of progenitors into CD45$^+$CD4$^+$CD3$^-$ LTi cells requires the presence of the transcription factor Ikaros (25) and the protein Id2 (18, 20), which is a member of the Id family. Id proteins are inhibitors of basic helix-loop-helix transcription factors and play an important role in the regulation of lineage

commitment and differentiation. In Id2-deficient mice, LTi cells are absent and LNs, PPs and NALT do not develop.

Another molecule required for the generation of LTi cells is the nuclear retinoic acid related-orphan receptor RORγt (19). Apart from promoting survival of double positive thymocytes, RORγt is expressed by LTi cells, and by proinflammatory IL-17$^+$ T helper cells (19, 26). In mice lacking RORγt, LTi cells are undetectable (19), LNs and PPs do not develop, and the thymus is abnormal (27). NALT development, however, is normal in these mice (28), suggesting that NALT development does not strictly require the presence of RORγt$^+$ LTi cells (18).

LTi cells co-express TNF-related activation-induced chemokine (TRANCE) and TRANCER (29). TRANCE-deficient mice have a lower number of LTi cells and LNs are absent in these mice (29-31). By overexpressing TRANCE, the number of LTi cells can be rescued (32). Moreover, TRANCE induces the upregulation of LTαβ on the surface of LTi cells (33). These data strongly suggest that TRANCE promotes the generation and regulates the function of LTi cells.

Altogether, LTi cells are a unique subset of cells derived from IL-7Rα$^+$ FL precursor cells, which can induce the development of SLO anlagen through interaction with LTβR expressed by mesenchymal cells (see also 1.1.3 and 1.1.4).

1.1.2. LTi cells in adult mice

In several studies, the question has been addressed if adult LTi cells exist, which may contribute to lymphoid tissue development/organization under normal and inflammatory conditions.

The accumulation of CD45$^+$CD4$^+$CD3$^-$ cells has been shown more than 10 years ago in helminth-infected adult mice (34). Mice infected with the parasite developed splenomegaly and hepatomegaly. The authors have shown that splenocytes enriched for CD4$^+$ cells and cultured in presence of the parasite downregulated T cell receptor (TCR) and CD3 and resembled large granular lymphocytes. The CD4-enriched population contained maybe both CD4$^+$CD3$^+$TCR$^+$ T cells and CD4$^+$CD3$^-$ TCR$^-$ LTi-like cells, the latter being selected by the parasite, the other dying. *In vivo*, flow cytometry analysis of spleen and liver of infected mice revealed an increased number of CD4$^+$CD3$^-$ cells compared to uninfected mice. It has been hypothesized

that these cells may play a role in maintaining the balance between host and parasite (34).

In a transgenic (tg) mouse model using GFP as a reporter gene inserted into the Rorc(γt) gene (35), clusters of RORγt$^+$ cells within CPs, and to a lesser extent in ILFs and in the subepithelial dome of PPs were observed 1-2 weeks after birth. RORγt$^+$ cells expressed c-Kit and IL-7Rα, but, apart from CD4, no other lineage marker. The phenotype of lin$^-$c-Kit$^+$IL-7Rα$^+$ cells was similar to fetal LTi cells. Adult lin$^-$c-Kit$^+$IL-7Rα$^+$ cells were therefore thought to be the adult counterpart of fetal LTi cells. In RORγ-deficient mice, neither intestinal lin$^-$c-Kit$^+$IL-7Rα$^+$, nor CPs and ILFs were found (35, 36), suggesting that lin$^-$c-Kit$^+$IL-7Rα$^+$ LTi-like cells in adult mice supported the formation of organized GALT in the intestine, including both CPs and ILFs. Indeed, a recent study shows that transfer of RORγt$^+$ LTi-like cells into RORγ-deficient mice that are devoid of CPs and ILFs, induces the development of organized structures in the gut (37). As transfer of wild type (WT) bone marrow (BM) into LTα$^{-/-}$ or $\gamma_c^{-/-}$ allows the reconstitution of CPs and ILFs, BM may be a source of LTi-like cell precursors (36, 37).

In the spleen of adult mice a population of cells resembling adult LTi-like cells has been described. CD4$^+$CD3$^-$ cells were located at the B/T interface in the marginal sinus and in B cell follicles. Histological analysis showed that at both places, CD4$^+$CD3$^-$ cells were in close contact with primed T cells. CD4$^+$CD3$^-$ cells expressed high levels of the T cell costimulatory molecules OX40L and CD30L, members of the TNF family. The receptors OX40 and CD30 were expressed by primed, but not naive T cells. Therefore, CD4$^+$CD3$^-$ cells may be potential candidate for providing co-stimulatory signals to primed T cells (21). OX40L and CD30L were exclusively expressed by adult but not fetal CD4$^+$CD3$^-$ cells after *in vitro* culture (38). However, addition of IL-7 to fetal LTi cells increased CD30L expression (39).

The adoptive transfer of WT splenocytes into LTα$^{-/-}$ mice was not able to restore splenic organization. Nevertheless, transfer of LTα$^{-/-}$ splenocytes into RAG$^{-/-}$ mice induces a normal organization (40), suggesting that a cell type present in RAG$^{-/-}$ mice was able to induce splenic organization in the presence of lymphocytes. Adult LTi-like cells express high levels of LTα and LTβ mRNA (40) and are therefore a good candidate. Indeed, fetal and adult LTi cells, but not lymphocytes or DCs were able to restore B/T segregation in the spleen of LTα-deficient mice (40).

Taken together, these data suggest that, in addition to their function in mucosal immunity and T cell priming, LTi cells may be important for splenic organization. Blocking LTαβ with a soluble LTβR-huFc resulted in loss of discrete B cell follicles and altered the marginal zone (41). These findings demonstrate that continuous LTβR signals are required to maintain the splenic organization.

During acute infection with lymphocytic choriomeningitis virus (LCMV), antiviral cytotoxic T cells destroy infected T cell zone stromal cells, leading to SLO disruption. The $gp38^+$ fibroblastic reticular cell (FRC) network in the white pulp is mainly affected. In consequence, the host becomes unable to respond to pathogens and loses immunocompetence. LCMV infection was shown to be followed by the accumulation of $CD45^+CD4^+IL\text{-}7R\alpha^+lin^-$ cells in spleen and LNs, probably due to proliferation of an existing pool of cells. The cell number arised the maximum at the peak of infection. In the absence of $CD45^+CD4^+IL\text{-}7R\alpha^+lin^-$ cells, the reorganization of the spleen was delayed. In $ROR\gamma^{-/-}$ chimeric mice, the injection of $CD45^+CD4^+IL\text{-}7R\alpha^+lin^-$ cells accelerated the restoration of the splenic architecture. These experiments show that LTi cells are important for SLO organization during the entire life. Continuous crosstalk with stromal cells is crucial for SLO maintenance as well as for restoration after tissue-damaging infections (42).

1.1.3. Mesenchymal organizer cells

By whole mount immunostainings, Adachi and colleagues have shown the presence of vascular cell adhesion molecule (VCAM)-1^+ cells in the gut, as early as embryonic day (E) 15.5 (14). In primitive anlagen, VCAM-1^+ cells and IL-$7R\alpha^+$ LTi cells co-localized, suggesting that they collaborated in generating PP anlagen. VCAM-1^+ cells have been called “organizer” cells and have been characterized in more details. Organizer cells express LTβR and other adhesion molecules, such as ICAM-1 and MAdCAM-1 (33). In addition, they co-express platelet-derived growth factor receptor α (PDGFRα) and PDGFRβ, suggesting a mesenchymal origin (43). Organizer cells also express TRANCE and produce IL-7 and the chemokines CXCL13, CCL19 and CCL21 (33, 44), which are potent chemoattractants for LTi cells (33, 43, 45). No cell surface marker specific for hematopoietic or endothelial cells could be detected on organizer cells. They are an active component of PP

organogenesis, with the capacity to recruit mature lymphocytes and organize lymphoid tissue compartmentalization.

Cupedo and colleagues have found a LN equivalent to PP organizer cells, co-expressing VCAM-1, ICAM-1 and MAdCAM-1 (33). They described two distinct types of organizer cells, thereby showing the diversity existing in VCAM-1^{+}ICAM-1^{+} cells (33). The proportion of VCAM-1^{high}ICAM-1^{high} cells was higher than VCAM-1^{int}ICAM-1^{int} cells in mesenteric compared to peripheral LNs, respectively (33), suggesting difference in the molecular requirements for mesenteric and peripheral LN development.

VCAM-1^{high}ICAM-1^{high} cells from E17.5 LNs and PPs have been shown to be distinct populations (46). Indeed, IL-7 expression is higher in LNs than in PPs, in contrast to the chemokines CXCL13, CCL19 and CCL21 which are higher in PPs than in LNs. However, the expression profile of these genes becomes comparable in LN and PP organizer cell populations of 4 day old mice.

Altogether, these results suggest that the mesenchymal organizer cells found in peripheral LNs, mesenteric LNs and PPs are different with respect to expression levels of various cell adhesion molecules and cytokines. This may explain why the deletion of one of these factors mostly affects the development of only some but not all SLOs.

1.1.4. Lymph node organogenesis

During embryogenesis, LNs form from budding of primitive lymphatic veins thereby forming lymph sacs imbedding stromal cells. Between E12.5 and E13.5, the early formation of LN anlagen consists of clusters of IL-7Rα^{+} cells with VCAM-1^{+} resident stromal cells. Studies in KO mice have shown that molecules of the TNF super-family are crucial for the development of LNs. Indeed, in the absence of LTα, LTβ or LTβR, LN development is blocked, indicating that the engagement of LTβR is an essential step for LN organogenesis (Figure 3). Indeed, injection of a LTβR agonist antibody (Ab) into LT$\alpha^{-/-}$ mice is able to restore LN development. LTi cells express high level of LT$\alpha\beta$ and are therefore a good candidate as the cellular source of LT$\alpha\beta$. Stimulation of the LTβR activates two NF-κB pathways. The classical pathway leads to the expression of VCAM-1, ICAM-1 and MAdCAM-1 and the alternative pathway to the production of the chemokines CXCL12, CXCL13 (BLC), CCL19 (ELC) and

CCL21 (SLC) (47). CXCL13 binds to CXCR5, the corresponding chemokine receptor expressed by LTi cells. Signalling through CXCR5 activates the integrin $\alpha4\beta1$ via an inside-out signal (17).The activated form of $\alpha4\beta1$ will then bind to VCAM-1, thereby leading to a firm adhesion between LTi and organizer cells. Since mice deficient for CXCR5 or CXCL13 lack most LNs, it is clear that chemokine/chemokine receptor family members are required for LN development.

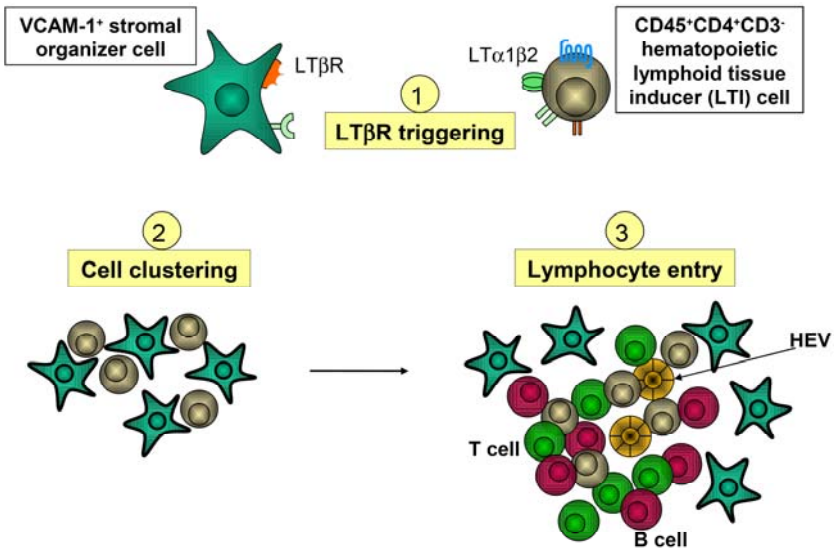


Figure 3. Development of SLOs starts with the cluster of VCAM-1^+ mesenchymal organizer and LTi cells. $CD4^+CD3^-$ LTi cells engage LTβR expressed by organizer cells. The LTβR signalling cascade induces the expression of VCAM-1 and the production of chemokines. The chemokine CXCL13 binds the chemokine receptor CXCR5, which triggers an inside-out signal leading to the activation of the integrin $\alpha4\beta1$. This integrin binds to its high affinity receptor VCAM-1 which most likely reinforces the interaction between both cell types (1). Firm adhesion of both cells leads to recruitment and cluster of more cells (2). Finally, HEVs are formed, that allow entry of lymphocytes and their compartmentalization into B and T cell areas (3).

1.1.5. Peyer's patch organogenesis

Adachi and colleagues have described the development of PPs in three steps (14). First, at E15.5, VCAM-1-expressing cells cluster and form spots that co-localize with ICAM-1^+ cells. PP formation is initiated at the proximal end of the intestine and proceeds towards the distal end. The second step in PP organogenesis takes place at E16.5-E17.0 when VCAM-1^+ICAM-1^+ spots are colonized by IL-$7R\alpha^+CD4^+CD3^-$ cells. The third and last step, which occurs postnatally, is the migration of mature T and B cells into these early PP anlagen.

Although IL-7R$\alpha^{-/-}$ and LTβR$^{-/-}$ mice do not develop PPs, nor have VCAM-1 spots, IL-7$^{-/-}$ mice have VCAM-1 spots but do not develop PPs. LT$\alpha^{-/-}$ mice were also reported to lack PPs (48, 49). Injection of LTβR agonist into LT$\alpha^{-/-}$ mice does not restore PP development, suggesting that other signalling pathways are involved.

1.2. Tertiary lymphoid organs

In contrast to SLO development, which is restricted to embryogenesis, tertiary lymphoid organs (TLOs) are inducible lymphoid structures that develop after birth. TLOs are lymphocytic cell accumulations arising through a process called “lymphoid neogenesis” in tissues affected by chronic inflammation (50). TLOs are not restricted to specific anatomical locations, and they can form in non-lymphoid organs. The neogenesis of lymphoid tissue is observed in many human chronic inflammatory diseases caused by autoimmune reactions or chronic allergy, microbial infections and chronic allograft rejections (51) (Table 1). It is currently unclear which role TLOs have in the pathogenesis of the chronic diseases (52, 53). On the one hand, TLO formation can be protective as shown in the case of lung infection with *Mycobacterium tuberculosis* (53). In this case, the infection promotes the development of lymphoid structure containing GCs. Given that B cell-deficient mice have enhanced susceptibility to *M.tuberculosis*, it suggests that B cells, and by extension TLOs, are important for the resistance against the infection. On the other hand, the immune response generated by TLOs can be harmful. Upon organ transplantation, TLO neogenesis can occur in the graft and prove the microenvironment for B cell differentiation, affinity maturation and production of autoreactive Abs involved in graft rejection (54).

Table 1.

Development of TLOs in chronic autoimmune, inflammatory and infectious diseases.

Disease	Affected organ
Chronic autoimmune diseases	
Rheumatoid arthritis	Joints
Multiple sclerosis (EAE)	Central nervous system
Sjögren's syndrome	Salivary glands
Hashimoto's thyroiditis	Thyroid gland
Grave's disease	Thyroid gland
Myasthenia gravis	Thymus
Diabetes	Pancreas
Other inflammatory diseases	
Ulcerative colitis	Large intestine
Crohn's disease (IBD)	Small intestine
Atherosclerosis	Arteries
Infectious diseases	
Influenza	Lungs
Mycobycterium tuberculosis	Lungs
Chronic Hepatitis C	Liver
Helicobacter-pylori gastritis	Gastric mucosa
Others	
Chronic GvHD	Transplant

1.2.1. TLOs: similarities and differences compared to SLOs

Lymphoid neogenesis is a dynamic process during which lymphocytic infiltrates evolve into aggregates that eventually organize into secondary B cell follicles with GCs and distinct T cell areas containing DCs and HEVs. The formation of GCs in TLOs indicates that immune responses can occur in these tissues. In some cases, a complete B cell maturation is observed in ectopic GCs, with Ag-driven clonal expansion. In rheumatoid arthritis (RA) and Sjögren’s syndrome, the generation of plasma cells in TLOs are associated with GCs, which is consistent with local Ag presentation, but it is not clear, whether plasma cells develop in or migrate to TLOs (47, 48, 51).

While TLOs share some morphological and functional properties with SLOs, the physiological events that induce the *de novo* formation of TLOs remain unknown.

There is evidence that, similar to SLO development, stromal cells play an important role in TLO formation (55). In case of chronic inflammation, local fibroblasts may lose their tissue function and differentiate into stromal cells producing homeostatic chemokines. This is the case in RA, where the fibroblast-derived chemokines CXCL12 and CXCL13 are overexpressed in the joints (55). This phenomenon of ectopic expression of chemokines is observed in many other inflammatory diseases, such as Sjögren's syndrome.

Altogether, these data suggest that in case of chronic inflammatory diseases, the tissue-specific stroma starts to express inappropriate chemokines that induce a lymphoid environment in a non-lymphoid organ (55).

1.2.2. TLO development in tg mouse models

TLO formation has been induced in several tg models in which inflammatory cytokines or lymphoid chemokines were expressed under the control of a tissue-specific promoter. For instance, LTα expressed under the control of the rat insulin promoter (RIP) led to inflammatory lesions in the pancreas and in the kidney (50). These structures were organized like SLOs and contained all cell subsets found in normal SLOs. Indeed, T cells, B cells, plasma cells, FDCs, as well as HEVs were present in RIP-LT mice. The formation of GC and the presence of isotype switched-B cells in these ectopic follicles suggest that these tissues were functional and responded to Ag. Ecopic follicles were not associated with the development of diabetes. Indeed, RIP-LT mice did not spontaneously develop tissue damage, unless infiltrating T cells were activated (50), thereby leading to tissue destruction and diabetes. Experiments with the expression of either LTα alone or both LTα and LTβ under the control of the RIP promoter show that, although LTα alone could initiate TLO organogenesis, simultaneous expression of both LTα and LTβ induces lymphoid neogenesis with larger infiltrates, a clearer separation of the B and T zones, large FDC networks, higher levels of CXCL13, CCL19 and CCL21 expression, and a strong expression of PNAd on the luminal surface of HEVs (56). CXCL13 was shown to upregulate LT$\alpha\beta$ expression by B cells, which in turn engage the LTβR on stromal cells. This led to increased expression of CXCL13 and further promoted follicle

development (57). These data establish that LTαβ and homeostatic chemokine expression are linked via a positive feedback loop that is critical for the development of SLOs and TLOs (57).

The overexpression of CCL21 or CXCL13 in non-lymphoid tissues was sufficient to recruit T or B cells and to promote lymphoid neogenesis (58, 59). For example, overexpression of CXCL13 in the pancreas of mice induced the formation of lymphoid tissue and the differentiation of HEVs allowing adoptively transferred lymphocytes to colonize the lymphoid infiltrate and segregate into B and T cell areas (59). Tg mice expressing CCL21 under the control of the thyroglobulin promoter developed ectopic organized lymphoid tissues in the thyroid gland. In this model, LT-expressing $CD4^+$ T cells were more important than B cells to initiate the development of lymphoid infiltrates and lymphatic vessels, through a process called lymphangiogenesis (60). Even present in high quantity, overexpression of CCL21 in the skin did not lead to the development of TLOs. This implies that differences exist in the mechanisms leading to the development of ectopic lymphoid tissues in various organs (58).

Considering that lymphoid chemokines are expressed in inflamed tissues without organized lymphoid tissue (61, 62), it is tempting to speculate that these chemokines are induced by inflammation prior to the formation of segregated B and T cell areas. Indeed, early expression of homeostatic chemokines may be independent of LTαβ or TNF, as seen in influenza-infected lungs of $LT\alpha^{-/-}$ mice (63). $LT\alpha^{-/-}$ mice were lacking peripheral LNs, but upon influenza infection, developed inducible lymphoid tissues in the lung. These lymphoid tissues were organized in T and B cell areas and allowed an immune response to occur, as demonstrated by the fact that the infection was cleared (63).

Altogether, studies of lymphoid neogenesis in human diseases and animal models show that at least two critical events seem to be required to promote TLO formation: firstly, inflammation and cytokine (LTαβ, TNF) expression and secondly lymphoid chemokine production by stromal cells. In addition, the development of HEVs may be required for the recruitment of lymphocytes (51).

1.2.3. Role of LTi cells in TLO development

Although LTi cells are clearly required for LN and PP development, a role in TLO development or maintenance remains to be elucidated. In a study using RIP-BLC tg mice, $CD4^+CD3^-$ LTi cells were the first hematopoietic cells recruited to the islets very early after birth (59). This suggests that LTi cells may be involved in the development of TLO that will eventually later develop at this place.

The transfer of neonatal LN-derived cells or sorted LTi cells to ectopic sites, like the skin, of newborn mice was followed by the *de novo* formation of lymphoid tissue. However, the transfer of LTi cells to adult mice only generated disorganized clusters of lymphocytes without FDCs or HEVs (64). This indicates that although neonatal LN-derived cells can induce the development of TLOs attracting mature lymphocytes, the environment in adult mice is inappropriate for lymphoid tissue neogenesis. This may be due to a lack of inflammation or a too low number of $LT\alpha\beta$-expressing LTi cells. B cells also express $LT\alpha\beta$, but apparently, this signalling is not sufficient to induce compartmentalization of B and T cell areas.

Recent studies using mice expressing CCL21 in the thyroid gland show that the first cells appearing in the thyroid gland were $CD4^+$ T cells and not LTi cells. Moreover, in CCL21 tg x $Id2^{-/-}$ mice, TLO formation was not affected (65). This study shows that TLO development in this mouse model does not require Id2-dependent LTi cells.

To summarize all the data obtained from various animal models, the development of TLOs is probably coordinated by many factors including the induction of chemokine expression and the influx of various hematopoietic cells.

1.2.4. TLOs and autoimmunity

TLOs often develop in autoimmune diseases at sites of chronic immune attack. The main autoimmune diseases and the respective affected organs are listed in Table 1.

In many cases, the formation of well-developed ectopic follicles in these sites correlates with increased severity of the disease and the local production of auto-Abs. For example, RA is an autoimmune disease characterized by the chronic inflammation of the joints leading to progressive destruction of cartilage and bone

(66). Patients with RA can be divided into different groups, with either diffuse lymphoid aggregates in their synovium, or B and T cell aggregates, or finally highly developed GCs, FDCs and segregated B and T cell areas (62, 67).

Mice expressing LCMV glycoprotein in pancreatic islets develop islet-associated lymphoid tissues and autoimmune diabetes upon immunization with DCs that display the cytotoxic T lymphocyte epitope from the LCMV glycoprotein (68). These data indicate that DCs also have an important role in lymphoid neogenesis (68). Indeed, DCs are an important source of chemokines and survival factors and may facilitate lymphocyte homing and compartmentalization in inflamed tissues (68). The role of the DCs could also be to prime T cells in spleen and LNs, which in turn would induce lymphoid neogenesis.

Sjögren's syndrome is an autoimmune disease characterized by infiltration of activated T cells around the duct in the salivary gland (69). Infiltrating leukocytes are mainly T cells, but substantial numbers of B cells and plasma cells are also present in inflamed tissues (70). These infiltrates can further develop into ectopic lymphoid tissue. The development of GCs has been reported and may contribute to disease progression (69). The formation of ectopic GCs in Sjögren's syndrome patients is, at least in part, driven by abnormal expression of cytokines and chemokines, such as TNF, LT, CXCL13 and CCL21 (71).

Autoimmune diseases are very complex and until now there are no evidences whether TLO development triggers the disease or is a consequence of it. From studies on mouse models, it seems that autoimmunity promotes the development of TLO in the affected organ, rather than resulting from it. As previously discussed, studies in tg mouse models suggest that cytokine- or chemokine-induced development of ectopic lymphoid tissue does not automatically lead to autoimmunity (59, 72). However, once established, TLO may contribute to the persistence of chronic stimulation and autoimmune reactions instead of clearing the disease.

1.2.5. TLOs and infection

The development of TLO can also occur as a response to local infections (table 1).

For example, it is well established that the lungs are a common site for the formation of TLOs called "inducible bronchus-associated lymphoid tissue (iBALT)" in response to acute or chronic lung infections (63) or in case of pulmonary disease or

pulmonary fibrosis (52). Infection with Influenza virus induces iBALT formation, consisting of B cell follicles with GCs and FDCs, T cell areas with $CD11c^+$ DCs, $PNAd^+$ HEVs and lymphatic vessels. IBALT participates in lymphocyte priming, and mice lacking all conventional SLOs except iBALT survive higher doses of influenza than WT mice (63). This implicates that iBALT is able to promote local protective immunity. In iBALT, as in many TLOs, CXCL13 is expressed on reticular cells in B follicles, and CCL21 is expressed on $PNAd^+$ HEVs. Upon lung infection, CXCL13 and CCL21 expression is induced by a LTα- and TNF-independent mechanism (63). However, in LT$\alpha^{-/-}$ or LTβR$^{-/-}$ mice, iBALT is not properly organized. This may be due to the lack of appropriate LTα-dependent stromal cells or HEVs, which are critically required for lymphoid tissue organization.

In summary, peripheral organs such as the lungs can react to viral infection by developing ectopic lymphoid tissue. Comparing data obtained from mouse models with TLO development, it is tempting to speculate that TLOs formed in response to viral infection are able to fight against the pathogen and to clear the infection. In contrast, TLOs formed in response to chronic inflammation or autoimmunity, may have a pathological role, worsening instead of healing the disease.

1.3. Interleukin 7

Human and mouse IL-7 have been cloned in the late 1980's (73, 74) and their genomic sequences share 81% homology. The murine IL-7 cDNA is 462 base pair long and it forms a protein of 154 amino acids, which molecular weight represents 14.9 kDa (74).

IL-7 is expressed by mesenchymal and epithelial cells in the gut, BM and thymus. In addition, IL-7 can also be expressed by fibroblasts, smooth muscle cells, keratinocytes and DCs following activation (75, 76). IL-7 can be secreted or presented on the cell surface by heparin sulfate and fibronectin (75). In tissue, IL-7 is associated with extracellular matrix. Importantly, IL-7 expression by hematopoietic cells has never been observed, suggesting that only non-hematopietic cells can produce IL-7.

IL-7 is a member of the IL-2/IL-15 cytokine family that signals through the common cytokine gamma chain (γ_c). In addition to γ_c, IL-7R is composed of IL-7Rα chain (74, 75).

In mice, the expression of IL-7Rα is limited to hematopoietic cells, mostly from the lymphoid lineage. NK, developing T and B cells (only in mice) and mature T cells express IL-7Rα. Both IL-7Rα and γ_c subunits are expressed by LTi cells. Moreover, in man, IL-7Rα is also expressed by intestinal epithelial cells and BM-derived macrophages (75, 76).

By binding to IL-7R, IL-7 triggers different signalling pathways, among them are phosphatidylinositol 3-kinase (PI3-kinase) and Src family tyrosine kinases. However, the most prominent one is the Janus kinase (Jak)/signal transducer and activator of transcription (STAT) pathway (Figure 4). Jak 1 and 3 are members of the Janus family of tyrosine kinases, which are important factors in cytokine receptor signalling pathways (76). Jak3 is constitutively associated with γ_c, and Jak1 is associated with IL-7Rα. Binding of IL-7 to IL-7Rα induces dimerization of the receptor with γ_c, on which IL-7 also possesses binding sites. Subsequently, Jak3 phosphorylates tyrosine residues on the cytoplasmic tail of IL-7Rα. Activated Jak1 and Jak3 then recruit and tyrosine-phosphorylate STAT5. This induces the dimerization and translocation of STAT5 to the nucleus, where transcription of IL-7 target genes occurs. TCRγ and B cell leukemia (Bcl) 2 are two examples of IL-7 targets (77).

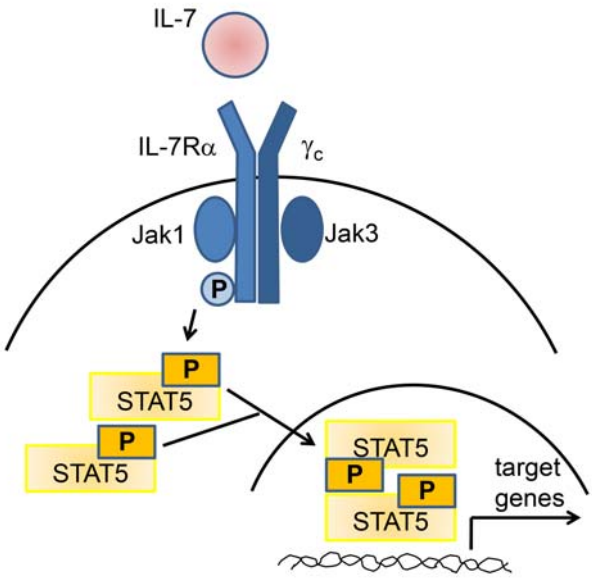


Figure 4. IL-7 signalling pathway. Upon binding of IL-7 on IL-7R, Jak3 phosphorylates tyrosine kinases on the cytoplasmic tail of IL-7Rα chain. This induces the recruitment of STAT5 and its phosphorylation. STAT5 dimerizes and translocates to the nucleus to start the transcription of IL-7 target genes.

1.3.1. Role of IL-7 in lymphopoiesis and lymphocyte homeostasis

IL-7 has first been defined as a hematopoietic growth factor implicated in immature B cell development and has been called lymphopoietin-1 (78). In mice both B and T cell developments are regulated by IL-7, whereas in humans, IL-7 seems to be required only for T cell development.

In the thymus, IL-7 is implicated in T cell development and in $\gamma\delta$ TCR rearrangement. In the periphery, IL-7 plays a role in survival/persistence of resting CD4 and CD8 T cells and their homeostatic turnover by increasing Bcl-2 expression. IL-7 may also be responsible for the differentiation of effector to memory T cells (79, 80). Therefore, IL-7 is required for the homeostasis of peripheral T cells.

In IL-7-deficient mice, B cell development is blocked at the pro-B cell stage, thymic cellularity is decreased 20-fold, $\alpha\beta$ T cell population is reduced, whereas $\gamma\delta$ T cells are nearly absent (79).

Tg expression of IL-7 under the MHC class II promoter leads to a dramatic expansion of immature B cells in the BM, spleen and LNs. A 30-fold increase in mature T cells is observed, but thymic development is not affected (81). In T-lymphopenic mice, the enhanced availability of IL-7 increases T cell recovery through reduction in apoptosis and increase in proliferation (82). These data have demonstrated that IL-7 has multiple effects on hematopoietic cells.

It has been recently shown that the biological activity of IL-7 can be increased by combining IL-7 with an anti-IL-7 Ab (83). These findings were based on a previous study by the same laboratory showing that IL-2 had a poor biological activity, probably due to its short lifespan, and binding IL-2 with anti-IL-2 mAb before *in vivo* administration induced a strong expansion of T and NK cells (84). In order to test whether IL-7R^{+} naive and memory subsets of T cells could undergo proliferation *in vivo*, administration of IL-7/anti-IL-7 Ab complexes has been performed (83). Treatment of the mice with IL-7/anti-IL7 Ab complexes induced a strong expansion of naive and memory T cells. Moreover, IL-7/anti-IL-7 Ab complexes could restore T cell development in IL-7$^{-/-}$ mice (83). IL-7/anti-IL-7 Ab complexes displayed 50 to 100-fold higher efficiency than IL-7 alone. Importantly, anti-IL-7 Ab had to be pre-bound to IL-7 to enhance the activity of the cytokine (83). The mechanism underlining the enhanced activity of IL-7 is likely its prolonged lifespan *in vivo*.

1.3.2. Role of IL-7 in lympho-organogenesis

Mice lacking IL-7, IL-7Rα or signalling component of IL-7R, such as Jak3 have severe defects in LN and PP organogenesis (45, 85-87) thus demonstrating that IL-7 is important during lymphoid organogenesis. Indeed, IL-7 produced by fetal organizer cells has a key function in inducing LTαβ expression by LTi cells (1, 44, 45).

Yoshida and colleagues have shown that the expression of LTαβ on IL-7Rα$^+$ LTi cells could be induced by both IL-7 and TRANCE (44). These data show that there is a partial overlap between IL-7 and TRANCE effects. TRANCER$^{-/-}$ and TRAF6$^{-/-}$ mice did not develop LNs, but PP development was normal. This suggests that TRAF6 is downstream of TRANCER, but not of LTβR, which is required for both LN and PP development. IL-7Rα$^+$ cells isolated from TRAF6$^{-/-}$ mice did not respond to TRANCE, but upregulated LTαβ expression in response to IL-7 (44). Administration of IL-7 to TRAF6$^{-/-}$ embryos, which lack the TRANCE signalling pathway, was sufficient to trigger LN genesis. Therefore, IL-7 is able to replace TRANCER signalling for LN induction, but the maturation into segregated compartments is not completed and requires TRANCER/TRAF6 signalling.

The role of IL-7 for LTi progenitor and LTi cell survival/proliferation remained unsolved until now, and was the subject of manuscript 1.

1.3.3. Role of IL-7 in autoimmune diseases

A role of IL-7 for disease progression was discussed in RA (88) and in other autoimmune diseases such as colitis (89, 90), multiple sclerosis (91) and diabetes (92).

In RA patients, elevated levels of IL-7 were found in the synovial fluid, produced by fibroblasts, macrophages, endothelial cells and DCs (88). As already mentioned in the section 1.2.4, patients suffering from RA developed TLOs in their inflamed joints. The activation of the IL-7R signalling pathway could therefore be related to the development of TLOs in RA patients, and further contribute to the progression of the disease (67).

Multiple sclerosis is the most common neurologic disease. It is an inflammatory autoimmune disease in which lymphocytes and macrophages infiltrate the central nervous system. Mouse models for experimental autoimmune encephalitis (EAE)

were used to understand the mechanisms occurring in multiple sclerosis. Among many other genes, an allelic variant of IL-7Rα was overexpressed in peripheral blood cells of patients suffering from multiple sclerosis (93).

In all these autoimmune diseases, the elevated level of systemic IL-7 could sustain autoreactive T cell responses, and permit the establishment of a specific microenvironment for chronic autoimmune diseases.

1.4. IL-7 overexpressing mouse models

The first IL-7 tg mouse has been generated in 1991 by Samaridis et al (94). They established a tg mouse model expressing IL-7 only in lymphoid cell compartments such as BM, spleen and thymus. For that, IL-7 cDNA was under the control of an Ig kappa light chain promoter and a heavy chain enhancer. This resulted in expression of IL-7 transgene by circulating B and T cells. IL-7 overexpression in these mice induced an increase in B cell precursor number in the BM, mature B cells in the spleen, as well as a thymocyte and peripheral T cell expansion (94).

In 1993, Rich and colleagues generated a tg mouse, where IL-7 cDNA was expressed under the control of an Ig heavy chain promoter and enhancer (95). They found expression of the transgene in the BM, LNs, spleen, thymus and skin. In addition to the previously reported lymphoproliferative disorder induced by IL-7 overexpression, they also observed a progressive cutaneous disorder with dermal lymphoid infiltrate (95).

A mouse with IL-7 expressed under the Srα promoter has been shown to develop chronic colitis (96). Immunohistological analysis revealed the presence of lymphoid infiltrates in the colonic lamina propria, mostly composed of $CD4^+$ T cells. The authors suggested that chronic inflammation in the colonic mucosa was mediated by a colonic epithelial cell-derived IL-7 (96).

In 1995, another IL-7 tg mouse was created, with the murine IL-7 expressed under the control of the MHC class II promoter (81). These mice allowed the analysis of the effects of IL-7 on the differentiation of fetal thymocytes, and on the development of the T cell repertoire. In these tg mice, IL-7 was produced by all MHC class II-expressing cells, including the thymic epithelium. IL-7 overexpression by MHC class II cells induced an accumulation of T cells and immature B cells in LNs. Due to the presence of more immature B cells in IL-7 tg compared to WT mice, LN and spleen

architecture were abnormal in IL-7 tg mice. In contrast, the thymus was not altered. Indeed, no difference was observed between WT and IL-7 tg thymus structure, nor in thymocyte development (81).

2. AIM OF THE STUDY

Our laboratory is investigating the role of hematopoietic cells in inducing lymphoid tissue formation, and the regulation of LTi cells and lymphoid tissue formation by cytokines such as IL-7 and thymic stromal lymphopoietin (TSLP). As main tools, various tg or KO mouse models are used.

During my PhD, two main questions were addressed.

Question 1:

Are $CD4^+lin^-$ cells in adult mice *bona fide* LTi cells and how are they regulated?

Question 2:

What are the roles of IL-7 and adult $CD4^+lin^-$ cells in ectopic lymphoid tissue formation under normal or inflammatory conditions?

In order to address these questions, we have created a mouse model with a systemic expression of IL-7. Mice expressing murine IL-7 under the control of the MHC class II promoter (81), called IE-IL-7, have been crossed to mice expressing the MHC class II transactivator (CIITA) under the control of the ubiquitous Srα promoter (97). In these double tg mice (termed H-IL-7), the ubiquitous synthesis of CIITA promotes the systemic expression of IL-7. Three to seven-folds more IL-7 transcripts are produced in H-IL-7 compared to IE-IL-7 mice. Alternatively to studying H-IL-7 mice, we treated WT or $IL\text{-}7^{-/-}$ mice with IL-7/anti-IL-7 Ab complexes.

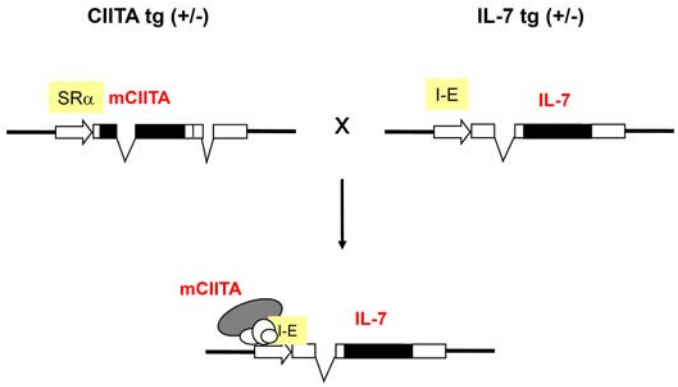


Figure 5. H-IL-7 mouse model. An heterozygous mouse expressing CIITA under the control of the Srα promoter has been crossed with an heterozygous mouse expressing murine IL-7 under the control of the MHC class II (I-E) promoter.

3. RESULTS

3.1. Review

Interleukin 7-induced lymphoid neogenesis in arthritis: recapitulation of a foetal developmental programme?

Daniela Finke, Sandrine Schmutz

Peer reviewed article

Interleukin 7-induced lymphoid neogenesis in arthritis: recapitulation of a foetal developmental programme?

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Summary

Chronic inflammatory diseases such as rheumatoid arthritis (RA) are associated with the *de novo* formation of organised lymphoid tissue in a subpopulation of patients. The aberrant expression of cytokines and chemokines by stromal cells plays an important role in recruitment and survival of effector cells of the immune system and the development of ectopic tertiary lymphoid organs (TLOs). TLOs may promote the persistence of inflammation and the recognition of self antigens. Recent studies in man and mice now indicate that interleukin 7 (IL-7) is implicated in the formation of TLOs and progression of chronic inflammation.

Key words: Interleukin 7; arthritis; inflammation; lymphoid tissue inducer cell; tertiary lymphoid organ

Introduction

IL-7 is a cytokine that uses the common gamma chain (γc) and the IL-7 receptor α (IL-7Rα) chain for signalling. It is primarily expressed by epithelial and stromal cells of various organs such as the thymus, bone marrow, intestine and skin [1]. IL-7 is required for T lymphocyte development and homeostasis in man and mice but only in mice it has an additional function in B cell development (figure 1). Apart from its role in differentiation and survival of lymphocytes, studies in knockout mouse models have identified IL-7 as a critical cytokine regulating secondary lymphoid organ (SLO) development. A specialized subset of IL-7R-expressing haematopoietic cells named "lymphoid tissue inducer" (LTi) cells has been identified as a key player in generating lymph nodes (LNs) and Peyer's patches (PPs) [2–5]. LTi cells form cellular aggregates with local stromal cells and interact via adhesion and TNF family member molecules. During this haematopoietic/mesenchymal crosstalk, the production of cytokines, chemokines and adhesion molecules leads to the recruitment of leukocytes and the organisation into lymphoid compartments.

It is well established that the development of SLOs is completed after birth. Chronic inflammation, however, is commonly associated with the *de novo* formation of ectopic lymphoid organs

Abbreviations

APRIL	a proliferation induced ligand
Blys	B lymphocyte stimulator
DC	dendritic cell
GC	germinal center
IFNγ	interferon γ
IL-7	interleukin 7
IL-7R	interleukin 7 receptor
JAK3	janus-activating kinase 3
LN	lymph node
LTαβ	lymphotoxin αβ
LTβR	lymphotoxin β receptor
LTi	lymphoid tissue inducer
MIP 1β	macrophage inflammatory protein β
NOD	non-obese diabetic
PP	Peyer's patch
RA	rheumatoid arthritis
RANKL	Receptor activator of nuclear factor κB ligand
SLO	secondary lymphoid organ
Th	T helper
TLO	tertiary lymphoid organ
TNF	tumour necrosis factor
VCAM-1	Vascular adhesion molecule 1

No financial relationships to declare.

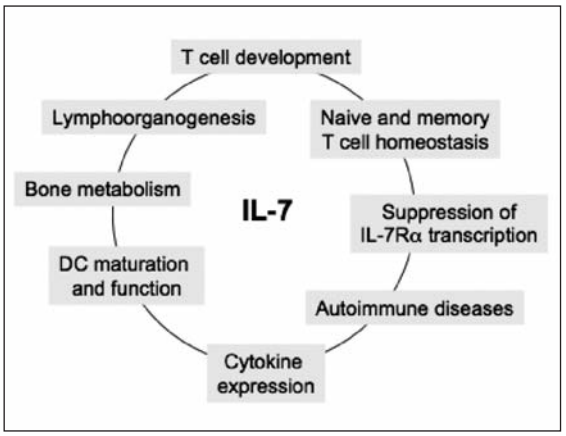

Figure 1

IL-7 has multiple effects on haematopoietic cells through induction of cytokines, survival and developmental signals. IL-7 is critical for the development of human T cells and for the homeostatic turnover of naïve and memory T cells. It also plays a role in dendritic cell (DC) development, maturation and function. Moreover, it is important in bone metabolism, during lymphoorganogenesis and can have an influence on autoreactive T cells leading to autoimmune diseases.

named "tertiary lymphoid organs" (TLO). The molecular mechanisms underlying the transformation of inflammatory infiltrates into TLOs are not completely understood, but studies in mice indicate that lymphoid organ development during ontogeny and inflammation shares some common features (figure 2). In both chronic inflammation and organogenesis, the activation of stromal cells leads to the release of molecules that regulate the recruitment, proliferation and survival of leukocytes. The establishment of a niche for incoming leukocytes is mediated by the collaboration of extracellular matrix components, adhesion molecules, cytokines and chemokines. Tumour necrosis factor (TNF) and lymphotoxin αβ (LTαβ) expressed by haematopoietic cells are critical cytokines acting on mesenchymal stromal cells and vascular endothelial cells and promote the establishment of lymphoid niches. Despite the success of anti-inflammatory treatment in RA, TLOs still persist. In this review we will highlight the role of IL-7 in SLO and TLO development and discuss its function in the progression of RA.

Development and remodelling of secondary lymphoid organs

SLO development in mice is orchestrated by LTαβ$^+$ LTi cells, which express CD4, c-Kit (CD117) and IL-7Rα (CD127), originate from the foetal liver and circulate during early foetal life before they enter peripheral tissues [6]. At sites of LN and PP anlagen, LTi cells interact with local lymphotoxin β receptor (LTβR)$^+$ mesenchymal organizer cells thereby inducing the expression of lymphoid chemokines such as CCL19, CXCL13 and CCL21 by the organizer cells and the colonisation with mature leukocytes [6]. In the absence of LTi cells or if components of the LTβR signaling pathway are blocked, LNs and PPs do not develop. Similarly, the formation of LNs and PPs is impaired in mice lacking lymphoid chemokines and chemokine receptors. This led to the current concept of a haematopoietic/mesenchymal crosstalk required for the formation and organisation of lymphoid tissue. Once SLO development has progressed to a stage where functional lymphoid compartments are established, signals via TNF family member molecules and chemokines help maintain a T cell/B cell segregation and germinal center (GC) reaction during immune responses [7].

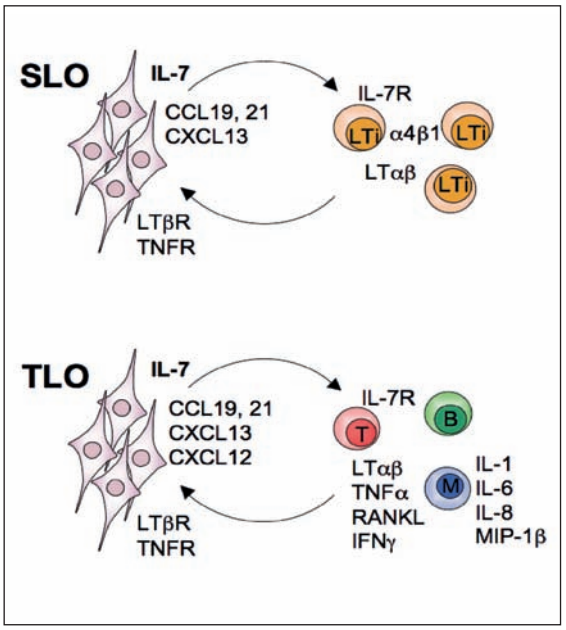

Figure 2

SLO and TLO development share common features. Chemokines, IL-7 and growth factors are provided by mesenchymal stromal cells (spindle formed). Haematopoietic cells (LTi cells, T cells, B cells, macrophages) produce cytokines and integrins, which promote stromal cell differentiation.

Mice lacking IL-7, IL-7Rα or Janus-activating kinase (JAK) 3, a signaling component of the IL-7R, have severe defects in LN and PP development suggesting a critical role of IL-7 in lymphoorganogenesis [8–10]. The precise nature of the signals provided by IL-7 was unsolved until now. We have recently shown that IL-7 induced the expression of LTαβ on LTi cells and amplified LTi cell numbers through inhibiting apoptosis [11]. In mice overexpressing IL-7 ubiquitously, lymphoid organs were hyperplastic and additional PPs and LNs were found. Ectopic LNs were connected to the lymphatic system and most probably developed from budding lymph sacs. The ectopic LNs were fully functional and supported normal T cell dependent B cell responses and GC reactions. Altogether, these data show that IL-7 is operative in the development of normal and ectopic lymphoid organs through increasing LTi cell number.

The development of SLOs during human ontogeny is a largely unexplored field and cells with LTi function have not been identified yet. In patients with JAK3-deficiency, RAG deficiency or X-linked agammaglobulinaemia, LNs are hypoplastic and formation of GCs does not occur [12] indicating that mature lymphocytes contribute to SLO organisation.

It is generally accepted that the developmental programme for SLO formation is completed after birth. In patients with chronic post-inflam-

matory and post-traumatic lymph stasis, however, the neogenesis of LNs from intralymphatic aggregates has been reported [13]. Inflammation and immune responses in LNs lead to fibroblast reticular cell hyperplasia followed by contraction after resolution of the immune activation. It is a matter of current research if the process of stroma hyperplasia and contraction during infection is triggered by molecular mechanisms that are also operative during the development of SLOs. Interestingly, a subset of adult $CD4^+CD3^-$ cells resembling foetal LTi cells was found to accumulate in reactive LNs of Helminth infected mice [14] and in the spleen of lymphocytic choriomeningitis virus-infected mice [15]. Adult $CD4^+CD3^-$ cells share some phenotypic marker with foetal LTi cells and appear to play a role in splenic organisation and immune responses [16]. Therefore, it is possible that LTi cells have a broader function as previously thought and act as inducers of lymphoid tissue during foetal and adult life.

Tertiary lymphoid organs in rheumatoid arthritis: role of IL-7

Chronic inflammatory diseases such as autoimmune diseases, chronic infections and chronic graft rejection are commonly associated with the formation of TLOs. These tissues resemble SLOs with segregation into T and B cell zones, dendritic cells (DCs), GCs, follicular dendritic cells, lymphatic vessels and high endothelial venules. Transgenic mice overexpressing lymphoid chemokines (CXCL13, CCL19, CCL21) or TNF family member molecules (LTαβ) under the control of a tissue-specific promoter, develop site-specific TLOs (for review see [17]). These data suggest that TLO development during chronic inflammation recapitulates a molecular programme used during foetal lymphoid organ development. The anatomical similarities between SLOs and TLOs have led to the hypothesis that TLOs provide the environment for generating chronic adaptive immune responses that contribute to disease progression. This concept was confirmed by investigating chronic organ transplant rejection in mouse and man where TLO formation promoted B and T cell mediated allograft rejection [18, 19].

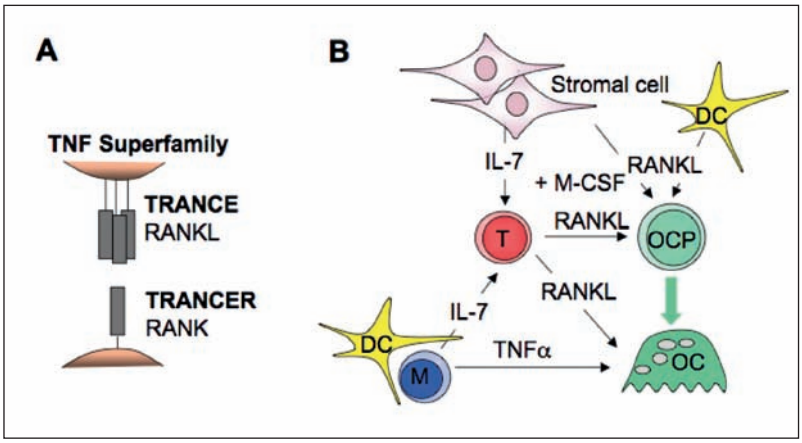

Figure 3

A. Receptor activator of nuclear factor κB ligand (RANKL, also named TRANCE) binds to its receptor RANK (also named TRANCER), a member of the TNF receptor superfamily.
B. IL-7-driven production of RANKL by T cells increases the pool of osteoclasts either indirectly by differentiation of osteoclast progenitors or directly by osteoclast proliferation. (DC: dendritic cell, M: macrophage, T: T lymphocyte, OCP: osteoclast progenitor, OC: osteoclast, TNFα: tumour necrosis factor α, M-CSF: macrophage colony stimulating factor).

RA is an autoimmune disease characterised by chronic inflammation of the joints leading to progressive destruction of cartilage and bone [20]. B cells, T cells, macrophages, synovial cells and endothelial cells producing proinflammatory cytokines are considered to be involved in the pathogenesis of RA. In contrast to the transient recruitment of leukocytes during the early phase of inflammation, in many but not all patients with established RA, fibroblast activation and hyperplasia lead to the establishment of TLOs in synovial lesions [21]. Fifty percent of patients form T/B cell aggregates, and half of them have synovial tissues containing B cell follicles with GCs [22, 23]. B cells isolated from these ectopic GCs undergo antigen-driven clonal expansion and somatic hypermutation [24] leading to memory B cells and autoantibody-producing plasma cells. The increased levels of B cell survival factors such as B lymphocyte stimulator (BLyS) and APRIL found in RA patients may further enhance these B cell responses [25]. Some T cells found in inflamed joints of RA patients have a diverse autoreactive T cell receptor repertoire [26]. Collectively, GCs in the synovia of RA patients may collect self-antigens, which can be presented to the adaptive immune system and stimulate autoreactive T and B cell responses.

There is evidence that the development of TLO in the synovia of RA patients is, analogous to SLO development, coordinated by the interaction of incoming $LT\alpha\beta^+$ haematopoietic cells with stromal cells (fibroblasts, endothelial cells). The activation of the LTβR signaling pathway in synovial fibroblasts and endothelial cells may lead to the inappropriate secretion of chemokines, growth and survival factors for leukocytes and the establishment of lymphoid structures. This concept is further supported by the fact that synovial tissues of RA patients overexpress LTα, LTβ, CXCL12, CXCL13, CCL21, and VCAM-1 [22, 23]. These molecules are also essential for the development of SLOs. Recent studies in a chronic arthritis mouse model reveal that in the absence of corresponding chemokine receptors (CXCR5, CCR7), TLOs fail to form followed by a signifi-

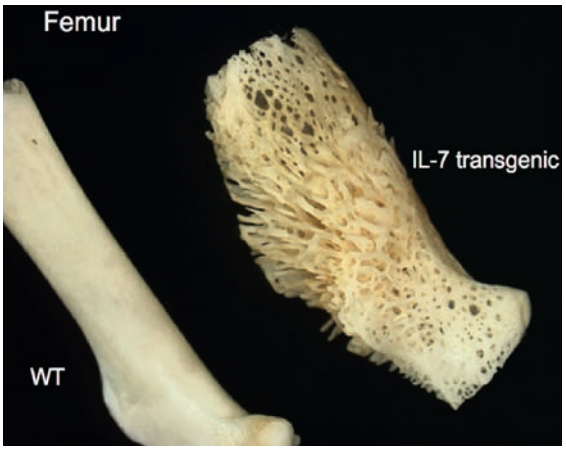

Figure 4

Femur of wild type (left) and IL-7 transgenic (right) mouse. IL-7 overexpression leads to osteolysis, lacuna formation and contraction in length.

cantly reduced joint destruction [27]. This strongly suggests that TLO development can contribute to the progression of the disease.

In RA patients, synovial fluid levels of IL-7 are strongly elevated [28, 29]. Fibroblasts, macrophages, endothelial cells and DCs in the synovia of RA patients produce IL-7 [30] (figure 3B). Interestingly, gene expression of RA synovia reveals that increased levels of IL-7, IL-7R and IL-7R signalling molecules are associated with the presence of TLOs [23]. The activation of the IL-7R signalling pathway may therefore play a role in TLO development analogous to its role in SLO development. Our studies in mice in which IL-7 overexpression induces the development of additional normal and ectopic lymphoid organs strongly support this paradigm of an IL-7-dependent mechanism of TLO formation [11].

Apart from a role in lymphoid tissue development, IL-7 is operative in bone loss through increased osteoclastogenesis mediated by T cells producing TNFα and the receptor activator of nuclear factor kB ligand (RANKL) [31, 32]. Both generalised and focal bone loss is found in patients with RA [33]. Higher levels of RANKL are detectable in synovial tissue of RA patients with active synovitis [34]. Activated T cells and RA stromal cells produce RANKL, which induces activation of RANK-expressing osteoclast progenitor cells and mature osteoclasts [35] (figure 3). Thus, studies in RA patients and animal models for arthritis highlight a role of IL-7 in secreting osteoclastogenic molecules. In line with this, in our IL-7 overexpressing mouse model, we observed a progressive osteolysis and bone remodelling irrespective of the gender (figure 4). Macroscopically we did not find signs of joint inflammation indicating that IL-7 alone is not sufficient to trigger the development of arthritis. It remains to be investigated whether IL-7 overexpressing mice are more susceptible to the development of experimentally induced arthritis. Altogether, IL-7 may have a dual function in the pathogenesis of RA through inducing TLO development and disturbing the bone metabolism. In addition, the local release of IL-7 in RA lesions might be the driving force for leukocyte survival and differentiation into potentially harmful effector cells. This is supported by the findings that synoviocytes from patients with RA stimulate the proliferation of Th1 cells through IL-7 [29, 36] and that IL-7-primed arthritogenic Th1 cells produce IFNγ and TNFα [28]. IL-7 also promotes cytotoxic T cell and Th2 responses [37]. Moreover, IL-7 can induce the secretion of IL-1α, IL-1β, IL-6, IL-8, macrophage inflammatory protein (MIP)-1β and TNFα by human monocytes [38–40]. The overexpression of TNFα in animals leads to the formation of TLO and the development of chronic arthritis, which may explain only one of the multiple mechanisms of TNF in the pathogenesis of the disease [41]. In turn, TNFα promotes the production of IL-7 by RA fibroblasts [36]. Despite considerable success in treatment of RA with anti-TNFα, a substantial proportion of patients do not respond and IL-7 persists upon anti-TNFα treatment [42] . In patients with RA refractory to anti-TNFα agents, the selective depletion of CD20-positive B cells with anti-CD20 antibodies (Rituximab) significantly reduces the activity of the disease in the majority of the patients [43–45]. Rituximab-treatment is more effective than switching to an alternative anti-TNF agent [46] suggesting that B cells have additional pathogenic functions in RA. The mechanisms, by which B cell depletion leads to a clinical improvement of RA, may rely on the effector function of B cells in antigen-presentation to T cells, the secretion of cytokines and the formation of TLO. This is supported by the finding that in rheumatoid synovium, LTβ-producing B cells are critical for T cell activation, production of IFNγ and IL-1β and formation of ectopic GCs [22, 47]. IL-7 can induce the expression of LTβ, which is critical for the development of ectopic GCs [11]. Finally, the induction of IL-7 by a TNF-independent mechanism can further contribute to establish T cell responses in TLOs. Therapeutic blockade of local IL-7 release or neutralisation of IL-7 protein may therefore have beneficial effects in established RA, but systemic immunosuppressive effects should also be taken into consideration. Altogether, TLO development in inflammatory RA shares some striking features with SLO development in mouse models. It is initiated by infiltrating haematopoietic cells, which activate local stromal cells. As a consequence of LTβ-dependent signals provided by haematopoietic cells, the stroma produces factors, which in turn help to establish and maintain inflammatory infiltrates. The local release of IL-7 may promote the chronic stimulation and survival of immune cells and the establishment of TLO that accounts for the progressive destruction of the tissue.

IL-7 and other autoimmune diseases

A role of IL-7/IL-7R in disease progression has also been proposed for other autoimmune diseases in humans or mouse models such as colitis [48], multiple sclerosis [49], diabetes [50], psoriasis [51] and sialitis in NOD mice [52]. Increased levels of systemic IL-7 where reported to directly sustain autoreactive T cell responses. Evidence for this comes from studies in mice where systemic IL-7 was essential for persistence of colitis [53]. The local release of IL-7 in chronically inflamed organs, however, may help establishing TLOs as previously discussed. In Sjögren's syndrome, the dysregulated expression of lymphoid chemokines together with the formation of TLOs has been observed [54, 55]. It is likely that conversion of fibroblasts into lymphoid stroma in the salivary gland is supportive of the high-affinity autoantibody production and the high incidence of B cell lymphomas associated with Sjögren's syndrome. Whether human LTi cells exist and contribute to TLO development in autoimmune diseases is still an open question but advances in engineering new models to study human haematopoietic progenitor cells may provide some tantalising clues. Altogether, autoimmunity is a multistep process requiring proinflammatory cytokines, growth factors, release of self-antigen and a *micromilieu* supporting the expansion and survival of self-reactive lymphocytes. Cytokines such as IL-7 may not only play an essential role in sustained T cell responses but also in establishing the microenvironment for chronic autoimmune responses.

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3.2. Manuscript 1

Ectopic Lymphoid-Organ Development Occurs through Interleukin 7-Mediated Enhanced Survival of Lymphoid-Tissue-Inducer Cells

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Immunity
Article

Ectopic Lymphoid-Organ Development Occurs through Interleukin 7-Mediated Enhanced Survival of Lymphoid-Tissue-Inducer Cells

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SUMMARY

Development of Peyer's patches and lymph nodes requires the interaction between $CD4^+$ $CD3^-$ IL-$7R\alpha^+$ lymphoid-tissue inducer (LTi) and VCAM-1^+ organizer cells. Here we showed that by promoting their survival, enhanced expression of interleukin-7 (IL-7) in transgenic mice resulted in accumulation of LTi cells. With increased IL-7 availability, de novo formation of VCAM-1^+ Peyer's patch anlagen occurred along the entire fetal gut resulting in a 5-fold increase in Peyer's patch numbers. IL-7 overexpression also led to formation of multiple organized ectopic lymph nodes and cecal patches. After immunization, ectopic lymph nodes developed normal T cell-dependent B cell responses and germinal centers. Mice overexpressing IL-7 but lacking either RORγ, a factor required for LTi cell generation, or lymphotoxin $\alpha_1\beta_2$ had neither Peyer's patches nor ectopic lymph nodes. Therefore, by controlling LTi cell numbers, IL-7 can regulate the formation of both normal and ectopic lymphoid organs.

INTRODUCTION

Peyer's patch (PP) and lymph node (LN) organogenesis is initiated through signaling events that require the interaction between two cellular partners of hematopoietic and mesenchymal origin (Adachi et al., 1997; Mebius et al., 1997; Yoshida et al., 1999). Adoptive transfer studies in mice have identified fetal hematopoietic $CD45^+$ $CD4^+$ $CD3^-$ cells expressing both IL-7Rα (CD127) and lymphotoxin (LT) $\alpha_1\beta_2$ as playing a pivotal role in lymphoid tissue induction (Finke et al., 2002; Fukuyama et al., 2002). PP development is initiated through the interaction of $CD4^+$ $CD3^-$ lymphoid tissue inducer (LTi) cells with VCAM-1-expressing mesenchymal cells. This cellular interaction, which involves integrins, lymphotoxin β receptor (LTβR), and tumor necrosis factor (TNF) receptor I (De Togni et al., 1994; Futterer et al., 1998; Kuprash et al., 2005), leads to cell-cluster formation, induction of chemokine production by mesenchymal cells, and subsequent recruitment of mature lymphocytes (Honda et al., 2001; Ngo et al., 1999). Consistent with this, the chemokine CXCL13 and its receptor have been identified as important factors in the development of PP and LN (Ansel et al., 2000; Forster et al., 1996).

A similar role for $CD4^+$ $CD3^-$ cells as inducers of peripheral LN development was shown (Eberl et al., 2004; Yoshida et al., 2002). LN development starts with the accumulation of IL-$7R\alpha^+$ cells at prospective sites of LN formation in E12.5 fetal mice (Yoshida et al., 2002). Through inhibition of the LTβR pathway, it could be shown that there is a time window (E11.5 to E16.5) for temporally and spatially coordinated LN development (Rennert et al., 1996).

The IL-7R-signaling pathway is required for the assembly of LTi and organizer cells during PP anlagen formation (Adachi et al., 1998). Accordingly, the single injection of a blocking IL-7R antibody (Ab) before E15.5 is sufficient to prevent PP but not LN development (Yoshida et al., 1999). Notably, several LN are still detectable in both IL-7Rα-deficient mice and mice lacking the IL-7R downstream signaling molecule JAK3, although PP are absent (Adachi et al., 1998; Luther et al., 2003). Altogether, these data imply that PP and LN development are differentially regulated and, in contrast to PP, that LN organogenesis is only partially dependent on IL-7Rα.

It is generally accepted that the developmental program for lympho-organogenesis is turned off after birth. However, in both reactive lymphoid hyperplasia and follicular hyperplasia, remodeling of lymphoid tissue has been observed. In addition, in inflammatory lesions of autoimmune diseases, allergic reactions, microbial infections, and chronic graft rejections, ectopic lymphoid tissues named "tertiary lymphoid organs" can develop (Aloisi and Pujol-Borrell, 2006; Drayton et al., 2006).

Neonatal LN cell suspensions containing LTi cells and stromal organizer cells injected intradermally into mice

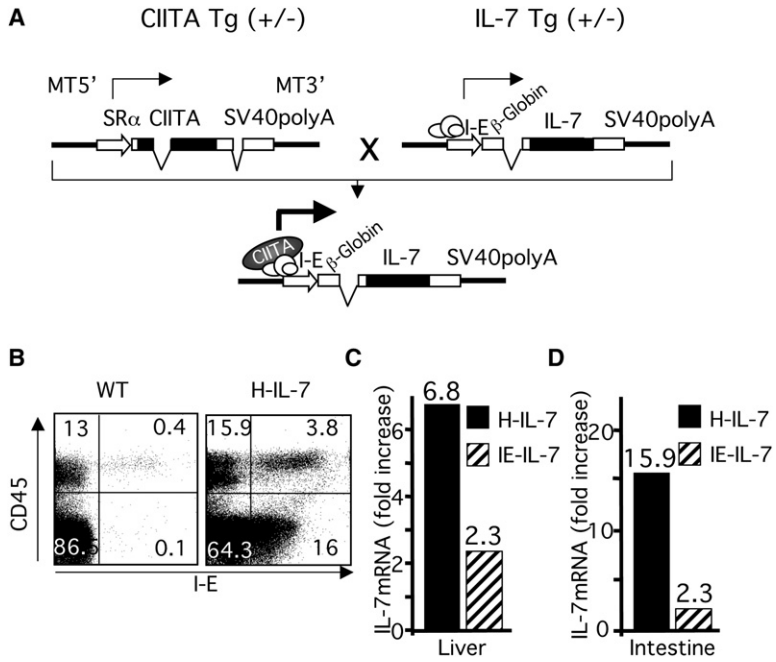


Figure 1. Monitoring of CIITA Tg and IL-7 Tg Expression in Fetal Mice

(A) Tg mice expressing CIITA ubiquitously through MT5′, MT3′, and SRα elements were crossed with MHC class II I-E IL-7 mice.

(B) The proportion of I-E$^+$ cells in the E 14.5 FL of WT and H-IL-7 mice was measured by flow cytometry. Shown are cytogram displays of cells stained with the indicated markers. Numbers in the quadrants indicate the percentage of cells expressing the respective marker. These data are representative for 5 independent experiments.

(C and D) IL-7 transcripts were quantified by real-time PCR in E14.5 fetal liver (C) and intestine (D). Values were normalized to TATA box-binding protein (TBP), and increase of IL-7 mRNA in H-IL-7 and IE-IL-7 compared to WT controls are shown. Values represent the mean of 3 to 5 mice.

are able to aggregate and form lymphoid-like structures (Cupedo et al., 2004). This study convincingly shows that LTi cells are able to reorganize lymphoid stroma after dissociation but it does not answer the question of whether LTi cells are able to generate de novo ectopic lymphoid organs.

In order to address this question, we have used IL-7 transgenic (Tg) mice expressing different amounts of IL-7. In these mice, LTi cell numbers were increased as a result of IL-7-dependent enhanced survival and, to a minor extent, proliferation of the cells. We observed multiple PP developing throughout the entire small intestine, and in addition, numerous organized ectopic LN (ELN) and cecal lymphoid patches (LP) were formed. Ectopic lymphoid tissue development was dependent on the presence of LTi cells and their expression of $LT\alpha_1\beta_2$. Our data demonstrate that de novo lympho-organogenesis is a highly plastic process, which can be regulated by IL-7, LTi cell numbers, and $LT\alpha_1\beta_2$ expression. These data shed new light on possible mechanisms involved in tertiary lymphoid tissue development in chronic inflammatory diseases.

RESULTS

Fetal IL-7 Tg Expression Results in Increased Number and Size of PP

To investigate the role of IL-7 in the formation of lymphoid tissue, we used Tg mice expressing different amounts of IL-7. In the first mouse type (IE-IL-7), murine IL-7 was expressed under the control of the MHC class II Eα promoter (Mertsching et al., 1996). In the second type, IE-IL-7 mice were crossed with animals expressing the MHC class II transactivator (CIITA) under the control of the ubiquitous SRα promoter (Figure 1A; Otten et al., 2003). The CIITA transactivator increases transcription from MHC class II promoters, and therefore, in double Tg mice (H-IL-7 mice), the ubiquitous synthesis of CIITA promoted systemic IL-7 Tg expression. In the E14.5 fetal liver (FL) of wild-type (WT) (non-Tg litter control) mice, the majority of cells were negative for MHC class II I-E and CD45 (Figure 1B). However, in H-IL-7 mice, 19.3% CD45$^+$ and 19.9% CD45$^-$ cells expressed I-E as a consequence of CIITA Tg expression in the FL. Accordingly, a 2.3- and 6.8-fold increase in total IL-7 transcripts was detectable in the FL of IE-IL7 and of H-IL-7 mice, respectively, as compared to WT controls (Figure 1C). In the fetal intestine, IL-7 transcripts were increased only 2.3-fold in IE-IL-7 but 15.9-fold in the H-IL-7 mice (Figure 1D). At all ages tested, IL-7 transcripts were more abundant in H-IL-7 Tg compared with IE-IL-7 Tg mice (data not shown).

We used IE-IL-7 and H-IL-7 mice to test the effect of enhanced IL-7 availability on the formation of PP in the intestine. Two VCAM-1$^+$ PP anlagen were detectable in the proximal small intestine of E16.5 WT and IE-IL-7 mice (Figure 2A and data not shown). In E16.5 H-IL-7 mice, a continuous band of VCAM-1$^+$ cells could be identified on the antimesenteric side of the entire small intestine (Figure 2A). Coexpression of ICAM-1 and VCAM-1 is characteristic of PP organizer cells (Adachi et al., 1997; Honda et al., 2001). By flow cytometry, we found a 5-fold increase in the percentage of VCAM-1$^+$ ICAM-1$^+$ organizer cells in the small intestine of E16.5 fetal H-IL-7 mice (Figure 2B). There was a clear correlation between high IL-7 expression and increased PP numbers with 6.6 ± 1.3 in WT and 8.0 ± 2.0 in IE-IL-7 but 31.4 ± 9.4 in H-IL-7 mice (Figure 2C). The increase in PP numbers was found in both the proximal and distal parts of the intestine (Figure 2D). PP of H-IL-7 newborn animals were larger in diameter, and in addition to isolated VCAM-1$^+$ spots, elongated clusters of VCAM-1$^+$ cellular aggregates could be

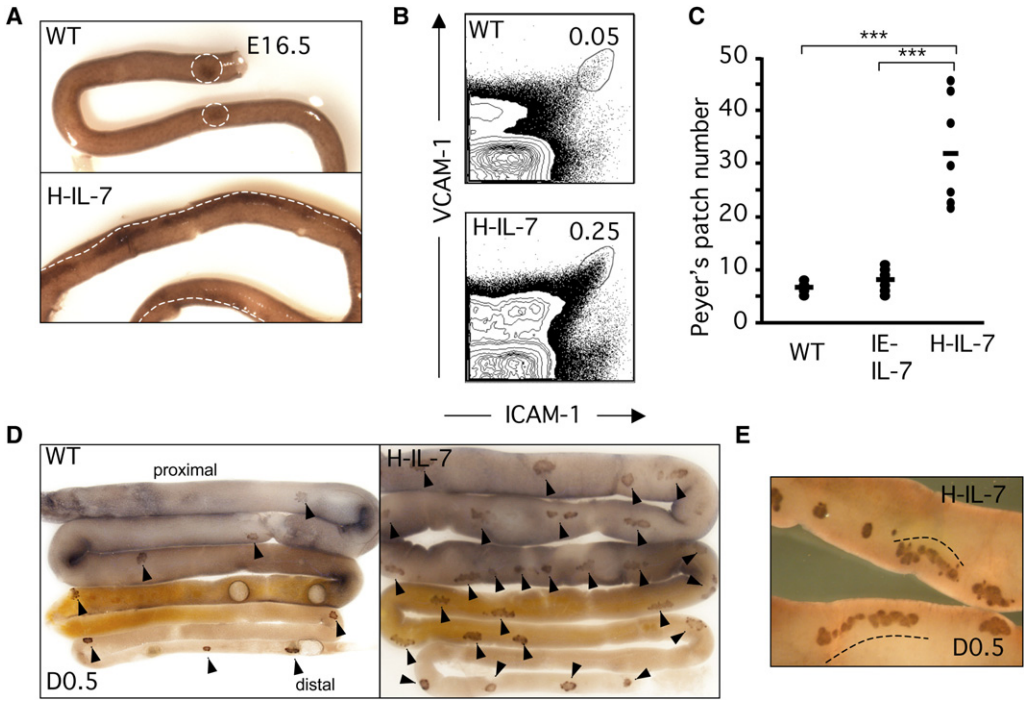


Figure 2. VCAM-1$^+$ Organizer Cells Expand in the Developing Gut of H-IL-7 Mice

(A) Expression of VCAM-1 in the gut of E 16.5 WT and H-IL-7 mice detected by whole-mount immunohistochemistry. Dotted lines show VCAM-1$^+$ cluster.

(B) The proportion of VCAM-1$^+$ ICAM-1$^+$ organizer cells in the E 16.5 gut was measured by flow cytometry. Number indicates the percentage of VCAM-1$^+$ ICAM-1$^+$ cells.

(C) Total PP number in neonatal WT (n = 10), IE-IL-7 (n = 12), and H-IL-7 (n = 8) mice determined by VCAM-1 whole-mount immunohistochemistry. Significantly different values are indicated: ***$p < 0.001$.

(D) PP localization in newborn intestine of WT and H-IL-7 mice, indicated by arrows.

(E) VCAM-1$^+$ cluster formation in newborn H-IL-7 mice indicated by dotted lines.

identified (Figure 2E). Equivalent structures were not detectable in IE-IL-7 or in WT mice. The phenotype of single CIITA Tg mice was identical to WT mice, demonstrating that CIITA overexpression alone had no effect on PP organogenesis (data not shown). Altogether, these data demonstrate that the whole length of the antimesenteric side of the gut was potentially capable of forming PP. The additional PP seen in H-IL-7 mice were still detectable in adults (Figure S1A in the Supplemental Data available online). The number of lymphoid follicles found in individual PP was markedly increased in both IE-IL-7 and H-IL-7 mice (Figure S1B).

IL-7 Induces the Development of ELN and Cecal LP

To enumerate the number of LN in mice differing in IL-7 expression, Chicago blue dye was injected into the footpads of adult H-IL-7, IE-IL-7, WT, and *Il7*$^{-/-}$ mice. In *Il7*$^{-/-}$ mice, the number of LN was largely reduced or absent relative to WT mice (Figure S2). In H-IL-7 mice, the size of LN was enlarged relative to WT mice (Figure 3A), and multiple ELN were found in various regions such as the axilla, mediastinum, and within the abdominal cavity (Figure 3A, Table 1; Figure S3). In IE-IL-7, some but not all ELN were found (Table 1). As expected, WT mice were completely free of ELN development. ELN contained a normal ratio of CD4$^+$ to CD8$^+$ T cells (Figure 3B). 10% to 12% of cells found in the LN of H-IL-7 mice were CD93$^+$ CD19$^+$ B cell precursors (Figure 3B) expressing c-Kit (CD117) but not IgM (data not shown). This is in agreement with previous studies demonstrating that in IE-IL-7 mice, the number of IL-7-dependent late pro-B, pre-BI, and pre-BII cells in peripheral lymphoid organs was dramatically increased (Mertsching et al., 1996). The absolute T and B cell numbers of ELN from H-IL-7 and inguinal LN (ILN) from WT mice were comparable (Figure 3C). In contrast, CD4$^+$ T cell and CD19$^+$ B cell numbers were 2-fold increased in ILN of H-IL-7 mice as compared to WT controls.

Immunohistochemical examination of ILN and ELN from adult H-IL-7 mice revealed that, like WT mice, there was a normal segregation into B cell follicles and paracortical T cell areas (Figure 3D). Intriguingly, in all H-IL-7 mice examined, multiple LPs were found in the proximal cecum (Figure 3E, middle two images) and colon (data not shown). In contrast to isolated lymphoid follicles or inflammatory infiltrates, these LPs were organized into B cell follicles interspersed with T cell zones (Figure 3E, bottom). LPs were clearly distinguishable from the well-described solitary lymphoid patch in the distal cecum of WT mice.

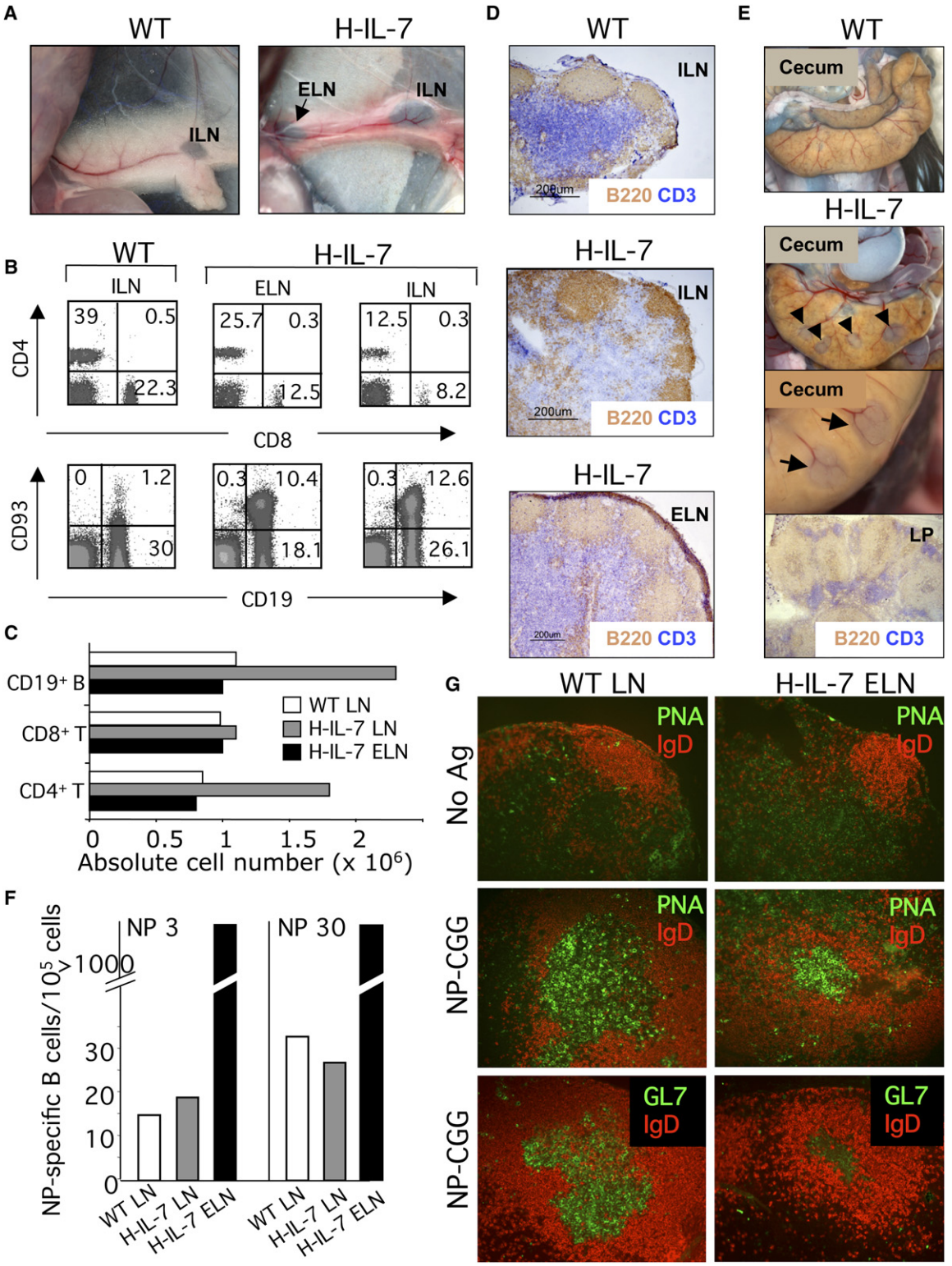


Figure 3. IL-7 Is Involved in the Development of Ectopic LN

(A) 4 days after s.c. injection of Chicago blue dye, the ILN of WT (left) and H-IL-7 (right) mice became apparent. An ELN (indicated by arrow) is shown in proximity to the ILN of H-IL-7 mice.

(B) LN single-cell suspensions of H-IL-7 and WT mice were analyzed by flow cytometry with the indicated monoclonal antibody. Data are representative of 5 experiments.

(C) Absolute cell numbers ($\times 10^6$) of T and B cells in WT LN (inguinal) and H-IL-7 LN (inguinal) and ELN are shown.

Table 1. Increased Availability of IL-7 Induces Ectopic LN

	Number of Positive Mice		
Ectopic LNs	WT	IE-IL-7	H-IL-7
Inguinal	0/12	2/11	11/13
Axillary	0/12	3/11	13/13
Mediastinal	0/12	2/11	12/13
Abdominal	0/12	1/11	13/13
Intercostal	0/12	0/11	9/13
Pancreatic	0/12	0/11	7/13
Cecal patches	0/12	3/11	13/13

4 days after Chicago blue injection into adult mice, quantitative assessment of the number of mice generating the indicated ectopic LN and cecal lymphoid patches was performed by stereomicroscopy. WT (n = 12) mice were compared with IE-IL-7 (n = 11) and H-IL-7 (n = 13) mice.

These results demonstrate that increased IL-7 availability resulted in the ectopic formation of organized LN and of intestinal LPs.

In order to test the functional activity of ELNs, mice were injected s.c. with the T cell-dependent antigen 4-hydroxy-3-nitrophenylacetyl hapten conjugated to chicken γ globin (NP-CGG) precipitated in Alum. 9 and 14 days after immunization, the numbers of low- and high-affinity Ab-producing cells were tested in draining and nondraining LNs. The number of specific Ab-producing cells in draining popliteal LNs and ELNs increased between day 9 and 14, whereas at no time tested were we able to detect NP-specific Ab-producing cells in nondraining LNs (data not shown). On day 14, draining popliteal LNs from WT and H-IL-7 mice generated comparable numbers of specific Ab-secreting cells after immunization, whereas the number of both NP-specific high (NP3)- and low (NP30)-affinity Ab-producing cells in ELN was dramatically increased (Figure 3F). Draining ELN contained germinal centers with PNA^+ and $GL\text{-}7^+$ IgD^- cells, indicating that normal T-B collaboration occurred in ELN of immunized H-IL-7 mice (Figure 3G). In order to determine immune responses at earlier time points, we injected mouse mammary tumor virus (SW) s.c. and measured Syndecan 1^+ class II^{lo} plasmablast and $V\beta6^+$ $CD4^+$ T cell responses on day 6 after immunization (Ardavin et al., 1999). The draining ELN and popliteal LN showed comparable responses (data not shown). Importantly, upon immunization with adjuvant, a procedure that induces a local inflammatory response, additional ELN were formed in H-IL-7 but not in WT mice. Whereas in most naive H-IL-7 mice, 1 ELN was found symmetrically at both sides of the inguinal region after injection, after immunization we found 3 to 4 additional ELNs exclusively at the site of injection. The immunization-induced ELNs contained a striking frequency of NP-specific B cells (data not shown). Independently of any immunization, we also found numerous ectopic lymphoid follicles in autoimmune target organs such as the pancreas and salivary gland (data not shown). Taken together, our data indicate that the increased availability of IL-7 led to the spontaneous and immunization-induced formation of tertiary lymphoid organs.

LTi Cells Are Required for De Novo PP and LN Formation in H-IL-7 Mice

In neonatal mice, RORγt, a nuclear orphan receptor, is preferentially expressed in LTi cells, and RORγ-deficient mice lack LTi cells (Eberl et al., 2004; Sun et al., 2000). To explore whether LTi cells were required for the de novo formation of additional PP and LN, we backcrossed H-IL-7 mice to RORγ-deficient mice. Whole-mount VCAM-1 immunostaining of newborn intestine from H-IL-7 RORγ-deficient mice revealed that PP development was completely blocked (Figure 4A, right). This was consistent with the absence of LTi cells in the spleen of neonatal H-IL-7 RORγ-deficient mice (Figure 4B, right). ELN were absent in H-IL-7 RORγ-deficient mice (Figure 4C), suggesting that their development was dependent on the presence of LTi cells.

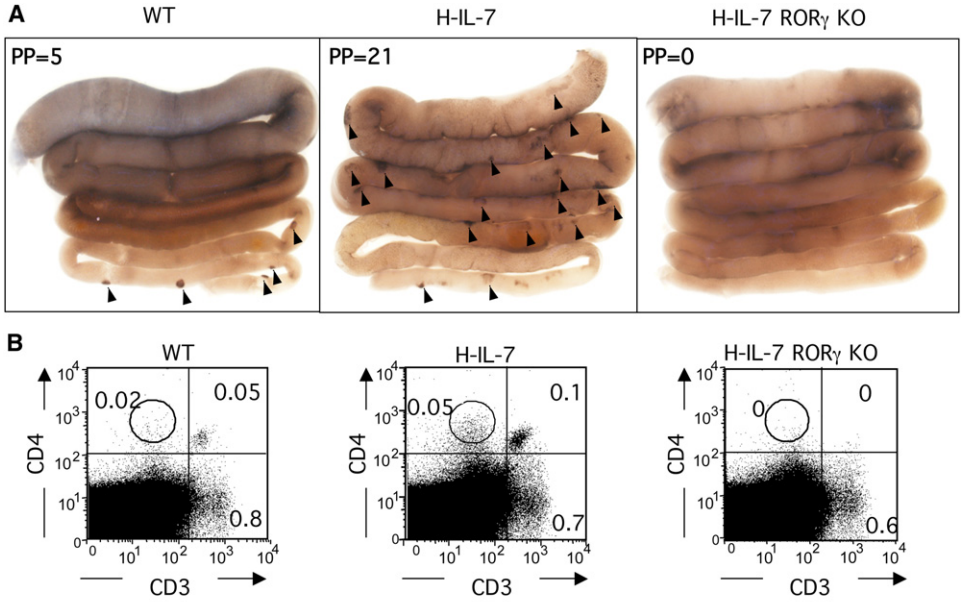
$LT\alpha_1\beta_2$ expressed by LTi cells is a crucial component in secondary lymphoid organ development. In order to test whether the effect of increased IL-7 availability was $LT\alpha_1\beta_2$ dependent, H-IL-7 mice were crossed to $Lta^{-/-}$ animals. As shown in Figure 4D, H-IL-7 $Lta^{-/-}$ mice were devoid of any ELN, thereby demonstrating that $LT\alpha_1\beta_2$ was required for the formation of ectopic lymphoid tissue.

LTβ transcripts were increased 3.8-fold in IE-IL-7 and 14.8-fold in the whole intestine of E16.5 H-IL-7 embryos, respectively (Figure S4A) at a time when the majority of $LT\alpha_1\beta_2$-expressing cells in the gut are LTi cells. FACS analysis showed that the mean fluorescence intensity of $LT\alpha_1\beta_2$ on LTi cells was increased to the same extent in both IE-IL-7 and H-IL-7 mice (Figures S4B and S4C). These data suggest that the increase in total LTβ transcripts in the intestine of H-IL-7 mice was the result of both increased $LT\alpha_1\beta_2$ expression and frequency of LTi cells.

H-IL-7 Mice Have Elevated Numbers of LTi Cells

The elevated amount of LTβ transcripts in fetal H-IL-7 mice prompted us to quantify LTi cell numbers in these mice. A 4-fold and 23-fold increase in mean number of LTi cells was observed in the spleen of E16.5 (1.8×10^3

(D) Immunohistochemistry of frozen sections of LN of 4-week-old WT mice and H-IL-7 with anti-B220 (brown) and-CD3 (blue) Ab.
(E) Multiple cecal LPs were identified in H-IL-7 mice (indicated by arrows). Immunohistochemical staining of cecal LPs with the indicated Ab is shown in the lower panel.
(F) Number of NP3 and NP30-specific Ab-secreting cells per 10^5 total LN (popliteal) cells from WT and LN (popliteal) and ELN cells from H-IL-7 mice 2 weeks after s.c. immunization with NP-CGG and alum (n = 3–5).
(G) Immunofluorescence staining of draining popliteal LN (WT) and ELN (H-IL-7) of mice 2 weeks after s.c. immunization with NP-CGG and alum. MAb specific for IgD (red) and GL-7 (green) as well as PNA (green) were used. Data are representative for analysis of three individual mice per group.



C

Ectopic lymph nodes		
WT	H-IL-7	H-IL-7 RORγ KO
0/7	3/3	0/3

D

Ectopic lymph nodes		
WT	H-IL-7	H-IL-7 $Lt\alpha^{-/-}$
0/3	4/4	0/3

Figure 4. RORγ and LTi Cells Are Required for PP Amplification in H-IL-7 Mice

(A) Whole-mount immunohistochemistry (anti-VCAM-1) of intestine isolated from neonatal WT (RORγ-heterozygous littermates), H-IL-7 (RORγ heterozygous), and H-IL-7 RORγ KO (RORγ-deficient) mice. PP anlagen are indicated by arrows. A mean of 5 PP was found in WT (n = 7), 21 PP in H-IL-7 (n = 3), and 0 PP in H-IL-7 RORγ KO mice (n = 3).

(B) Flow cytometry analysis of single-cell suspensions from neonatal spleen of WT, H-IL-7, and H-IL-7 RORγ KO mice. The circular gates were used to calculate the percentage of $CD4^+CD3^-$ cells among total lymphocytes.

(C) The number of ELN found in individual mice is shown. 3 to 7 mice per group were analyzed.

(D) H-IL-7 mice were backcrossed to $Lta^{-/-}$ mice and the number of ELN was determined. 3 to 4 mice per group were analyzed.

cells) and neonatal (2.3×10^4 cells) H-IL-7 mice (n = 6) relative to WT controls (n = 6) (Figure 5A). Similarly, a significant increase in mean LTi cell number was also observed in LN of H-IL-7 mice (n = 6) (Figure 5B). LTi cell numbers were not significantly elevated in fetal and neonatal IE-IL-7 mice. Our results were further supplemented by analysis of the gut of newborn (D 3.5) mice. We observed a massive colonization of the H-IL-7 small intestine by LTi cells (Figure 5C, top right). These LTi cells were not found within clusters of $CD3^+$ T cells (Figure 5C, top), but were forming tight follicles before $B220^+$ B cells accumulated in these segregated areas (Figure 5C, bottom left). In contrast to $CD4^+$ cells, VCAM-1^+ organizer cells were not expressing IL-7Rα, making it unlikely that organizer cells were directly responsive to IL-7 (Figure 5C, bottom middle and right).

We have previously shown that adoptively transferred LTi cells from WT mice are able to restore PP formation in neonatal *Cxcr5*$^{-/-}$ mice (Finke et al., 2002). In order to test whether $CD4^+CD3^-$ cells from H-IL-7 mice were functional LTi cells, sorted $CD4^+CD3^-$ cells were adoptively transferred into neonatal *Cxcr5*$^{-/-}$ mice. Transfer of 9000 sorted cells was sufficient to induce the development of 10 PP in *Cxcr5*$^{-/-}$ mice, a number comparable to that in WT mice (Figure S5A). The PP-like follicles in reconstituted *Cxcr5*$^{-/-}$ mice, however, were smaller as compared to PP in WT mice, probably as a result of inefficient colonization with CXCR5-deficient B cells. One of the hallmarks of LTi cells is their expression of RORγt transcripts. Therefore, to confirm that the $CD4^+CD3^-$ cells found in H-IL-7 mice were LTi cells, we determined the amount of RORγt transcripts in sorted LTi cells from neonatal spleen of H-IL-7 mice (Figure S5B). As negative control, $CD4^+$ T cells from H-IL-7 mice were used, and as positive control, total thymocytes from WT animals were used. RORγt transcripts were found in $CD4^+CD3^-$ cells and, as expected, in thymocytes. Similarly, thymocytes from H-IL-7 mice were positive for RORγt transcripts (data not shown). Altogether these data show that $CD4^+CD3^-$ cells found in high numbers in H-IL-7 mice are functional LTi cells.

IL-7 Is a Survival Factor for Fetal Progenitor and LTi Cells

It was reported that IL-7Rα$^+$ lin$^-$ FL cells were progenitors of LTi cells and that they expressed α4β7 integrin (Mebius

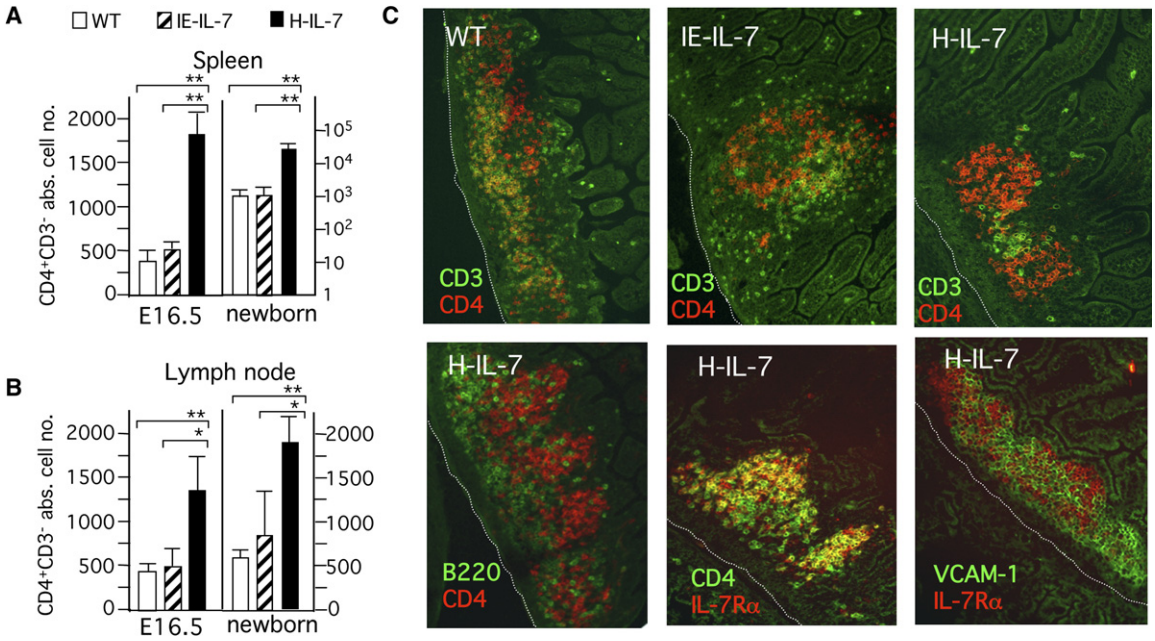


Figure 5. IL-7-Dependent Increase in LTi Cell Numbers

(A) Flow cytometry of splenocytes from E16.5 fetal and newborn (n = 6) WT, IE-IL-7, and H-IL-7 mice. A live gate was used to exclude dead cells. Shown are mean values ± SD. Significantly different values of absolute cell numbers are indicated: **p ≤ 0.01, *p < 0.05. For newborn spleen (right) a logarithmic scale was used.

(B) The absolute number of $CD4^+$ $CD3^-$ LTi cells in mesenteric LN (n = 6) from WT, IE-IL-7, and H-IL-7 mice.

(C) Immunohistochemistry of serial frozen sections from small intestinal PP of WT, IE-IL-7, and H-IL-7 mice 3.5 days after birth stained with the indicated Ab. The antimesenteric side of the intestine is highlighted by a dotted line. Data are representative for analysis of 3 to 4 mice per group.

et al., 2001; Yoshida et al., 2001). To explore whether these FL progenitors were already expanded in H-IL-7 mice, we examined the absolute number of $\alpha4\beta7^+$ FL cells in the E14.5 FL. Comparing fetal WT with H-IL-7 mice (E14.5), the absolute number of $\alpha4\beta7^+$ FL cells was increased more than 2-fold (Figure 6A). We could clearly discriminate these FL progenitors from early B cell-committed precursors, which were $B220^{hi}$ and $CD19^{hi}$. To determine whether the increase in $\alpha4\beta7^+$ progenitors was the result of IL-7-driven proliferation, we pulsed E14.5 pregnant mice with bromodeoxyuridine (BrdU) and after 1.5 hr labeling determined BrdU incorporation into $\alpha4\beta7^+$ progenitor cells by flow cytometry (Figure 6B). No difference in proportion of proliferating cells was observed when WT mice (34.9% of gated cells, mean fluorescence intensity [MFI] 36) were compared with H-IL-7 mice (31% of gated cells, MFI 32.7). To determine the rate of proliferating LTi cells, a 5 hr in vivo BrdU pulse of newborn animals was performed. The proportion of labeled LTi cells in the spleen of WT mice (13%, MFI 92) and H-IL-7 mice (8%, MFI 70) were not significantly different.

In order to investigate whether IL-7 promoted the survival of LTi cells and their progenitors, sorted $\alpha4\beta7^+$ cells isolated from FL of E14.5 WT mice were cocultured with ST-2 stromal cells. In the absence of added IL-7, the absolute number of $\alpha4\beta7^+$ cells at day 9 of culture dramatically dropped, and cells completely disappeared in cultures containing a blocking anti-IL-7Rα Ab (Figure 6C, left). We were able to detect commitment of $\alpha4\beta7^+$ cells into LTi cells provided IL-7 was present in culture (Figure 6C, right). In order to study the direct effect of IL-7 on survival of LTi cells, sorted $CD4^+CD3^-$ cells from newborn spleen of H-IL-7 mice were cultured on stromal cells. In the absence of added IL-7 and even more strikingly in cultures containing anti-IL-7R Ab, LTi cell numbers were significantly decreased (Figure 6D). Similar results were obtained from studies with WT LTi cells (data not shown). To address the question of whether cells became apoptotic in the absence of IL-7, $\alpha4\beta7^+$ FL cells from E14.5 WT mice were sorted and cultured in the presence of IL-7. After 7 days, IL-7 was withdrawn and 2 days later, Annexin V and 7AAD labeling of cells was performed. Our results show that removal of IL-7 significantly increased the percent Annexin V^+ $7AAD^+$ apoptotic $\alpha4\beta7^+$ FL cells and their LTi progeny (Figure 6E). These data were in agreement with the observation that in fetal H-IL-7 mice, the expression of FAS, a proapoptotic protein, was reduced (data not shown). In order to confirm the effect of IL-7 on LTi cell survival rather then proliferation, we tested carboxy fluorescein succinimidyl ester (CFSE)-labeled hanging-drop cultures of neonatal WT splenocytes. In the absence of IL-7, cell recovery of LTi cells was reduced by 37% already at day 1 of culture (Figure 6F, right). In contrast, the profiles of CFSE fluorescence intensity at day 1 revealed no significant difference in the number of cell divisions with or without IL-7 (Figure 6F, left). Flow cytometry of 3-day cultures revealed that (1) all LTi cells had a reduced CFSE fluorescence intensity within 3 days indicating cell division and

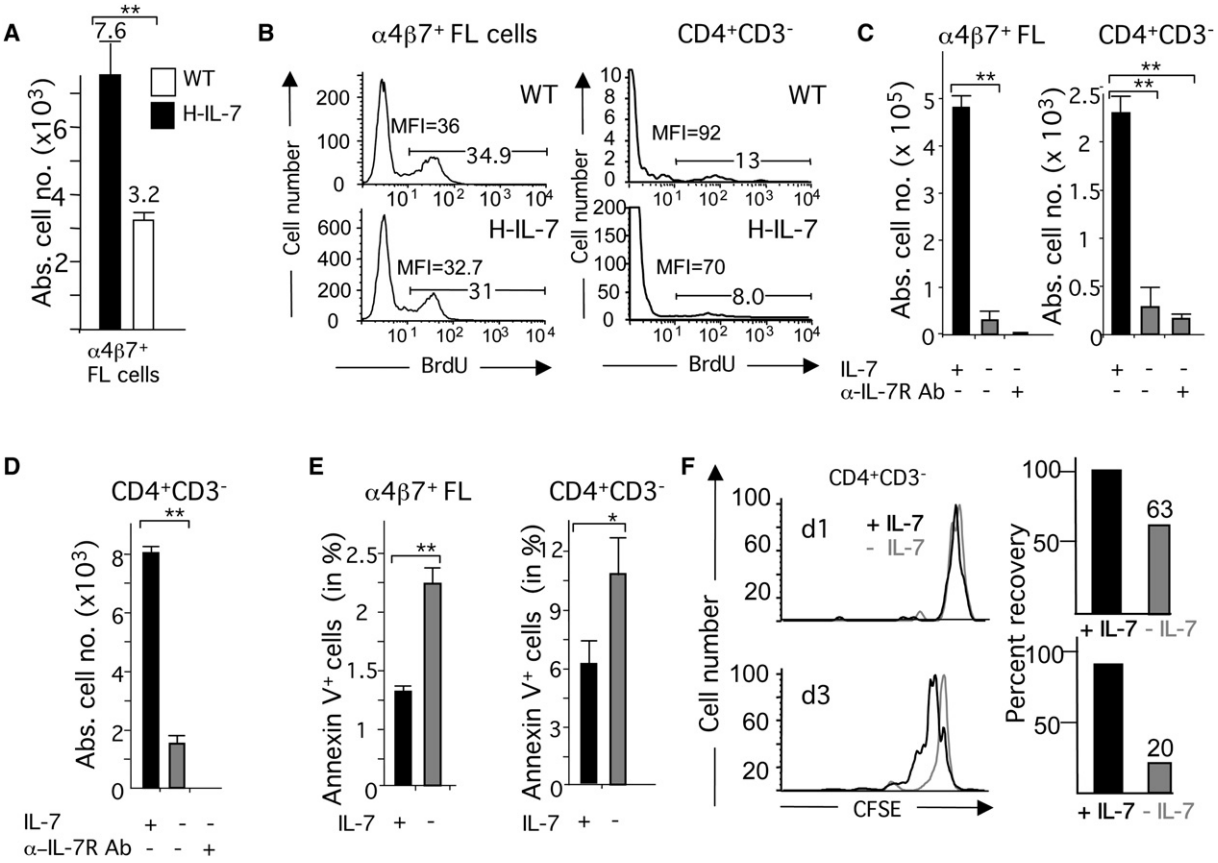


Figure 6. IL-7 Promotes the Survival of α4β7⁺ FL Progenitor Cells and of LTi Cells

(A) The absolute number of α4β7⁺ cells calculated with flow cytometry of E14.5 FL from WT (n = 4) and H-IL-7 mice (n = 6). Shown are mean values ± SD.

(B) Flow cytometric analysis of BrdU incorporation into (left) α4β7⁺ cells derived from E14.5 FL (WT versus H-IL-7) and (right) splenic CD4⁺ CD3⁻ LTi cells derived from neonatal spleen (WT versus H-IL-7), after in vivo pulse with BrdU. The FACS profile is representative for 3 independent experiments with 3 to 5 mice per group.

(C) 1.8 × 10⁵ sorted α4β7⁺c-Kit⁺ lin⁻ FL cells from WT mice (E14.5, n = 8) were cultured on ST-2 stromal cells with or without IL-7 supernatant or alternatively with blocking anti-IL-7Rα Ab. After 9 days, the absolute cell number of α4β7⁺c-Kit⁺ cells and CD4⁺CD3⁻ LTi cells was determined. The experiment was repeated three times. Data represent mean values ± SD. **p < 0.01.

(D) 5 × 10³ sorted CD4⁺CD3⁻ LTi cells of newborn H-IL-7 mice (n = 6) were cultured, and cell recovery was determined at day 15 of culture. Data represent mean values ± SD.

(E) Sorted α4β7⁺c-Kit⁺ cells from FL of E14.5 WT mice (n = 8) were cultured for 7 days in medium containing IL-7 followed by incubation with or without IL-7. 48 hr later, Annexin V labeling of 7AAD⁺ cells was determined in both α4β7⁺c-Kit⁺ and CD4⁺CD3⁻ LTi cells. Data represent mean values ± SD.

(F) CFSE profiles of gated CD4⁺CD3⁻ LTi cells among neonatal splenocytes from WT mice (n = 6) cultured for 1 and 3 days as hanging drops was determined by flow cytometry (left). Dead cells were excluded from analysis by means of 7AAD. Right panel shows the mean percent recovery of CD4⁺CD3⁻ LTi cells after culture of splenocytes from six neonatal WT mice.

(2) approximately half of the LTi cells cultured in the presence of IL-7 underwent one more division. We could not fully exclude, however, that in the absence of IL-7, cycling cells preferentially died. Altogether, our data indicate that IL-7 was crucial for the survival of LTi cells and their fetal liver progenitors.

DISCUSSION

In this study, we have shown that IL-7 was a growth factor that directed survival of LTi cells. Increased in vivo availability of IL-7 in Tg mice led to the accumulation of LTi cells and the formation of additional lymphoid organs. Mice overexpressing IL-7 but lacking either RORγ, a factor required for LTi cell generation, or $LT\alpha_1\beta_2$ normally expressed by LTi cells, had neither PP nor ELN. Taken together, in this model, de novo organogenesis was clearly dependent on IL-7, LTi cell number, and LTβR signaling.

During the past years, extensive studies have been performed to better understand the pathogenic mechanism for ectopic lymphoid tissue development, so-called "tertiary lymphoid tissue" at sites of chronic inflammation. A key role of IL-7 in immunopathology of persistent inflammation has been discussed (Hartgring et al., 2006). Mice carrying an IL-7 Tg fused to an immunoglobulin heavy chain promoter/enhancer or an SRα-driven IL-7 Tg

develop diffuse T lineage infiltrates in the skin (Rich et al., 1993) and the colon (Watanabe et al., 1998), respectively. In humans, ectopic lymphoid follicles are found in patients with rheumatoid arthritis, and disease development positively correlates with high concentrations of serum and synovial IL-7 (Takemura et al., 2001; van Roon et al., 2005). Moreover, both IL-7 and its receptor are implicated in multiple sclerosis pathogenesis (Booth et al., 2005; Traggiai et al., 2001). A causal link between IL-7 production and tertiary lymphoid tissue formation has not been investigated so far.

Here we report that increased availability of IL-7 promoted de novo formation of PP and LN through LTi cells, because in H-IL-7 RORγ-deficient mice, which lack LTi cells, PP and ELN were completely absent and no induction of $VCAM^+$ stromal cells was observed. Consistent with this, transfer of LTi cells isolated from neonatal H-IL-7 mice into newborn *Cxcr5*$^{-/-}$ mice induced the formation of PP-like structures, thereby confirming their functional properties.

In normal mice, neonatal LN suspensions injected intradermally can aggregate and form lymphoid-like structures (Cupedo et al., 2004). This is in line with numerous studies in mice demonstrating a striking capacity of lymphoid cell suspensions, e.g., from fetal thymic lobes, to grow and reaggregate at ectopic sites (Anderson and Jenkinson, 2001). In Tg mice, the expression of TNF family member molecules and chemokines driven by a pancreas-specific promoter is followed by the development of intrapancreatic tertiary lymphoid tissue (for review see Drayton et al., 2006). In these studies, the intriguing question remained: are LTi cells responsible for the spontaneous formation of lymphoid tissue at these ectopic sites? Our data clearly demonstrated that in a mouse model with increased IL-7 availability, multiple LN developed spontaneously at ectopic sites in an LTi cell-dependent manner, and furthermore, these ELN had a normal architecture, were connected to afferent and efferent lymphatics, and were functional in that they participated in T cell-dependent B cell responses. In addition, we showed that upon immunization with adjuvant, a procedure that induces an inflammatory response, multiple ELN harboring Ab-producing cells were generated at sites of injection. This indicated that in H-IL-7 mice, functional tertiary lymphoid follicles were formed at sites of inflammation. Consistent with the formation of tertiary lymphoid tissues in patients with autoimmune diseases, naive H-IL-7 mice spontaneously developed tertiary lymphoid follicles in target organs for autoimmune reactions such as pancreas and salivary gland.

Compared to H-IL-7 mice, IE-IL-7 mice formed similar numbers of follicles per PP but no additional PP, and fewer ELN and cecal LP. These results suggested that either the amount of IL-7 required for the formation of ELN and increase in lymphoid follicle numbers was below the amount required for the organogenesis of additional PP, or alternatively that the sites or onset of IL-7 expression in IE-IL-7 mice differed from H-IL-7 mice, thereby not allowing de novo formation of PP during embryonic development. In addition, it is possible that the increased number of follicles seen in IE-IL-7 and H-IL-7 mice was due to the IL-7-driven increase in B and T cells, rather than an effect during organogenesis. In agreement with this, local IL-7 Tg expression in enterocytes of *Il7*$^{-/-}$ mice on a (B6x129 Ola)F_1 hybrid background harboring some residual PP in the jejunum and ileum could partially restore intestinal lymphocyte compartments and PP numbers in the distal small intestine but was unable to fully restore PP number or size to that found in WT mice (Laky et al., 2000).

In a recent paper, the CCL21-driven formation of lymphoid structures in the thyroid gland was reported to occur independently of LTi cells but required the entry of mature T cells (Marinkovic et al., 2006). It is likely that, depending on the model and stimuli, other cell subsets can contribute to the formation of ectopic tertiary lymphoid tissues. Various cell subsets, such as B cells, T cells, and NK cells, can express substantial amounts of $LT\alpha_1\beta_2$ and, hence, may have a pivotal role in ectopic lymphoid tissue formation. In agreement with this, the contribution of T and NK cells to postnatal LN development has been reported (Coles et al., 2006). It remains to be elucidated which additional signals, e.g., proinflammatory cytokines or chemokines, are required for an LTi-independent mechanism of tertiary lymphoid-tissue formation.

In fetal WT mice, IL-7 is produced by intestinal $VCAM\text{-}1^+$ organizer cells (Nishikawa et al., 2003). We observed a striking capacity of the entire small intestine of fetal H-IL-7 mice to generate a continuous band of $VCAM\text{-}1^+$ organizer cells on the antimesenteric wall before birth. The fact that large numbers of LTi cells clustered to form aggregates may explain the coalescence of VCAM-1 stainings in neonates. Alternatively, there was an active mechanism that allowed migration of VCAM-1 organizer cells into the aggregates. This clearly demonstrates that $VCAM\text{-}1^+$ PP anlagen induction in the embryo was not fixed to particular anatomical sites. A molecular feedback loop between IL-7-producing $VCAM\text{-}1^+$ PP organizer cells and IL-7-responsive LTi cells might further contribute to cluster formation and lympho-organogenesis. Indeed, IL-7 can induce $LT\alpha_1\beta_2$ expression by LTi cells, which is mandatory for the engagement of LTβR on the organizer cells, and for further lymphoid stroma differentiation (Honda et al., 2001; Yoshida et al., 2002). Consistently, we observed that $LT\alpha_1\beta_2$ expression was upregulated on LTi cells of both IE-IL-7 and H-IL-7 mice. Nevertheless, only in H-IL-7 mice was an increase in PP number (5-fold) observed, suggesting that increased $LT\alpha_1\beta_2$ expression alone was not sufficient to generate additional PP. In *Lta*$^{-/-}$ H-IL-7 mice, ectopic LN and PP were undetectable, indicating again that the de novo formation of lymphoid tissue was strictly dependent on $LT\alpha_1\beta_2$-expressing cells.

A direct role for IL-7 in LTi cell survival had not yet been investigated. Here, we demonstrated that IL-7 was crucial for the survival and subsequent cell division of LTi cells and its FL progenitors. By using mice with either high or intermediate IL-7 Tg expression, we observed that cell numbers were amplified only if a high amount of IL-7 was available.

In the FL of E14.5 mice, IL-7R$^+$ cells have been identified as progenitors for LTi cells (Mebius et al., 2001; Yoshida et al., 2001). In H-IL-7 mice, we found a 2-fold increase in IL-7R$^+$ $\alpha4\beta7^+$ FL cell numbers and a 3-fold increase in the amount of IL-7 transcripts. We did not observe a significant in vivo effect of increased IL-7 availability on the rate of proliferating $\alpha4\beta7^+$ FL progenitors and LTi cells. In vitro, the presence of IL-7 for 1 day did not change the rate cell divisions but substantially improved LTi cell recovery. In the absence of IL-7 for 3 days, a further increase in the proportion of apoptotic cells was observed. IL-7 also had an effect on the rate of proliferation of LTi cells in vitro at day 3, because approximately 50 percent of the cells cultured in medium supplemented with IL-7 underwent one more cell cycle. We cannot exclude that dividing cells preferentially died without IL-7, thus explaining the difference in numbers of cycling cells. Both survival and subsequent cell division explain the in vivo and in vitro amplification of $\alpha4\beta7^+$ FL progenitors and LTi cells. Likewise, differentiation of $\alpha4\beta7^+$ FL progenitors into CD4$^+$ CD3$^-$ LTi cells was dependent on the presence of IL-7. It is likely that IL-7 delivers antiapoptotic signals to LTi cells, thus promoting their survival. In line with this, the expression of antiapoptotic *Bcl* family members such as *Bcl-2* and *Bcl-X* has been recently reported in LTi cells of fetal spleen after a 5 day in vitro culture period in the presence of IL-7 (Kim et al., 2006).

Our data clearly show that the process of lymphoid-organ formation is highly plastic and can be influenced by the increased expression of IL-7 leading to the expansion of the pool of LTi cells through preventing apoptosis. The degree of lymphoid-tissue formation was tightly dependent on the amount of IL-7. Our findings extend the previously defined role of IL-7 for PP development to a more general function of IL-7 in inducing lympho-organogenesis. In addition, they suggest that organized tertiary lymphoid organs can develop, provided that IL-7 and sufficient numbers of LT$\alpha\beta$-expressing LTi cells are available.

EXPERIMENTAL PROCEDURES

Mice

CIITA IL-7 double-transgenic mice (H-IL-7) were generated by crossing heterozygous CIITA Tg females (Otten et al., 2003) with heterozygous IL-7 Tg (IE-IL-7) males (Mertsching et al., 1996). *Lta*$^{-/-}$ mice were originally generated by De Togni and colleagues (De Togni et al., 1994). *Cxcr5*$^{-/-}$ mice were originally generated by Foerster and colleagues (Forster et al., 1996). RORγ-deficient mice were kindly provided by D. Littman (Sun et al., 2000). *Il7*$^{-/-}$ mice (von Freeden-Jeffrey et al., 1995) were at least 8 times backcrossed to C57BL/6 mice. For visualizing LN, 1% Chicago sky blue 6B (Sigma-Aldrich) diluted in PBS was injected s.c., and mice were analyzed 30 min or 4 days later. Mice were housed under standard conditions in a pathogen-free mouse facility. The study received the approval of the Cantonal Veterinary Office of the city of Basel, Switzerland.

Adoptive Cell Transfer and Immunization

Sorted CD4$^+$CD3$^-$ cells were injected intravenously (i.v.) into newborn *Cxcr5*$^{-/-}$ mice as previously described (Finke et al., 2002). For in vivo immunization, adult age-matched WT and H-IL-7 mice were immunized with one single s.c. injection of 50 μg of the T-dependent B cell antigen NP-CGG (Biosearch Technologies) Alum precipitate. 1 and 2 weeks later, mice were sacrificed, and draining as well as non-draining LN were analyzed for specific T-dependent B cell responses.

ELISPOT Assay

96-well Nunc Maxisorp plates were coated with 50 μl of NP-conjugated bovine serum albumin (NP-NP$_3$ BSA and NP$_{33}$-BSA at a concentration of 5 μg/ml [Biosearch Technology Inc. Novato, CA]). After blocking with PBS/1% BSA, plates were incubated with various dilutions of LN cell suspensions, followed by washing and incubation with biotinylated goat anti-mouse Ig (CALTAG) overnight. After incubation with streptavidin alkaline phosphatase (Boehringer Mannheim) in PBS/Tween, substrate solution (5-Bromo-4-chloro-3-indolyl-phosphate (Sigma) dissolved in 1-Amino-2-Methyl-1-Propanol (Sigma) was added and stopped 4 hr later.

Flow Cytometry Analysis and Cell Sorting

Single-cell suspensions from fetal intestine (E16.5) were obtained by incubating the intestine in PBS containing 1 mg/ml dispase (GIBCO) for 15–25 min at 37°C. Single cells derived from spleen, LN, intestine, or FL were incubated with the anti-mouse FcγRII MAb (clone 2.4G2) before incubation with a mix of fluorochrome-conjugated Ab. Sample acquisition was performed with a FACScalibur (Becton Dickinson). Cells were sorted with a FACS Aria (Becton Dickinson).

Antibodies

MAb either biotinylated or conjugated to fluorochromes (FITC, PE, PE-Cy5, PE-Cy7, APC) were directed against the following mouse Ag: CD45 (30-F11, Biolegend), MHC II (M5.114.15.2, Biolegend), CD3 (145-2C11, eBioscience), CD4 (H129, Biolegend), CD8 (53-6.7, Biolegend), CD11c (N418, eBioscience), CD19 (6D5, Biolegend), B220 (RA36B2, Biolegend), CD93 (AA4.1, eBioscience), IgD (1.19), Peanut Agglutinin (Vector Laboratories), GL-7 (Ly-77, PharMingen), VCAM-1 (429, eBioscience), LTβR Ig (kind gift from J. Browning, Biogen, Cambridge, MA), $\alpha4\beta7$ (DATK 32, BD Bioscience), c-Kit (CD117) (ACK2, eBioscience), and IL-7Rα (A7R34, eBioscience). Lineage cocktail mix contained MAb specific for CD3, CD8, CD11c, CD11b (M1/70, Biolegend), CD19, B220, Gr-1 (RB6-8C5, eBioscience), and NK1.1 (PK136, eBioscience).

Analysis of Cell Proliferation and Cell Death

250 μg and 1.8 mg of BrdU diluted in PBS was injected i.p. into newborn and E14.5 pregnant adult mice, respectively. 5 or 1.5 hr later, mice were sacrificed, splenocytes harvested from newborn mice, or alternatively FL cells cell suspensions were derived from E14.5 embryos, and surface staining with MAb was performed as described before. Cells were fixed, permeabilized, and stained with FITC-conjugated anti-BrdU Ab (BD Pharmingen) or isotype controls according the manufacturer's instruction (BD Pharmingen). In order to determine cell divisions of LTi cells, total splenocytes from neonatal C57BL/6 mice were labeled with 0.2 μM CFSE (Invitrogen) for 10 min at 37°C. After washing in PBS 10% FCS, cell suspensions were plated in a final concentration of 5 × 10^5 cells/30 μl in Terasaki plates, and plates were inverted to generate hanging-drop cultures. 1 or 3 days later, cells were harvested and analyzed by flow cytometry.

In order to determine the rate of apoptosis in FL and LTi cells cultured with or without IL-7, FACS-sorted $\alpha4\beta7^+$ c-Kit$^+$ lin$^-$ cells were cultured on irradiated (2000 Rad) ST-2 stromal cells in IMDM/FCS supplemented with 3% IL-7 supernatant. After 7 days, IL-7 was withdrawn from half of the plate and replaced by medium without IL-7. 48 hr later, cells were harvested and analyzed with the Annexin V and 7AAD labeling kit from BD Pharmingen according to the manufacturers' protocols. In brief, 10^6 cells were surface stained with various Ab and resuspended in Annexin V binding buffer, followed by adding Annexin V-Cy5 and 7AAD. After 15 min incubation of the cell suspension at room temperature, cells were analyzed by FACS Calibur.

Cell Culture

FACS-sorted $\alpha4\beta7^+$c-Kit$^+$ lin$^-$ FL cells (E14.5) and neonatal CD4$^+$ lin$^-$ LTi cells were cultured in IMDM/FCS supplemented with antibiotics (penicillin/streptomycin, ciproxine, kanamycin), 0.5 mg insulin per 100 ml, 0.1% mercaptoethanol, and 1% of nonessential amino acids on irradiated (2000 Rad) ST-2 stromal cells with or without IL-7 supernatant (3%) or with blocking anti-IL-7Rα Ab (clone A7R34; 50 μg/ml).

RT-PCR and Quantitative Real-Time PCR Analysis

For RT-PCR of RORγt, the following primers were used (forward [F], reverse [R]): RORγt [F] ACCTCCACTGCCAGCTGTGTGCTGTC, [R] TCATTTCTGCACTTCTGCATGTAGACTG. The cycling conditions for RORγt RT-PCR were: 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 30 s at 65°, and 45 s at 72°C. The primers for the TATA box binding protein (TBP) were used: [F] CCATTCTCAAACTCTGACCAC3, [R] CCGTGGCTCTCTTATTCTCAT. The cycling condition for TBP RT-PCR were: 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Quantitative real-time PCR was performed with the ROTOR-GENE RG-3000 with the sensiMix (dT) (Quantace). The following primers were used: IL-7 [F] GATAGTAATTGCCCGAA TAATGAACCA, [R] GTTTGTGTGCCTTGTGATACTGTTAG, LTβ [F] AATGCTTCCAGGAATCTAGCC, [R] CCAAGCGCCTATGAGGT, TBP [F] CGTGAATCTTGGCTGTAAACT, [R] GTCCGTGGCTCTCTTATTCT.

The cycling conditions for IL-7 real-time PCR were: 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 15 s at 60°C, and 20 s at 72°C. The conditions for LTβ real-time PCR were: 10 min at 94°C, followed by 40 cycles of 10 s at 95°C, 5 s at 60°C, and 6 s at 72°C. The relative expression of target genes was measured in triplicate and normalized to the expression amount of TBP. The fold difference (as relative mRNA Tg expression) between samples derived from H-IL-7, IE-IL-7, and WT mice was calculated by the comparative C_T method ($\Delta\Delta C_T$) and the received values were illustrated.

Immunohistochemistry and Immunofluorescence Microscopy

Immunostainings of frozen sections and whole-mount immunohistochemistry analysis of fetal (E16.5) and newborn intestine was performed as previously described (Finke et al., 2002). For immunofluorescence staining, 10 μm acetone-fixed cryosections of neonatal intestine or adult LN were incubated with the primary rat anti-mouse Ab, followed by incubation with the secondary goat anti-rat Ig Cy3 (Jackson ImmunoResearch) and by incubation with biotinylated rat anti-mouse Ab in PBS. Sections were stained with Streptavidin Alexa 488 (Molecular Probes) in PBS/1% BSA and finally embedded in Fluorsave (Calbiochem).

Statistical Analysis

To evaluate statistically significant differences, we used unpaired two-tailed Student's t test. p values less than 0.05 were considered statistically significant.

Supplemental Data

Five figures are available at http://www.immunity.com/cgi/content/full/26/5/643/DC1/.

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Supplemental Data

Ectopic Lymphoid-Organ Development Occurs through Interleukin 7-Mediated Enhanced Survival of Lymphoid-Tissue-Inducer Cells

Dominik Meier, Caroline Bornmann, Stephane Chappaz, Sandrine Schmutz, Luc A. Otten, Rhodri Ceredig, Hans Acha-Orbea, and Daniela Finke

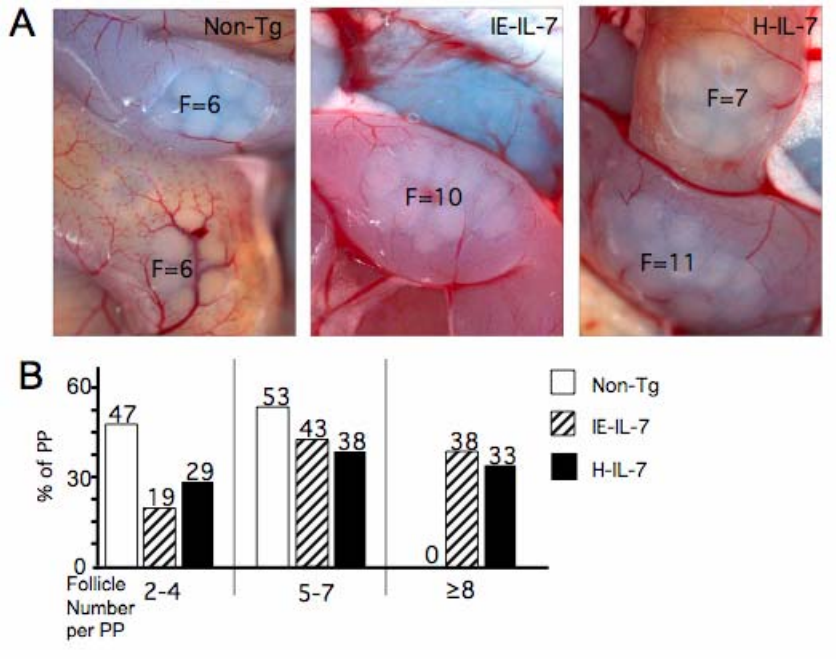

Figure S1. IL-7-Dependent Increase in PP Follicle Number
(A) Microscopic inspection of adult PP following Chicago blue injection in WT, IE-IL-7 and H-IL-7 mice. F, follicle number is indicated in the figure. (B) The percentage of individual PP with an average follicle number of 2-4, 5-7 of more than 8 is shown. Data were obtained from analyzing WT (n=8), IE-IL-7 (n=5) and H-IL-7 (n=5) mice.

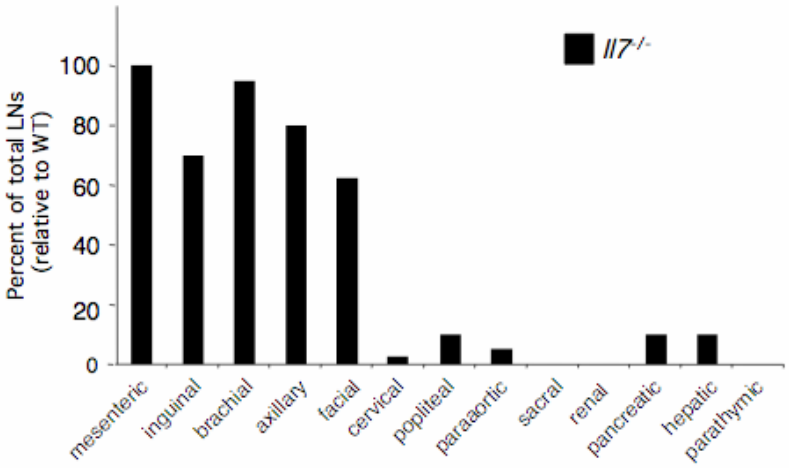

Figure S2. LN Development Is Impaired in *Il7*$^{-/-}$ Mice
4 days after s.c. injection of Chicago blue dye, the number of indicated LN in adult *Il7*$^{-/-}$ mice (n=10) was assessed, and the percentage relative to WT numbers (100%) was calculated.

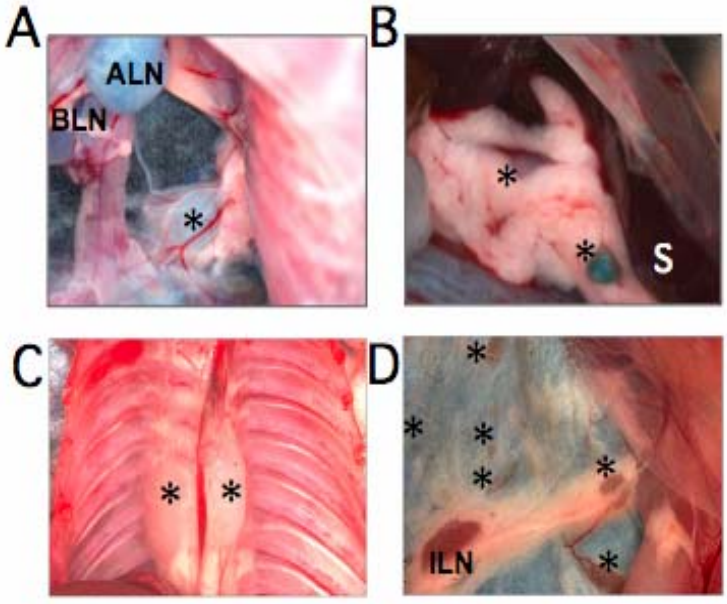

Figure S3. Ectopic LN Development in H-IL-7 Mice
In H-IL-7 mice with or without Chicago blue injection, ectopic LN (indicated by asterisk) are shown in the (A) axilla, (B) pancreas, (C) intercostal region and (D) randomly distributed in the deep subcutaneous region. ALN, axillary LN; BLN, brachial LN; ILN, inguinal LN; S, spleen.

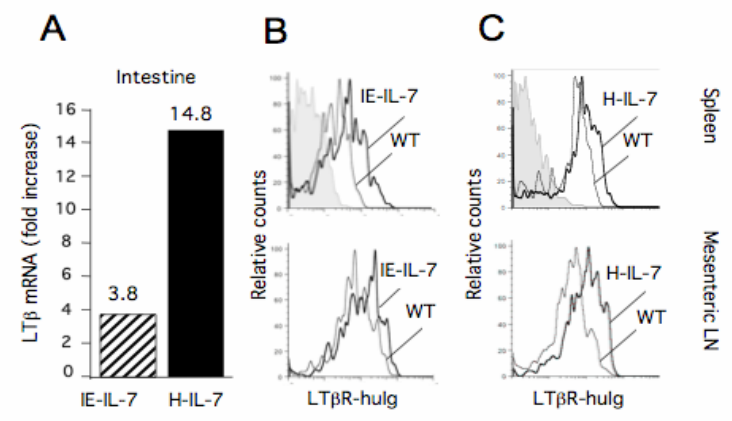

Figure S4. IL-7 Promotes the Expression of LT$\alpha_1\beta_2$ by LTi Cells In Vivo
(A) LTβ-specific quantitative real time-PCR of total RNA isolated from the intestine of E16.5 fetal IE-IL-7 and H-IL-7 mice. The fold difference of LTβ mRNA comparing the indicated mice with WT controls is shown. (B) Gated on $CD4^+$ $CD3^-$ LTi cells, LTβR-Fc staining of *ex vivo* isolated splenocytes and mesenteric LN of newborn IE-IL-7 (bold) versus WT mice (thin), and (C) H-IL-7 (bold) versus WT mice (thin) is shown. LTi cells were negative for the lineage marker B220, CD19, CD11c, CD11b and NK1.1. Mean fluorescence values for LT$\alpha_1\beta_2$ were: 60 and 178 for IE-IL-7 spleen and LN, and 107 and 137 for age-mached H-IL-7 mice spleen and LN versus 21 and 109 for spleen and LNs in WT mice, respectively. Data are representative for 3 experiments. As negative controls, splenocytes derived from *Lt$\alpha^{-/-}$* mice were used (shaded).

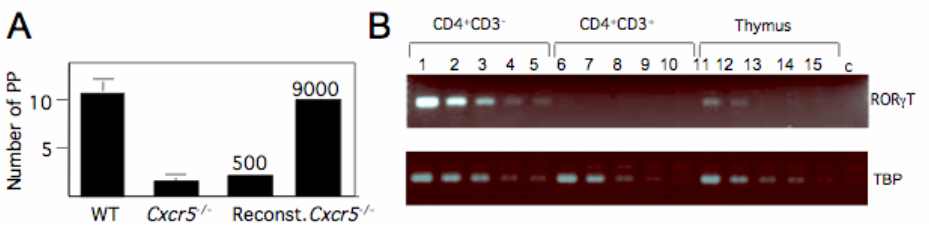

Figure S5. $CD4^+CD3^-$ Cells from H-IL-7 Mice Are Functional LTi Cells
(A) 3 weeks after i.v. adoptive transfer of 500 or 9000 sorted $CD4^+CD3^-$ cells from spleen of fetal H-IL-7 mice into neonatal *Cxcr5$^{-/-}$* mice, VCAM-1 immunohistochemical analysis of recipient intestine was performed. As controls, untreated WT mice and *Cxcr5$^{-/-}$* mice are shown. (B) RT-PCR of RNA isolated from FACS-sorted $CD4^+$ $CD3^-$ LTi cells, and $CD4^+$ $CD3^+$ T cells derived from neonatal H-IL-7 mice and from total thymus of litter controls. The linear range of the RORγt –specific RT-PCR is shown by serial 2-fold dilutions of RNA. TATA box binding protein (TBP) was used as house-keeping gene in the RT-PCR. For $CD4^+$ $CD3^-$ LTi cells, RNA isolated from 1,800 cells (lane 1) to 112 cells (lane 5), for $CD4^+$ $CD3^+$ cells, RNA from 900 (lane 6) to 56 cells (lane 10) and for the thymus, RNA from 450 cells (lane 11) to 28 cells (lane 15) was used. C shows the H_2O control.

*3.3. Manuscript 2**

Interleukin 7 regulates the peripheral pool of adult RORγ$^+$ lymphoid tissue inducer cells

Sandrine Schmutz, Nabil Bosco, Stephane Chappaz, Onur Boyman, Hans Acha-Orbea, Rhodri Ceredig, Antonius Rolink, and Daniela Finke

*This manuscript is in revision

Interleukin 7 regulates the peripheral pool of adult RORγ$^+$ lymphoid tissue inducer cells[1]

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Running title: Regulation of adult LTi cells

Keywords: Interleukin 7, lymphoid tissue inducer cell, lympho-organogenesis, spleen.

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Abstract

During fetal life, $CD4^+CD3^-$ lymphoid tissue inducer (LTi) cells are required for lymph node and Peyer's patch development in mice. In adult animals, $CD4^+CD3^-$ cells are found in low numbers in lymphoid organs. Whether adult $CD4^+CD3^-$ cells are LTi cells and are generated and maintained through cytokine signals has not been directly addressed. Here, we show that adult $CD4^+CD3^-$ cells adoptively transferred into neonatal $CXCR5^{-/-}$ mice induced the formation of Peyer's patches demonstrating for the first time their *bona fide* LTi function. Increasing IL-7 availability in wild type mice either by IL-7 transgene expression or treatment with IL-7/anti-IL-7 complexes increased adult LTi cell numbers through *de novo* generation from bone marrow cells and increased survival and proliferation of LTi cells. Our observations demonstrate that adult $CD4^+lin^-$ cells are LTi cells and that the availability of IL-7 determines the size of the adult LTi cell pool, which can trigger lymphoneogenesis.

Introduction

During fetal life, the development of secondary lymphoid organs in mice is determined by the interactions between lymphotoxin (LT)$\alpha_1\beta_2^+$CD45$^+$CD4$^+$CD3$^-$ LTi cells and mesenchymal LTβR$^+$VCAM-1$^+$ organizer cells (1, 2). The first lymph node (LN) and Peyer's patch (PP) anlagen in fetal mice develop between embryonic day E13.5 and E15.5 from clusters of LTi and organizer cells (3, 4). The generation of LTi cells is dependent on the helix loop helix protein Id2 and the retinoic acid related orphan receptor RORγ (5, 6). In the absence of LTi cells or if the LTβR signalling pathway is perturbed, LNs and PPs do not form (7, 8). Moreover, IL-7 has an important role in LN and PP development (9) and we have recently shown that this relies on the IL-7-dependent survival of foetal LTi cells and their fetal liver (FL) progenitors (10). The number and localization of secondary lymphoid organs is developmentally fixed in mice. By increasing the pool of fetal LTi cells *in vivo* through IL-7 transgene expression this restriction can be overcome leading to the formation of additional ectopic lymphoid organs.

Among CD3$^-$ cells, RORγt is an exclusive marker of fetal LTi cells (11). Fate mapping experiments with ROR(γt) $^{+/GFP}$ mice led to the conclusion that LTi cells persist in the gut of adult mice (12). In wild type (WT) adult mice, CD4$^+$CD3$^-$ cells were found in the spleen where they help organize the architecture and optimize immune responses (13, 14). In addition, CD4$^+$CD3$^-$ cells accumulate in the spleen of LCMV-infected mice (15). Neonatal and adult splenic CD4$^+$CD3$^-$ cells display similar genetic profiles distinguishing them from other cells. Both express LTα, TNFα, c-Kit (CD117), IL-7Rα (CD127) and TNF receptors (TNFRII, TRANCER, HVEM) (16) but in contrast to adult cells, fetal LTi cells express neither CD30L nor OX40L (17) unless treated *in vitro* with IL-7 (17) or TNFSF15 (16), respectively. The mechanism for the generation and persistence of adult CD4$^+$CD3$^-$ cells and their possible function as inducers of newly formed lymphoid organ remains to be addressed.

In the present study, we firstly sought to determine whether adult $CD4^+CD3^-$ cells have *bona fide* LTi activity and secondly, whether they could be expanded by increased availability of IL-7 either in transgenic or IL-7/anti-IL-7 complex-treated mice (18). Herein, we show that adult $CD4^+CD3^-$ cells are functional LTi cells, which are dependent on RORγt and IL-7.

Materials and Methods

Mice and *in vivo* treatment

H-IL-7, ROR$\gamma^{-/-}$, CXCR5$^{-/-}$ and IL-7$^{-/-}$ mice were previously described (10, 19). H-2k bcl-2 transgenic mice were originally generated in the laboratory of I.L. Weissman (20) and kindly provided by A. Trumpp. Immune complexes containing 10 μg recombinant human IL-7 (R&D) and 50 μg anti-IL-7 neutralizing monoclonal antibody (mAb) (M25) were i.p. injected into mice at day 0, 2 and 4, and mice were analyzed at day 7. If indicated, IL-7/anti-IL-7 complex treatment was prolonged over 3 wks. FACS-sorted LTi cells were i.v. injected into neonatal CXCR5$^{-/-}$ mice as described (19). Splenocytes, LTi, BM or FL (E13.5) cells were i.v. injected into sublethally (600 Rad) irradiated mice (RAG$^{-/-}\gamma c^{-/-}$, IL-7R$\alpha^{-/-}$ mice). Mice were housed under standard conditions in a pathogen-free mouse facility. The study received the approval of the Cantonal Veterinary Office of the city of Basel, Switzerland.

Antibodies

Abs either biotinylated or conjugated to fluorochromes (FITC, PE, PECy5, PeCy7 or APC) , purchased from BioLegend, eBioscience or BD Pharmingen, were used against the following mouse antigens: CD4 (clone: RM4-5), c-Kit/CD117 (2B8), CD3 (145-2C11), CD8 (53-6.7), TCRαβ (H57-597), TCRγδ (UC7-13D5), CD11c (N418), CD19 (6D5), B220 (RA3-6B2), NK1.1 (PK136), Gr-1 (RB6-8C5), TER119 (TER119), IL-7Rα (A7R34), CD18 (M18/2), β1 integrin (HMb1-1), α4β7 integrin (DATK32), Sca-1 (D7), CD44 (IM7), CD62L (MEL-14), LTβR-huFc (gift from J. Browning), TRANCE (IK22/5), TRANCER (R12-31), ICAM-1 (3E2), CD184 (2B11/CXCR4), CD45.1 (A20), CD45.2 (104), VCAM-1 (429), IL-7 (Lot number AWR01, R&D), M25 (mouse anti-human, Amgen). Secondary Abs were streptavidin (purchased from BioLegend) conjugated to FITC, PE, PECy5, PECy7 or APC, HRP-

conjugated goat anti-rat IgG (Biosource), Alexa Fluor 488 donkey anti-goat IgG (Invitrogen), Cy3-conjugated goat anti-rat IgG (Jackson ImmunoResearch).

Flow cytometry and cell sorting

10^6 cells derived from various lymphoid organs were stained with mAbs using standard protocols (10). Cells were resuspended in PBS containing 3% FCS, stained with biotinylated or fluorochrome-conjugated Abs and analyzed using a FACScalibur (Beckton Dickinson & Co). To analyse for the presence of LTi cells, total splenocytes from 6-8 wk old mice were depleted of erythrocytes, stained with biotinylated anti-CD4 Ab and enriched using MACS separation (Miltenyi Biotec). MACS-enriched cells were labelled with anti-CD4, anti-c-Kit and a cocktail of Abs specific for CD3, CD8, CD11c, CD19, B220, TCRαβ, TCRγδ, Gr-1, NK1.1 and TER119 (lin-cocktail). Data were analysed with the FlowJo software (Tree Star). A FACSaria (Becton Dickinson & Co) was used to sort adult LTi cells.

Reverse Transcriptase (RT)-PCR

RORγt- and TBP-specific RT-PCR were carried out as previously described (10).

In vitro* culture and proliferation assay *in vitro* and *in vivo

FACS-sorted LTi cells from H-IL-7 spleen were cultured on primary splenic stroma (15) in IMDM/FCS supplemented with antibiotics (penicillin/streptomycin, ciproxine, kanamycin), 0.5 mg insulin per 100 ml, 0.1% β-mercaptoethanol, 1% non essential amino acids without or with blocking anti-IL-7Rα Ab (clone A7R34; 50 μg/ml). The presence and number of LTi cells was analyzed by flow cytometry after 8 days.

To determine whether LTi cells proliferate in the presence of IL-7, sorted LTi cells from $RAG^{-/-}$ spleens were labelled with 1.25 μM CFSE (10 minutes at 37°C) and cultured on

primary splenic stroma (15) with or without IL-7. After 7 days cells were analyzed by flow cytometry. To test *in vivo* proliferation, 10^8 $CD4^+$ MACS-enriched CFSE-labelled splenocytes were adoptively transferred into irradiated mice.

Immunofluorescence and immunohistochemistry

Tissues were snap-frozen in Tissue-Tek O.C.T Compound (Sakura). 5-8 μm sections were fixed in acetone and rehydrated in PBS. For intracellular staining, slides were blocked in PBS 0.2% gelatine (porcine skin, type A, Sigma) 1% donkey serum for 30 minutes and then incubated with primary Ab in PBS 0.2% gelatine overnight at 4°C. For surface staining, tissue sections were blocked in PBS 5% goat serum 1% BSA and then incubated with primary Ab for 1 hour at room temperature. Sections were washed and incubated with secondary Ab for 45 minutes. For enzymatic immunohistochemistry, peroxidase activity was developed using 3,3'-Diaminobenzidine tetrahydrochloride (Sigma). Slides were embedded in Moviol or Medimount (Medite).

Results and Discussion

Adult $CD4^+CD3^-$ cells are LTi cells

We investigated the LTi activity and number of $CD4^+CD3^-$ cells in normal and IL-7 overexpressing IL-7/CIITA double transgenic (referred to as H-IL-7) adult mice. The percentage of $CD4^+CD3^-$ cells was increased 5-fold (Fig. 1A) and their number 30-fold in the spleens of H-IL-7 mice (Fig. 1B). In addition, adult $CD4^+CD3^-$ numbers were increased in LNs (Fig.1C and D). IL-7 protein was present in VCAM-1^+ $CD45^-$ cells in the splenic red pulp and was substantially increased in H-IL-7 mice (Supplementary Fig. S1A and B). Altogether, our findings demonstrate that increased IL-7 availability was associated with increased $CD4^+CD3^-$ cell numbers in adult mice.

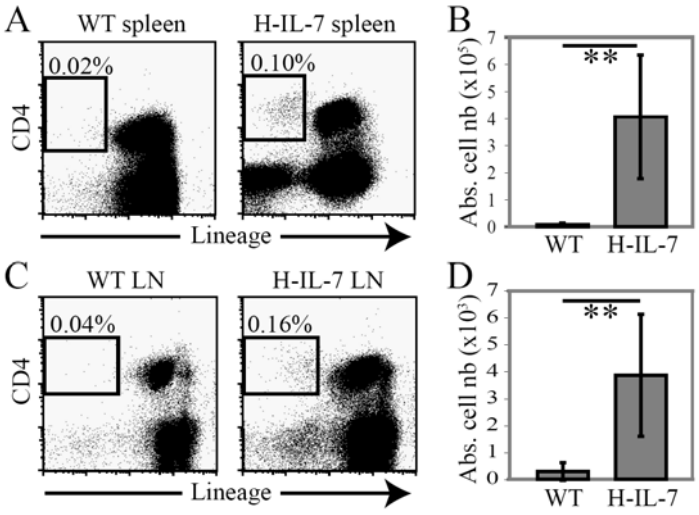

Figure 1. Adult $CD4^+lin^-$ cells are found in adult mice. (A) Flow cytometry of total splenocytes from adult mice revealed that the percentage of $CD4^+lin^-$ cells in H-IL-7 was 5 fold increased compared to WT (0.1% and 0.02%, respectively). Lineage cocktail contained Abs against CD3, CD8, CD11c, CD19, B220 and NK1.1. **(B)** The absolute number of splenic $CD4^+lin^-$ cells was 30 fold increased in H-IL-7 compared to WT ($3.7x10^5$ and $1.1x10^4$ cells, respectively). (n=12 mice per group, **p<0.05, Student *t* test) **(C)** $CD4^+lin^-$ cells represented 0.16% of total inguinal LN cells in H-IL-7 mice (n=6) compared to 0.04% cells in WT. **(D)** The absolute cell number of $CD4^+lin^-$ cells was 19 fold increased in H-IL-7 as compared to WT LN. (n=5 mice per group, **p<0.05, Student *t* test)

H-IL-7 mice were used as a source for isolating and characterizing adult $CD4^+CD3^-$ cells. Adult $CD4^+CD3^-$ cells expressed IL-7Rα, CD18, c-Kit, β1 integrin, α4β7 integrin, CD44 and

were negative for CD62L (Fig. S2A). Furthermore, they expressed LTαβ, tumor necrosis factor related activation-induced cytokine (TRANCE) and TRANCER (Fig. S2B). Transcripts for RORγt were found in adult $CD4^+CD3^-$ cells (Fig. S2C). Hence, freshly-isolated fetal and adult cells share a common phenotype *in vivo* (Table I). Our data add new information to a previous study demonstrating that *in vitro* cultured fetal and adult $CD4^+CD3^-$ cells display similar genetic fingerprints (16). In contrast to a previous report (17) we were unable to detect significant levels of OX40L and CD30L in adult $CD4^+CD3^-$ cells (Table I). This discrepancy may rely on the fact that in our study we investigated freshly isolated cells whereas in other studies, adult $CD4^+CD3^-$ cells were cultured overnight before testing.

Surface antigen	Expression	
	Newborn	Adult
CD45	+	+
IL-7Rα	+	+
c-Kit	+	+
Sca-1	+	-
β1	+	+
α4β7	+	+
ICAM-1	+	+
CXCR4	+	+
CD18	+	+
CD44	+	+
CD62L	-	-
LTαβ	+	+
TRANCE	+	+
TRANCER	+	+
CD30L	-	-
OX40L	-	-

Table I. This table shows the surface expression profile of fetal and adult LTi cells in H-IL-7 mice.

To investigate the function of adult $CD4^+CD3^-$ cells, we adoptively transferred them into $CXCR5^{-/-}$ newborn mice. $CXCR5^{-/-}$ mice lack almost all PPs unless neonatally reconstituted with fetal LTi cells from WT origin (19). Hence, this assay was established to monitor the functional activity of LTi cells in the gut. Recipient mice were analyzed 3 weeks after adoptive transfer by anti-VCAM-1 immunohistochemistry of the whole intestine. Here, we show that PP numbers in $CXCR5^{-/-}$ mice were restored in direct proportion to the number of transferred adult $CD4^+CD3^-$ cells (Fig. 2). 10^6 total splenocytes from WT animals were unable

to restore PP formation in CXCR5$^{-/-}$ mice suggesting that among this number of cells, insufficient LTi cells were present. These data demonstrate for the first time that CD4^{+}CD3^{-} cells from adult mice are *bona fide* LTi cells. In a previous study it was shown that adult CD4^{+}CD3^{-} cells help reorganize the splenic architecture after LCMV infection (15). This effect appeared to be mediated by accelerated restoration of the splenic stromal cell compartment. In addition, a role of adult CD4^{+}CD3^{-} cells in creating organized T/B segregation in the adult spleen was proposed (14). Our study clearly shows that adult LTi cells can mediate the *de novo* generation of lymphoid tissue. In line with this, H-IL-7 mice developed not only ectopic LNs and additional PPs but also formed tertiary lymphoid organs in non-lymphoid organs such as salivary glands (unpublished observations). Altogether, adult CD4^{+}CD3^{-} cells have lymphoid tissue-inducing activity, which may contribute not only to lymphoid tissue organization but also to the neoformation of secondary and tertiary lymphoid organs.

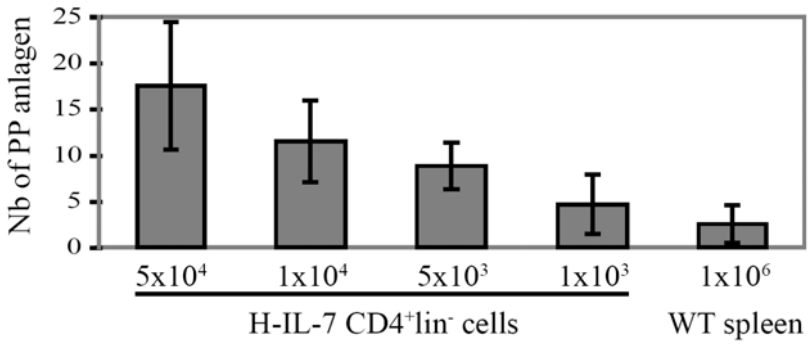

Figure 2. Adult CD4^{+}lin^{-} cells function as LTi cells. Various numbers of sorted LTi cells from H-IL-7 adult mice were i.v. transferred into CXCR5$^{-/-}$ newborn mice. As controls, 1x10^{6} WT splenocytes were injected. 3 weeks after transfer, the number of PPs was determined by anti-VCAM-1 immunohistochemistry of serial frozen sections (n=3 mice per group).

Adult LTi cells are strictly dependent on IL-7 and RORγt

In order to test if adult LTi cells and their precursors respond to IL-7, we treated WT and IL-7$^{-/-}$ mice with IL-7 or with a combination of IL-7 plus anti-IL-7 mAb M25. IL-7/anti-IL-7

complexes have been shown to display 50- to 100-fold higher biological activity than free IL-7 (18). In line with this, within 7 days, the *in vivo* administration of IL-7/anti-IL-7 complexes significantly increased splenic LTi numbers in WT (Fig. 3A) and IL-$7^{-/-}$ (Fig. 3B) mice. IL-7 treatment alone had no significant effect. Under steady state conditions, splenic $CD4^{+}CD3^{-}$ cell numbers in WT and IL-$7^{-/-}$ mice were comparable. Because IL-$7^{-/-}$ mice have no peripheral LNs, LTi cells mainly accumulated in the spleen. Our *in vivo* data prompted us to speculate that IL-7 increased the number of adult LTi cells through supporting survival and/or proliferation. Indeed, survival of sorted splenic LTi cells cultured for 8 days was reduced by around 50% in the presence of neutralizing anti-IL-7R mAb (Fig. 3C). IL-7 had a slight effect on the proliferation of LTi cells *in vitro (*Fig. 3D) and after adoptive transfer into $RAG^{-/-}\gamma c^{-/-}$ mice *in vivo* (Fig. 3E). In $RAG^{-/-}\gamma c^{-/-}$ mice, adoptively transferred LTi cells proliferated vigorously even when no IL-7/anti-IL-7 complexes were injected. It is likely that in $RAG^{-/-}\gamma c^{-/-}$ mice, elevated levels of IL-7 and/or additional factors supported their *in vivo* proliferation, which was increased after treatment with IL-7/anti-IL-7 complexes. Overexpression of either IL-7 or bcl-2 did not rescue LTi numbers in the spleen of adult ROR$\gamma^{-/-}$ mice, thus demonstrating their strict dependence on RORγt (Fig. 3F). Additionally, we also demonstrated that the amount of IL-7 was able to regulate the pool of adult LTi cells.

Fetal LTi cells have been reported to originate from multilineage FL precursor cells (21, 22) whereas, postnatally, the BM is the major site of hematopoiesis. Although BM chimera experiments have given indirect evidence that the BM harbours cells, or precursors, which can help organizing the spleen, clear data about the generation of peripheral $CD4^{+}CD3^{-}$ LTi cells from BM are still missing. In order to test if IL-7 can promote the generation of adult $CD4^{+}CD3^{-}$ LTi cells from BM cells, we transplanted BM from adult WT (Ly5.1) mice into irradiated IL-7R$\alpha^{-/-}$ (Ly5.2) recipients and treated the mice with IL-7/anti-IL-7 complex or left them untreated. As controls we injected FL cells from E13.5 (Ly5.1) WT mice into

irradiated IL-7R$\alpha^{-/-}$ (Ly5.2) recipients. As expected, in control mice receiving FL cells, a significant percentage of donor LTi cells (4.7 percent of lin^- donor cells) was found in the spleen whereas donor LTi cells from WT BM were almost undetectable (0.8 percent of lin^- donor cells) (Fig. 4). IL-7/anti-IL-7 complex treatment, however, significantly enhanced the generation of LTi cells (2.7 percent of lin^- donor cells) from the BM. Mixed chimera experiments confirmed that FL cells were almost 10-fold more efficient at generating LTi cells as compared to BM cells (data not shown). This is likely a result of a stronger proliferation of fetal hematopoietic cells (23). As previously shown, administration of BrdU to pregnant mice labelled fetal LTi cells (10). Seven weeks after continuous treatment of pregnant mice (E12.5-E19.5) with BrdU, all LTi cells in the offspring had lost BrdU, whereas 20% of maternal LTi cells were $BrdU^+$ (data not shown). Therefore, LTi cells in fetal and adult mice differ in their proliferative status.

Collectively, our data show that $CD4^+CD3^-$ cells from adult mice are *bona fide* LTi cells and that increased availability of IL-7 enlarges the adult LTi cell pool through survival and proliferation of pre-existing LTi cells and the *de novo* generation of LTi cells. *In vivo*, LTi cells develop from BM precursors relatively inefficiently unless additional IL-7 is provided. Considering the role of LTi cells in normal and ectopic lymphoid tissue development (10), adult LTi cells may play an important role in chronic inflammatory diseases such as rheumatoid arthritis, where local IL-7 availability is increased (24).

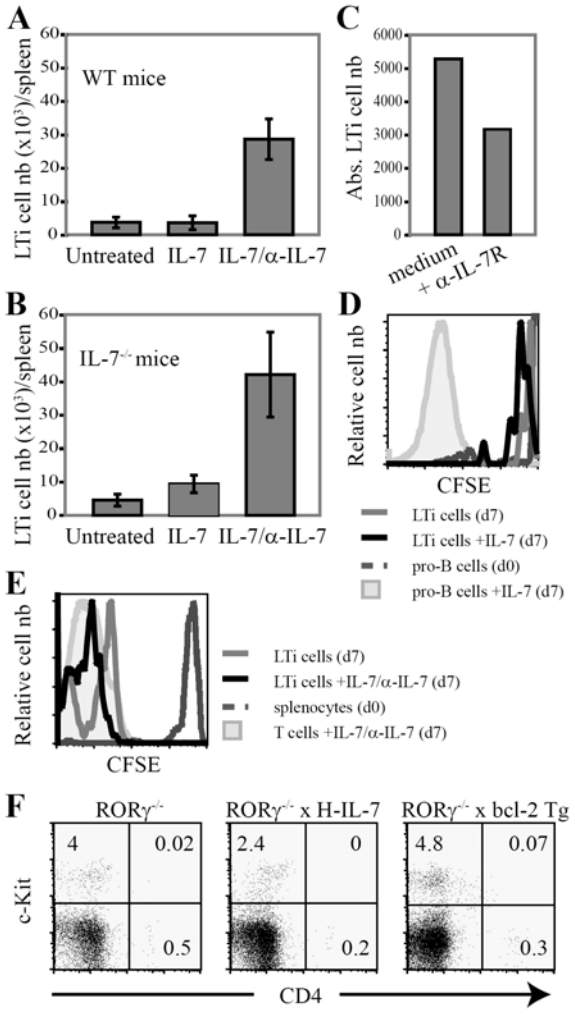

Figure 3. LTi cells are enriched in mice treated with IL-7/anti-IL-7 complexes. 6-8 wk old **(A)** WT or **(B)** IL-$7^{-/-}$ mice were either left untreated, treated with IL-7 or with IL-7/anti-IL-7 complexes. 7 days later, LTi cell numbers were significantly higher in treated than in untreated mice. Data are representative of 2 independent experiments with at least 5 mice per group. **(C)** LTi cells isolated from H-IL-7 spleen were cultured for 8 days without or with neutralizing anti-IL-7Rα Ab. **(D)** CFSE-labelled LTi cells or pro-B cells from WT mice were cultured for 7 days with or without IL-7. **(E)** CD4-enriched WT splenocytes were CFSE-labelled and injected into irradiated $RAG^{-/-}\gamma c^{-/-}$ mice. Mice were either left untreated or treated with IL-7/anti-IL-7 complexes and analyzed 7 days later. **(F)** Flow cytometry of splenocytes from $ROR\gamma^{-/-}$, $ROR\gamma^{-/-}$ x H-IL-7 and $ROR\gamma^{-/-}$ x bcl-2 transgenic mice. Cells were stained with a lin-cocktail, c-Kit and CD4. Gated on lin^- cells, the percentage of CD4 and c-Kit-expressing cells is shown. The density blots are representative for 3 mice per group.

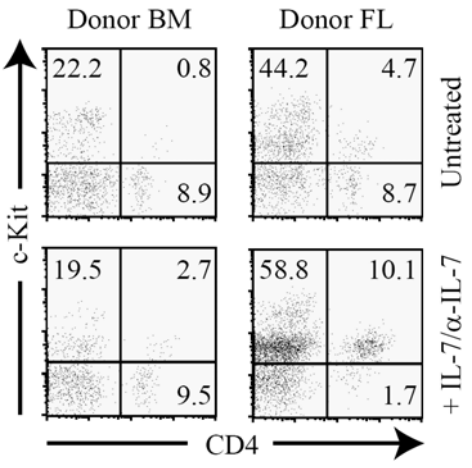

Figure 4. BM and FL can give rise to LTi cells *in vivo*. $1.5x10^6$ Ly5.1 BM cells or $5x10^5$ Ly5.1 FL cells were injected into irradiated IL-$7R\alpha^{-/-}$ recipients either left untreated or treated with IL-7/anti-IL-7 complexes. 3 weeks later, splenocytes were analyzed by flow cytometry. Shown are representative data of 3 mice after gating on lin^- donor splenocytes.

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Disclosures

Except for O.B., who is a shareholder in Nascent Biologics Inc., the other authors have no conflicting financial interests.

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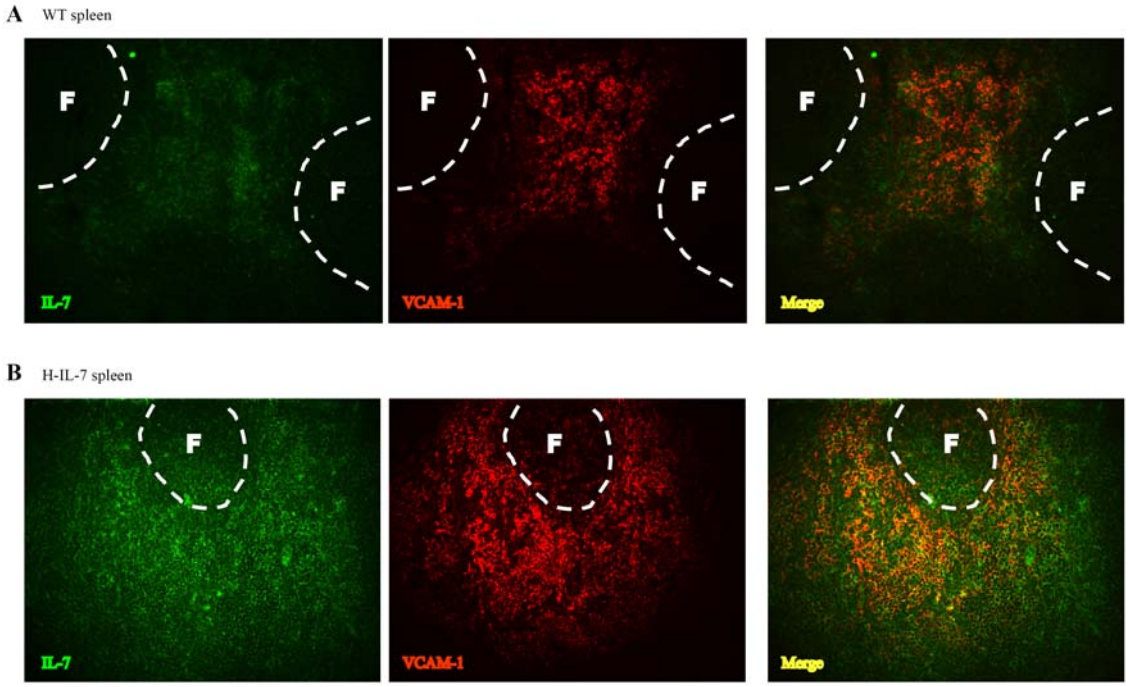
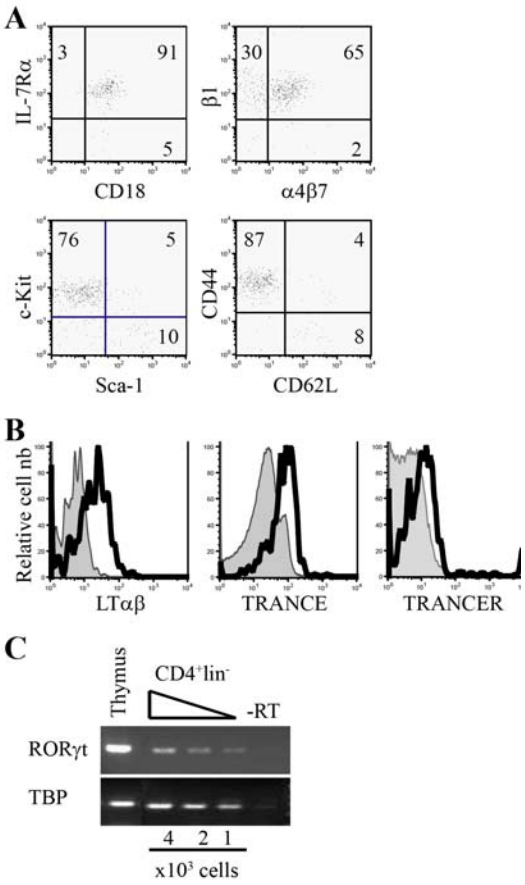
Supplementary Figures



Supplementary Figure 1. IL-7 is expressed by VCAM-1$^+$ cells in the splenic red pulp. Immunofluorescence of frozen spleen sections from 6-8 wk old **(A)** WT and **(B)** H-IL-7 mice using goat anti-mouse IL-7 Ab and rat anti-mouse VCAM-1 Ab. IL-7 was detected with an Alexa Fluor 488 donkey anti-goat IgG Ab (green) and VCAM-1 with a Cy3-conjugated goat anti-rat IgG Ab (red). F = Follicle.



Supplementary Figure 2. Adult CD4$^+$lin$^-$ cells share similar phenotype with fetal LTi cells. (A) Flow cytometry analysis of H-IL-7 adult spleen showed that CD4$^+$lin$^-$ cells expressed IL-7Rα, CD18, β1 and α4β7 integrins, c-Kit and CD44. **(B)** Gated on CD4$^+$lin$^-$ cells, all cells expressed LTαβ, TRANCE and TRANCER (bold line). Negative controls (filled) are LTα$^{-/-}$ splenocytes (left panel), CD19$^+$ B cells (middle panel) and CD4$^+$ T cells (right panel). **(C)** RT-PCR of sorted CD4$^+$lin$^-$ cells from spleen and thymocytes from adult H-IL-7 mice showed expression of RORγt. Serial dilutions of cDNA were performed and the house keeping gene TATA box binding protein (TBP) was used as internal control.

*3.4. Manuscript 3**

Increased availability of IL-7 triggers tertiary lymphoid tissue development in mice

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*This manuscript is in preparation

Increased availability of IL-7 triggers tertiary lymphoid tissue development in mice

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Abstract

The development of tertiary lymphoid organs (TLOs) often occurs at sites of chronic infection and inflammation. Until now, the mechanism for TLO development has not been solved. Here, we show that IL-7 overexpression in mice (H-IL-7 mice) leads to the formation of diffuse and organized TLOs in various non-lymphoid organs such as salivary gland and lung. The presence of naive follicular B, T and dendritic cells in TLOs correlates with the expression of the homeostatic chemokines CXCL13 and CCL21. Studies in LT$\alpha^{-/-}$ and ROR$\gamma^{-/-}$ mice reveal that the organization of TLOs into B and T cell compartments, but not the generation of diffuse TLOs, may require the presence of LTβ-expressing LTi cells. Furthermore, the infection with Leishmania major triggers the *de novo* formation of ectopic lymph nodes (LNs) in H-IL-7 but not in WT mice, thus demonstrating a synergistic effect of inflammation and IL-7 in TLO development. Altogether, we firstly showed that IL-7 regulates the development of TLOs and secondly, that IL-7 and inflammation collaborate in *de novo* formation of ectopic LNs.

Introduction

During embryogenesis, the development of secondary lymphoid organs (SLOs), such as LNs and Peyer's patches (PPs), results from interactions between $CD45^+CD4^+CD3^-$ lymphoid tissue inducer (LTi) cells and vascular cell adhesion molecule 1 $(VCAM\text{-}1)^+$ mesenchymal organizer cells (1). In mice lacking LTi cells, such as RORγ- or Id2-deficient mice, the formation of LNs and PPs is absent (2, 3). LTi cells express lymphotoxin (LT) αβ (4), which is required for LN and PP development (5-7). The signalling via the lymphotoxin β receptor (LTβR) expressed by the organizer cells leads to the activation of the NFκB pathway and the consecutive production of the homeostatic chemokines CXCL13, CCL19 and CCL21 (8). These chemokines control lymphocyte recruitment and localization in segregated compartments of lymphoid organs (9).

In human chronic inflammatory diseases such as Sjögren's syndrome, Hashimoto thyroiditis, rheumatoid arthritis and multiple sclerosis the accumulation of lymphocytes in the inflamed organ often leads to the development of tertiary lymphoid organs (TLOs). In addition, chronic infection and chronic graft rejection trigger lymphoid neogenesis in non-lymphoid organs (10, 11). The target organ undergoes complete remodelling with lymphoid stroma producing chemokines, thereby recruiting lymphocytes (12). The TLO microarchitecture contains some of the hallmarks of SLOs, such as B and T zone segregation, germinal centres (GCs) and high endothelial venules (HEVs). Mouse models with tissue-specific overexpression of chemokines have shown that chemokines, by controlling the influx of lymphocytes, can induce the generation of TLOs (12, 13). For example, LTα expressed under the control of the rat insulin promoter (RIP) leads to the formation of inflammatory lesions in the pancreas and in the kidney (14), suggesting that LTα might play a pivotal role in TLO development (15). Tg mice with expression of CCL21 under the control of the thyroglobulin promoter develop ectopic organized lymphoid tissues in the thyroid gland. In

this model, LT-expressing $CD4^+$ T cells seem to be more important than B cells to initiate lymphangiogenesis (16). In some cases, lymphoid chemokines are expressed in inflamed tissues that do not have yet organized lymphoid tissue (17, 18), suggesting that these chemokines are induced by inflammation prior to the formation of segregated B and T cell areas. Early expression of homeostatic chemokines may be independent of LTαβ or TNF, as seen in influenza-infected lungs of $LT\alpha^{-/-}$ mice (19).

Whether LTi cells play a role in TLO formation is still unknown. More than ten years ago, an accumulation of $CD4^+CD3^-$ cells has been observed in the spleen and liver of mice infected with helminth parasite (20). In a study using RIP-BLC tg mice, $CD4^+CD3^-$ LTi cells were the first hematopoietic cells recruited to the islets (12), suggesting that LTi cells may be involved in TLO development. However, recent studies using mice expressing CCL21 in the thyroid gland show that the first cells appearing in the thyroid gland are $CD4^+$ T cells and not LTi cells. Moreover, in CCL21 tg x $Id2^{-/-}$ mice, TLO formation was not affected (21), demonstrating that TLO development in the thyroid gland does not require LTi cells.

Little is known about the role of Interleukin 7 (IL-7) and IL-7 receptor (IL-7R) α in TLO development and autoimmune diseases. In patients with rheumatoid arthritis, ectopic lymphoid follicle formation coincides with increased expression of IL-7 and IL-7Rα/IL-2Rγ (22), suggesting that IL-7 might be important for TLO formation. The IL-7R pathway might be involved in the development of multiple sclerosis (23, 24) as the progression of the disease positively correlates with concentrations of IL-7 in the serum and the synovial tissue (18, 25). In patients with Sjögren's disease, the development of lymphocyte infiltrates in the salivary gland is associated with increased mRNA expression of IL-7 (26). Altogether, these results suggest that IL-7 may play a role in TLO development in organs affected by autoimmune diseases. We have previously shown the importance of IL-7 for LN and PP development. IL-7-deficient mice lack some peripheral LNs and IL-7 overexpressing mice develop additional

LNs and PPs (27). While data from clinical studies suggest a link between IL-7 availability and TLO formation, it is unclear whether increased availability of IL-7 is responsible for the formation of TLOs.

Here we show that IL-7 overexpression leads to a massive generation of TLOs in the salivary gland, liver, pancreas, stomach and lung. The expression of homeostatic chemokines in the salivary gland, probably leading to the influx of T and B lymphocytes, might trigger TLO formation. Adult LTi cells and B cells are the major producers of LTαβ in these mice. Both populations are increased in numbers and are found in lymphoid infiltrates of the salivary gland, suggesting implication of both subsets in the formation of TLOs. Finally, we show that infection with Leishmania major leads to TLO formation and increase in LTi cell number.

Altogether, these data show that the increased availability of IL-7 regulates the development of TLOs, and that upon infection, overexpression of IL-7 induces the *de novo* formation of lymphoid tissue, probably by increasing the pool of LTi cells.

Results

TLOs develop in non-lymphoid organs of IL-7 tg mice. To test if increased availability of IL-7 induced the development of TLOs, we analyzed non-lymphoid organs from mice expressing IL-7 transgene under the control of the Srα and I-E promoter (H-IL-7 mice (27)). In H-IL-7 but not in wild type (WT) or CIITA tg mice, we found several TLOs in the salivary gland (Fig. 1A, B and data not shown), the liver, pancreas, stomach, lung and brain (Supplementary fig. 1 and data not shown). TLOs were organized in segregated B and T cell regions (Fig. 1C) containing $PNAd^+$ high endothelial venules (HEVs) (Fig 1D). TLOs were surrounded by LYVE-1^+ lymphatic vessels (Fig. 1D) indicating that they were connected to the lymphatic vasculature. In addition, we found diffuse perivascular and periductal infiltrates composed of B, T cells and dendritic cells (DCs) (Fig. 1E, F). Of note, in these diffuse perivascular TLOs, $CD4^+CD3^-CD11c^-$ cells were detectable in close association with DCs (Fig. 1F and data not shown). We have previously reported that fetal and adult $CD4^+CD3^-$ cells isolated from H-IL-7 mice are functional LTi cells ((28) and Schmutz, in revision). To better understand the relative contribution of LTi, B and T cells to TLO development, we analyzed the salivary glands of H-IL-7 mice 2 days, 2 and 4 weeks after birth. B cells entered the salivary gland of H-IL-7 mice around 2 weeks after birth (Fig. 1Gii), whereas LTi cells were already present by day 2 (Fig. 1Hi), their number increasing with time (Fig. 1Hii, Hiii). Organized lymphoid tissue with B and T cell zones did not develop before 4 weeks of age (Fig. 1Giii). These data strongly suggest that LTi cells colonized non-lymphoid organs before B and T cells and before segregation into B and T cell zones had occurred.

Lymphocytes in TLOs of H-IL-7 mice are follicular-like B cells and naive T cells. To study if TLOs were composed of cellular subsets typically found in SLOs, we analyzed single cell suspensions from total salivary glands of H-IL-7 and WT control mice. Around 60% of

the $CD11c^+$ DCs isolated from the salivary gland of H-IL-7 mice were $CD8\alpha^-$ $CD11b^+$ DCs and around 5% were $CD8\alpha^+CD11b^-$ DCs (Fig. 2A). $CD19^+$ B cells could be subdivided into $B220^{high}$ and $B220^{low}$ cells (Fig. 2B). $B220^{high}$ B cells were mainly follicular $CD23^+CD21^+$ B cells (Fig. 2C). $B220^{low}$ B cells were likely short lived plasmablast B cells which preferentially migrate to secretory glands (29). Accordingly, we identified few plasmablasts in both H-IL-7 and WT control mice (Fig. 2B and data not shown) ruling out a role of these cells in TLO development. Of note, immunofluorescence staining showed IgA^+ B cells in H-IL-7 salivary glands, as well as in WT salivary glands (Fig. 2H and data not shown). However, these IgA^+ cells were different from B cells present in TLOs, which were negative for IgA (Fig. 2H). $CD4^+$ and $CD8^+$ T cells in the salivary gland of H-IL-7 mice expressed CD45RB and CD62L but were negative for CD25 and CD69 (Fig. 2D-G). This phenotype corresponds to naive T cells. Naive lymphocytes require CD62L to enter SLOs via HEVs. As shown before, HEVs were present in the TLOs of H-IL-7 salivary glands. Altogether our data indicate that SLOs and TLOs share the same subsets of hematopoietic cells, namely lymphocytes and DCs.

Lymphoid chemokines and inflammatory cytokines are produced in TLOs. SLO stromal cells produce chemokines, which specifically recruit naive B and T cells thereby generating organized B and T cell zones (9). Our findings suggested that in H-IL-7 mice, incoming B and T cells responded to chemokines secreted by TLO stromal cells. To study this, we determined the expression of CCL19, CXCL13 and lymphoid stromal marker in the salivary glands of H-IL-7 mice. Both chemokines were expressed at places where TLO development was observed (Fig. 3A, B). $Gp38^+$ stromal cells could be discriminated from $LYVE\text{-}1^+$ lymphatic and $CD31^+$ blood vessel endothelial cells (Fig.3C and data not shown). Altogether, these data suggest that ectopic chemokine expression in TLOs could contribute to

the entry and localization of B and T cells. In addition to lymphoid chemokines we observed that several cytokines implicated during inflammatory responses were expressed at a higher level in H-IL-7 salivary glands compared to WT (Fig.3D). For example, IL-1α and IL-1β, which are pro-inflammatory cytokines, were more expressed in H-IL-7 salivary glands than in WT. IL-17, IL-23 and IL-27 are pro-inflammatory cytokines and were also induced in H-IL-7 mice, as well as IL-16 and CCL5, which play a role in recruiting leukocytes to inflammatory sites. Surprisingly, IL-7 expression was low in H-IL-7 salivary gland.

Altogether, these data show that H-IL-7 mice develop TLO and provide an environment that favours inflammatory reactions in non-lymphoid organs.

Naive but not activated T cells home to TLOs. To test if naive or activated T cells migrate to TLOs in H-IL-7 mice, we adoptively transferred CFSE-labelled α-CD3-stimulated or freshly isolated T cells from OT-1 TCR tg mice. Mice were on a RAG-$2^{-/-}$ background to avoid any effect of endogenous TCR expression. 14 hours or 1 week after adoptive transfer, mice were sacrificed and sections of the spleen and salivary glands were analyzed by immunofluorescence for the presence of $CFSE^{+}$ donor cells (Fig. 4 and data not shown). Only naive donor T cells migrated to the sites of TLOs in the salivary gland of H-IL-7 mice (Fig. 4). Altogether, these data suggest that chemokines expressed in the salivary gland are able to recruit naive T cells.

LTαβ signalling is required for the organization of TLOs

To assess whether LTi cells determine the development of TLOs, we crossed H-IL-7 mice with ROR$\gamma^{-/-}$ mice. The development of tertiary lymphoid tissue was not affected in these mice (data not shown). This means that formation of tertiary lymphoid tissue can still occur in absence of LTi cells.

The activation of LTβR on stromal cells by LTαβ-expressing cells is required for SLO development (7). In order to test if LTβR–mediated signals were involved in the formation of TLOs, we crossed H-IL-7 mice on a $LT\alpha^{-/-}$ background. As shown in Fig 5A, TLOs represented as diffuse infiltrates around the ducts, were detectable in the salivary gland of H-IL-7 x $LT\alpha^{-/-}$ mice. These data demonstrate that IL-7 overexpression may be sufficient to induce the recruitment of lymphocytes to non-lymphoid organs, but that the formation of organized TLOs was dependent on LTαβ-expressing cells.

To identify the cells expressing LTαβ in the salivary gland of H-IL-7 mice, we analyzed single cell suspension from spleen of adult H-IL-7 mice. LTi cells and B cells clearly expressed LTαβ in H-IL-7 (Schmutz et al., in revision and Fig. 5B). Importantly, T cells from H-IL-7 mice were negative for LTαβ (Fig. 5B). Taken together, our findings suggest that both LTi cells and B cells could engage LTβR, which was crucial for the formation of organized TLOs.

In order to know whether LTi cells required the presence of lymphocytes to colonize the salivary glands of H-IL-7 mice, we crossed H-IL-7 mice to $RAG^{-/-}$ mice. By immunofluorescence, LTi cells were detectable in H-IL-7 x $RAG^{-/-}$ salivary gland sections (Fig. 5C), showing that the presence of LTi cells in the salivary glands was independent of lymphocytes.

TLOs develop at sites of Leishmania major infection in H-IL-7 mice. In humans, TLOs develop spontaneously at sites of chronic infection and inflammation (10). To test if IL-7 could trigger the development of TLOs in inflamed tissue, we infected susceptible BALB/c, B6 litter controls and H-IL-7 (B6) mice into the footpad with Leishmania (L) major. The increase in footpad scale relative to non-infected controls (referred to as ILS (inflammatory lesion size)) was determined 6 weeks after injection. In WT mice, the ILS was between 0.3 to

0.5 mm (data no shown) confirming previous data on the resistance of B6 mice against L. major (30). H-IL-7 mice, however, developed a significantly larger ILS as compared to WT controls (data not shown). Of note, LN-like TLOs developed next to the draining popliteal LN in H-IL-7 mice, but not in WT mice (Fig. 6A). Moreover, the size of the spleen increased with infection (Fig. 6A). Immunohistochemistry of TLOs revealed the formation of germinal centres (GCs), whereby size and number were comparable to GCs found in draining popliteal LNs of infected mice (Fig. 6B). The structure of the TLOs was comparable to draining WT and H-IL-7 LNs (Fig. 6C).

In addition, there was a significant increase in the absolute number of LTi cells in the draining LNs of infected WT and H-IL-7 mice versus non-infected controls (Fig. 7A). In contrast, the number of LTi cells in the spleen of infected mice was similar to non-infected mice (data not shown). From these data we can conclude that the s.c. infection with L. major induces the *de novo* formation of functional TLOs provided increased amount of IL-7 is available.

In the popliteal LN, the ratio between the percentages of $CD4^+$ and $CD8^+$ T cells were similar between non-infected and infected mice, regardless of the strain (Fig. 7B). But the total percentage of T cell decreased in WT mice as the percentage of $CD19^+$ B cells increased (Fig. 7B). Moreover, the absolute numbers of $CD4^+$, $CD8^+$ and $CD19^+$ cells increased in infected WT mice, as well as in infected H-IL-7 with only a minor increase in $CD19^+$ B cell numbers (Fig. 7C).

Discussion

The formation of TLOs is a hallmark of autoimmune diseases and has been observed in several mouse models. Overexpression of various lymphoid homeostatic chemokines, such as CXCL13 and CCL21, has been studied. These data clearly show the potential of these chemokines to induce TLO formation in non-lymphoid organs. However, the role of cytokines, such as IL-7 remained unknown. Here we studied the effects of systemic expression of IL-7 in an IL-7 overexpressing mouse model. In H-IL-7 mice, the development of TLOs was observed in non-lymphoid organs. Ectopic lymphoid tissues found in non-lymphoid organs were organized with B and T cell areas, probably segregated due to the expression of lymphoid chemokines. Moreover, TLOs formed in the salivary glands also contained LTi cells. Kinetic analysis of TLO development in the salivary gland of H-IL-7 mice showed that LTi cells colonized the salivary gland before lymphocytes. This happens accordingly to the development of LNs during early life, where LTi cells are the first cells colonizing the anlage, followed by B cells and later on T cells (31). Large numbers of LTi cells may enter peripheral organs followed by the induction of CXCL12 on the endothelium, leading to influx of B and T cells. Another possible scenario is that lymphocytes infiltrate non-lymphoid organs randomly and hence induce chemokine expression on stroma and endothelium followed by further influx and organization.

DCs were detected in TLO, where they might present self-antigens to T cells, thus triggering the development of ectopic lymphoid tissue, as proposed in a model of autoimmune diabetes (32). B cells found in the salivary gland of H-IL-7 mice had a follicular phenotype, although a population of plasmablast-like cells were found in H-IL-7 and WT salivary glands. $B220^{low}$ plasmablast-like cells found in the salivary gland of both H-IL-7 and WT mice were probably IgA-producing B cells, which are in steady-state conditions found in glands (29).

T cells with a naive phenotype were found in the salivary gland. Interestingly, the transfer of either naive or activated T cells revealed that only naive T cells migrated to the salivary glands to places where TLOs were already present.

Cell suspension of H-IL-7 salivary gland did not express IL-7, as shown by the cytokine array. However, by quantitative Real-Time PCR, we observed a significant increase in IL-7 mRNA level in H-IL-7 salivary glands compared to WT salivary glands (data not shown), suggesting post-transcriptional modifications. By immunofluorescence, we showed the presence of CXCL13 in TLOs. On the cytokine array, CXCL13 was low in H-IL-7 salivary glands. This can be due to a diluting effect. CXCL12, however, was more expressed. This corresponds to data we obtained by immunofluorescence. Indeed, in H-IL-7 salivary glands, CXCL12 was ubiquitously expressed associated with endothelial cells.

In H-IL-7 x ROR$\gamma^{-/-}$ mice, lacking LTi cells, TLO development was not affected. This means that IL-7 is sufficient to induce the neoformation of lymphoid tissue. However, since H-IL-7 x LT$\alpha^{-/-}$ mice only developed diffuse TLOs, LT$\alpha\beta$ signalling seems to be critically required for TLO organization. This signal could be provided by LTi or B cells, which are the two populations expressing LT$\alpha\beta$ in H-IL-7 spleen. In H-IL-7 mice, T cells do not express LT$\alpha\beta$, which is in contradiction with studies showing T and B cells expressing LT$\alpha\beta$ and playing a role in TLO formation. However, alternative ligands for LTβR, such as LIGHT for example, may be important for TLO organogenesis (33, 34).

TLO formation in H-IL-7 mice is similar to what happens in patients suffering from Sjögren syndrome (35). Sjögren syndrome is an autoimmune disease characterized by lymphocytic infiltrates in the salivary glands, followed by a decrease in the secretory response. Various mouse models have been used to study a particular aspect of Sjögren syndrome (reviewed in (36)). For example, in NOD mice, inflammatory reaction is observed, with B and T cell infiltrates in the salivary glands and a decrease in saliva flow (37). Human

studies on Sjögren syndrome patients show that the organization level of the salivary gland infiltrates correlated with chemokine expression (38).

In a model of parasite infection, we infected WT and H-IL-7 mice with L. major. Already two weeks after infection, H-IL-7 mice developed ectopic LNs in the region of the popliteal LN, which is the draining LN upon footpad infection with L. major. Moreover, the number of LTi cells significantly increased upon infection, in H-IL-7 mice, but also in WT mice. This increase could help regulating the infection. A recent study shows that during acute infection with Lymphocytic choriomeningitis virus (LCMV), the pool of LTi cells increases at the peak of infection, accelerating the restoration of the splenic architecture (39).

Altogether, this study shows that the ectopic overexpresson of IL-7 is able to induce the *de novo* formation of lymphoid tissue at ectopic sites. Moreover, overexpressing IL-7 could be a useful tool to study the effect of increased availability of a cytokine in the development of autoimmune diseases.

Methods

Mice

H-IL-7 mice were obtained by breeding heterozygous CIITA tg mice (40) with heterozygous IL-7 tg mice (41). OT-I tg mice were kindly provided by J-Kirberg. ROR$\gamma^{-/-}$ mice were kindly provided by D. Littman (2, 42). LT$\alpha^{-/-}$ mice were originally generated by De Togni et al. (42). RAG-/- mice were kindly provided by G.Holländer and J. Kirberg.

Antibodies

Abs either biotinylated or conjugated to fluorochromes (FITC, PE, PECy5, PeCy7 or APC) , purchased from BioLegend, eBioscience or BD Pharmingen, were used against the following mouse antigens: CD4 (clone: RM4-5 or H129), c-Kit/CD117 (2B8), CD3 (145-2C11), CD8 (53-6.7), TCR$\alpha\beta$ (H57-597), TCR$\gamma\delta$ (UC7-13D5), CD11c (N418), CD19 (6D5), B220 (RA3-6B2), NK1.1 (PK136), Gr-1 (RB6-8C5), TER119 (TER119), CD21, CD23 (B3B4, Biolegend), CD44 (IM7), CD122(TM-b1), CD25 (PC61), CD45RB (16A), CD62L (MEL-14), CD69 (H1.2F3), CD11b (M1/70), F4/80 (BM8), LTβR-Fc (gift from J. Browning), PNAd (MECA-79), Lyve-1 (polyclonal, RELIA Tech Gmbh), CD31 (*390), IgD (1.19), PNA (Vector Laboratories), CXCL13 (R&D), CCL19 (R&D), gp38 (R&D), VCAM-1 (429). Secondary Abs were streptavidin (purchased from BioLegend) conjugated to FITC, PE, PECy5, PECy7 or APC, Cy3-conjugated goat anti-rat IgG (Jackson ImmunoResearch), Alexa Fluor 488 donkey anti-goat IgG (Invitrogen), Alexa Fluor 488 goat anti-hamster IgG (Invitrogen), Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) and streptavidin Alexa 488 (Molecular Probes).

Immunohistochemistry and Immunofluorescence

For Haematoxylin Eosin staining, slides were stained in Haematoxylin (Medite) for 4 minutes, rinsed in tap water, incubated 5 seconds in 70%EtOH 1%HCl, rinsed in tap water for 10 minutes, stained in Eosin (Medite) for 10 seconds, briefly washed in tap water and finally dehydrated in EtOH 70%, 95% and 100%. Slides were incubated 5 minutes in xylenes (Sigma) before embedding in Pertex (Medite) for microscopical analysis.

For Immunofluorescence staining tissue samples were snap frozen in O.C.T Compound. 5 μm sections were fixed in acetone, blocked and incubated with primary Abs for 1 hour. After

washing, secondary Abs were added and incubated for 30 minutes. The slides were washed and mounted with Glycerol and 1,4-Diazabicyclo(2,2,2)octane (Sigma).

Flow cytometry

10^6 single cells derived from the spleen were resuspended in PBS containing 3% FCS and were stained with a mix of biotinylated or fluorochrome-conjugated Abs (see Ab section). Data were acquired using a FACScalibur (Beckton Dickinson & Co) and analysed with the FlowJo software (Tree Star).

Isolation of cells from the salivary glands

Salivary glands were isolated from anesthetized and PBS-perfused mice. They were cut in small pieces and incubated in 1 ml collagenase IV (10mg/ml, Sigma) for 1 hour at 37°C (agitating). Tissues were further homogenized with syringe and needle. The reaction was stopped by adding large volume of PBS 3% FCS. Cells were filtered before labelling with Abs for flow cytometry analysis.

Mouse Cytokine Array Panel A Array Kit (R&D)

This experiment was performed in agreement with the protocol given by R&D.

Salivary gland cell suspensions were obtained by digestion of the salivary glands with collagenase (see previous section).

Adoptive Cell Transfer

For adoptive transfer of CFSE-labelled OT-I-derived T cells, splenocytes from OT-I Tg mice were depleted for erythrocytes. Splenocytes were labelled with CFSE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester, Molecular Probes) prior to i.v. injection into either H-IL-7 or WT recipients. Spleen and salivary gland of the recipient mice were analyzed after either 14h or 1 week by flow cytometry and immunofluorescence.

Infection with Leishmania major

Mice were infected s.c. in both footpads and the lesion sizes were followed until analysis. Mice were sacrificed for long term experiment after 7-8 weeks.

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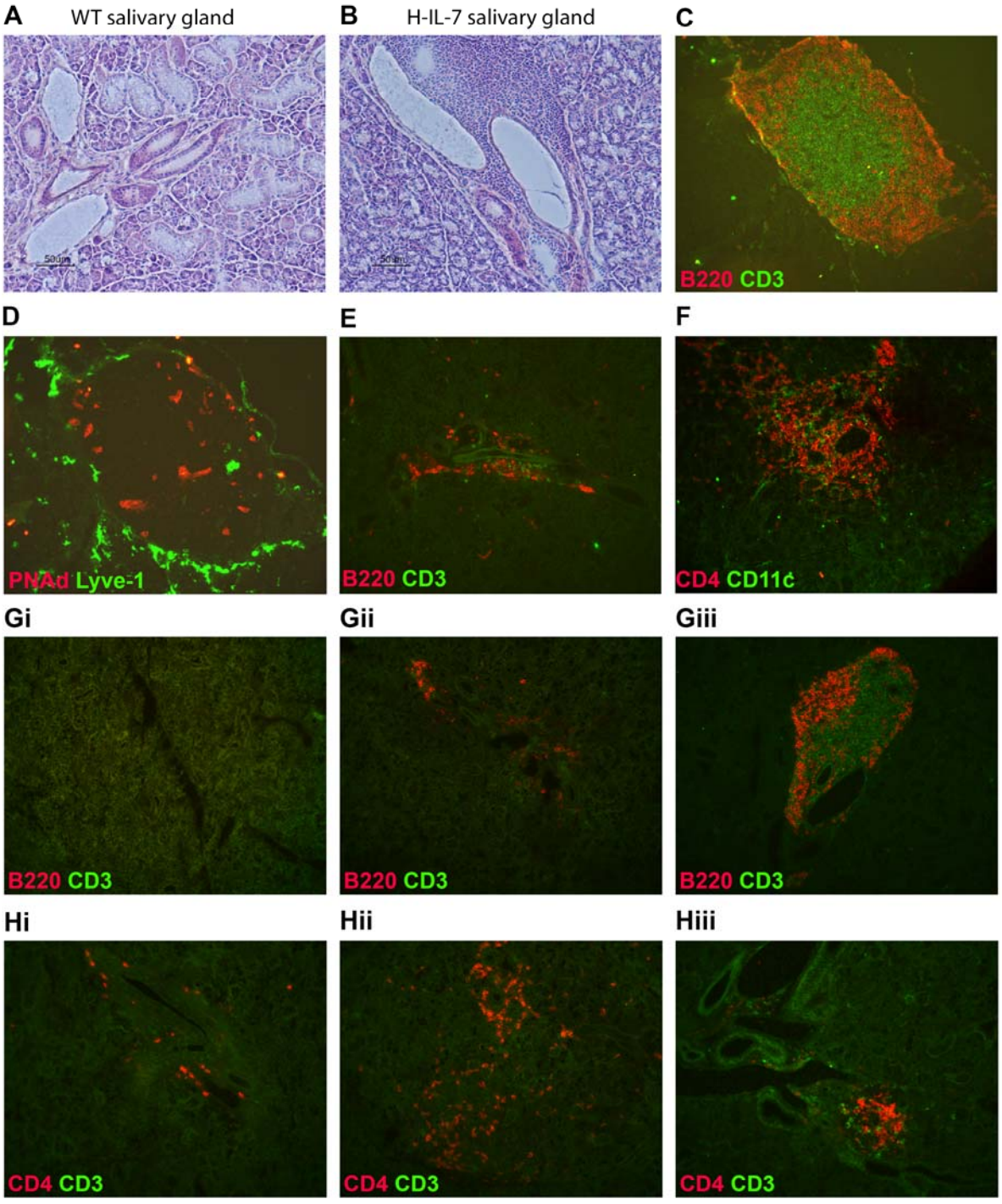


Figure 1. Organized TLO develop in the salivary glands of H-IL-7 mice. (A, B) The formation of lymphoid tissue is observed on frozen sections of H-IL-7 salivary gland, but not of WT mice. **(C)** TLOs contain B220$^+$ B cells and CD3$^+$ T cells organized in segregated areas. **(D)** PNAd$^+$ HEVs develop and Lyve-1$^+$ cells surround the newly formed lymphoid tissue. **(E, F)** Diffuse B and T cells, as well as CD11c$^+$ DCs, are found in the perivascular region of H-IL-7 salivary glands, CD4$^+$CD3$^-$CD11c$^-$ cells are also detected. **(G)** Lymphoid infiltrates, mostly B cells, are found in the salivary glands of H-IL-7 mice after 2 weeks of age **(Gii)**. After 4 weeks, they can form organized TLOs, with segregated B and T cell areas **(Giii)**. **(H)** In the salivary glands of 2 day-old H-IL-7 mice **(Hi)**, CD4$^+$CD3$^-$ cells are already present and their number increases with time **(Hii, Hiii)**.

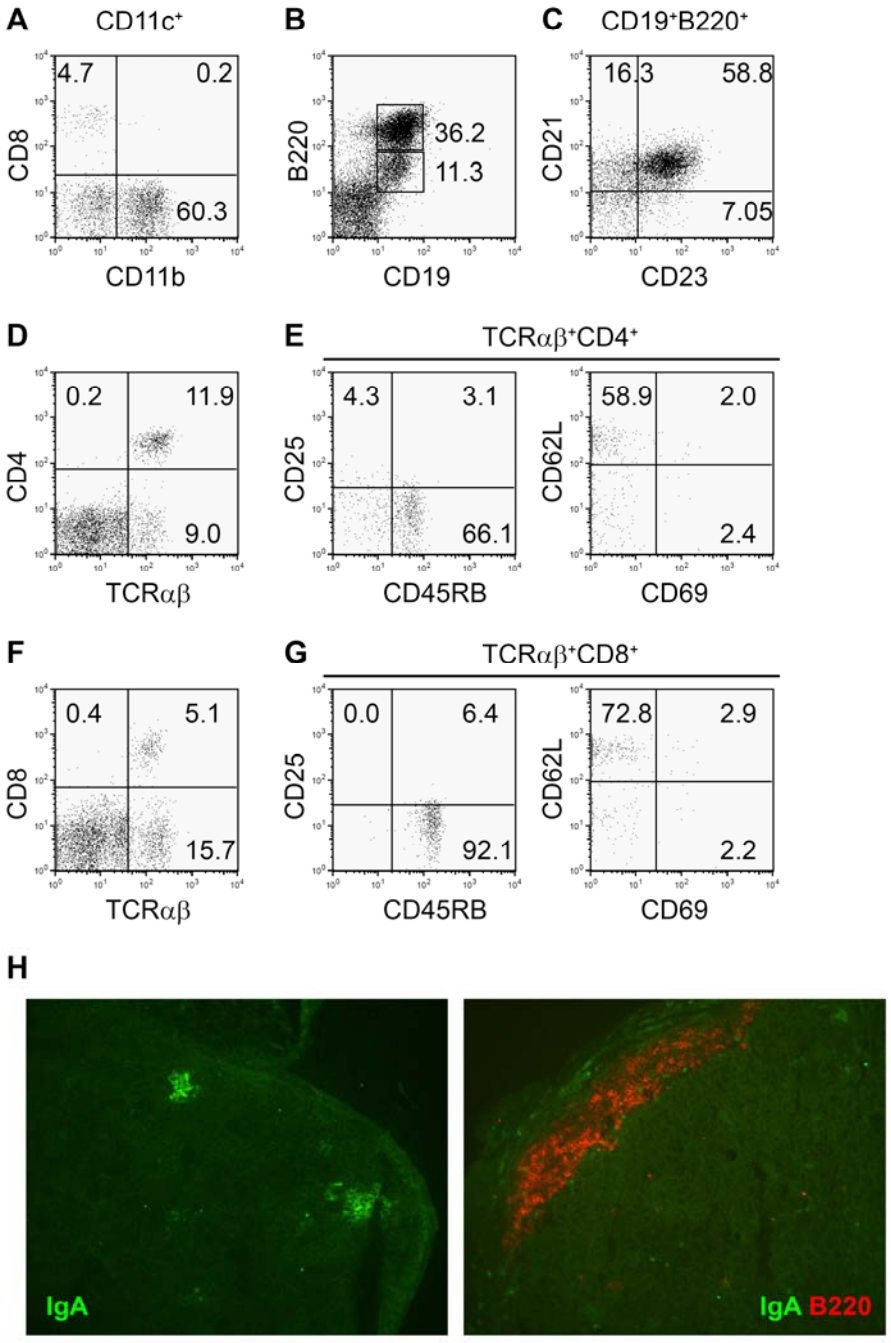


Figure 2. Follicular-like B cells and naive T cells are infiltrating the salivary glands of H-IL-7 mice. (A-G) Cell suspensions from the H-IL-7 salivary glands were analyzed by flow cytometry. **(A)** DCs were found to express more CD11b than CD8. **(B, C)** $CD19^+B220^+$ B cells were $CD21^+CD23^+$ follicular B cells. **(D-G)** Both $CD4^+$ and $CD8^+$ T cells were positive for CD45RB and CD62L, but negative for CD25 and CD69. **(H)** IgA^+ B cells are present in H-IL-7 salivary glands, but are different from $B220^+$ B cells present in TLOs.

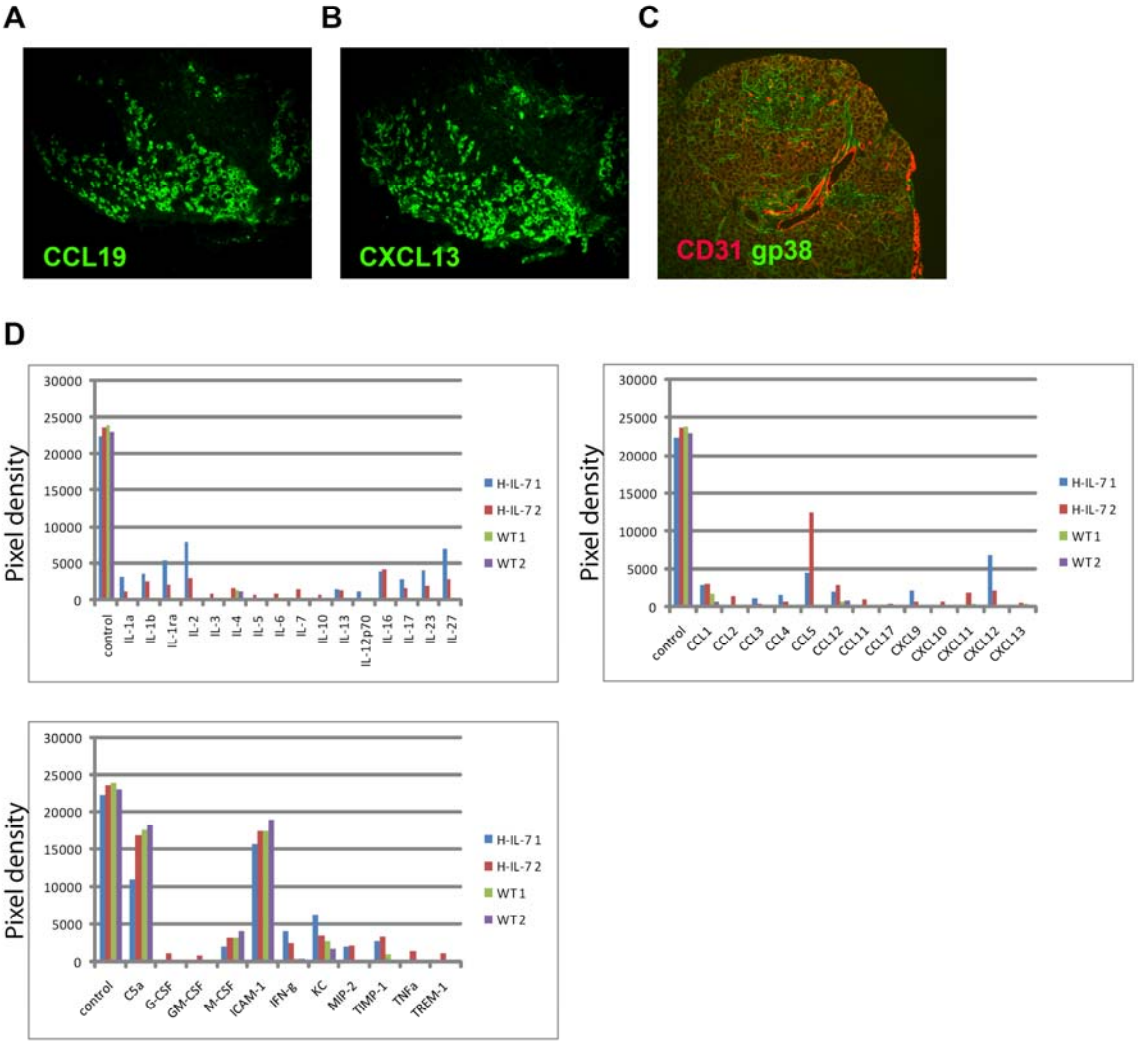


Figure 3. Homeostatic chemokines are produced at places where TLO develop. (A, B) The chemokines CXCL13 and CCL19 were detected in the salivary gland of H-IL-7 mice at places where lymphoid infiltrates develop. **(C)** Gp38^{+} lymphoid stromal cells are also present. **(D)** Cytokine array of cell suspensions obtained from H-IL-7 and WT salivary glands showed that mostly inflammatory cytokines were induced in H-IL-7 salivary glands.

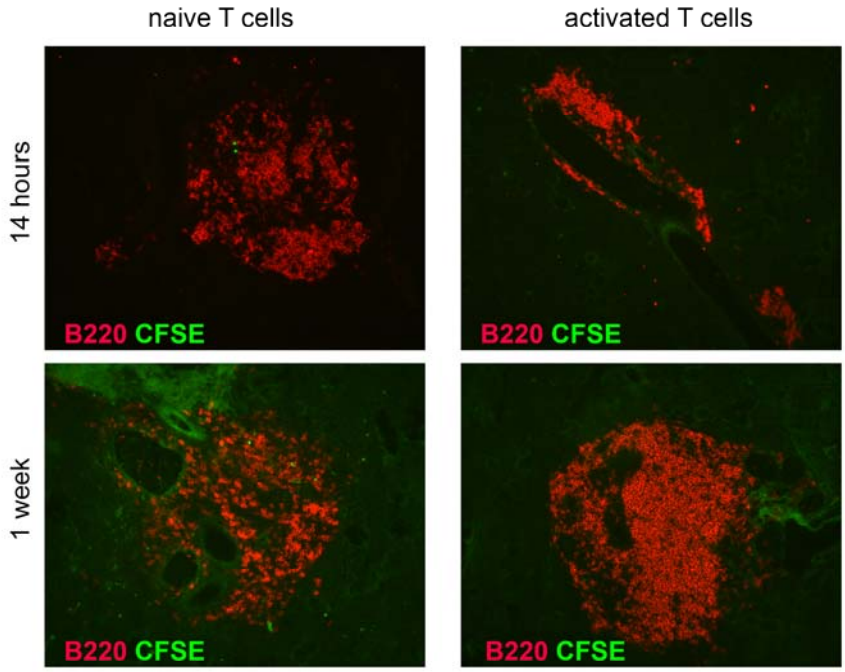


Figure 4. Only naive T cells are able to migrate to the salivary gland of H-IL-7 mice. Transfer of naive versus activated OT-I-derived T cells showed that only naive OT-I T cells could migrate to the salivary glands of H-IL-7 mice. These cells are found in the salivary gland at places where TLO were formed.

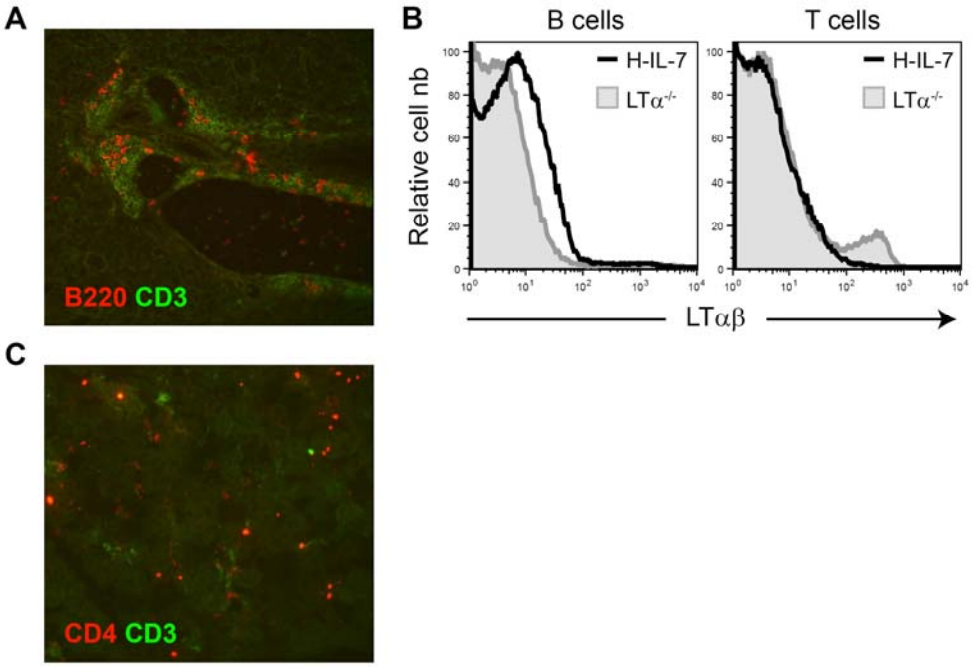


Figure 5. TLO formation is dependent on LTαβ. (A) Diffuse infiltrates of B and T cells are present in H-IL-7 x LTα$^{-/-}$ salivary glands. **(B)** Splenic B cells expressed LTαβ, whereas T cells were negative for LTαβ. **(C)** In H-IL-7 x RAG$^{-/-}$ mice, LTi cells are found in the salivary glands.

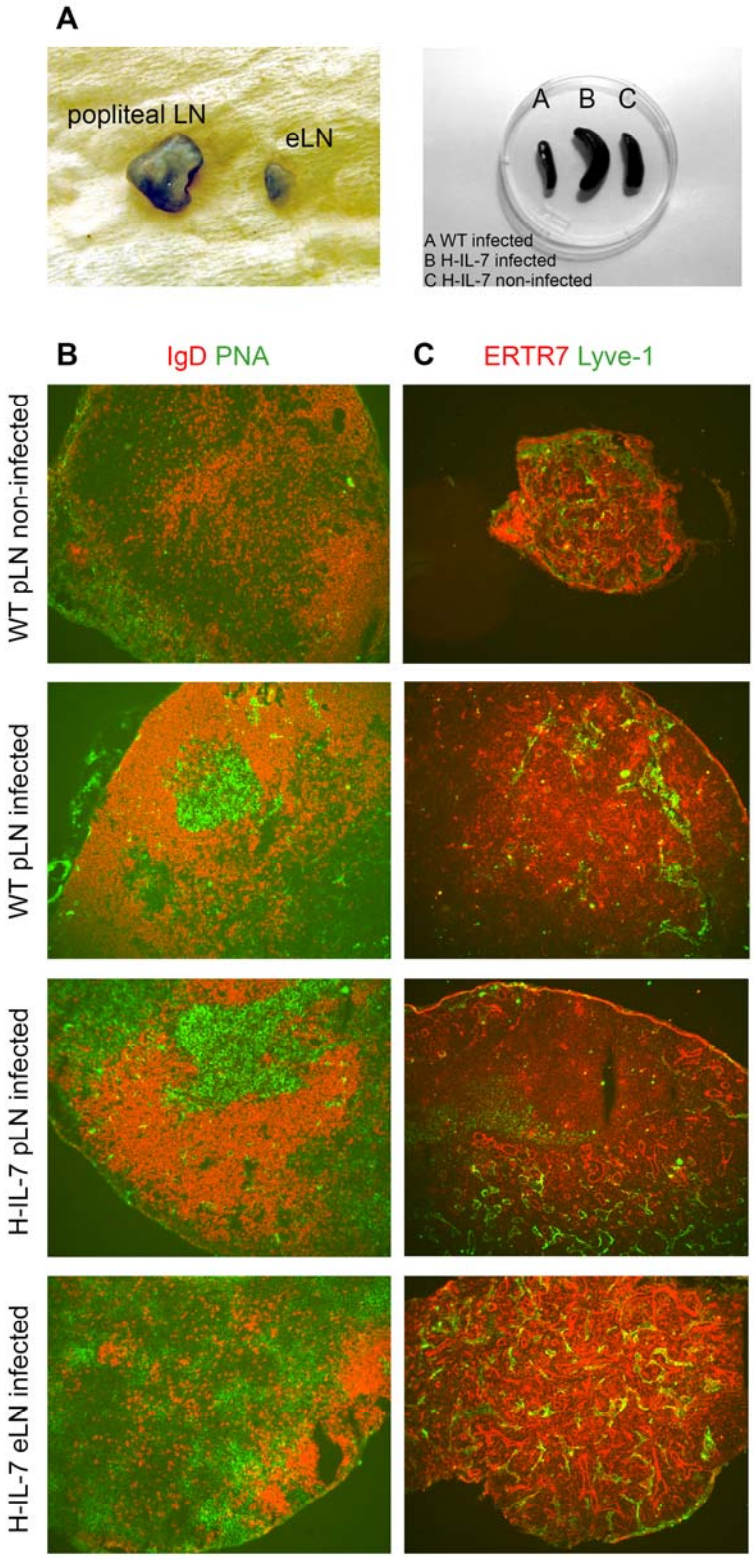


Figure 6. L. major infection induces the formation of additional LNs. (A) Infection of H-IL-7 mice with L.major induced the development of additional LNs in the popliteal region. The size of the spleen was increased in infected H-IL-7 mice. **(B)** Ectopic LNs, popliteal draining LNs from WT and H-IL-7 infected mice developed GCs, represented by $PNAd^{+}$ B cells. **(C)** The microarchitecture of ectopic LN is similar to popliteal LN.

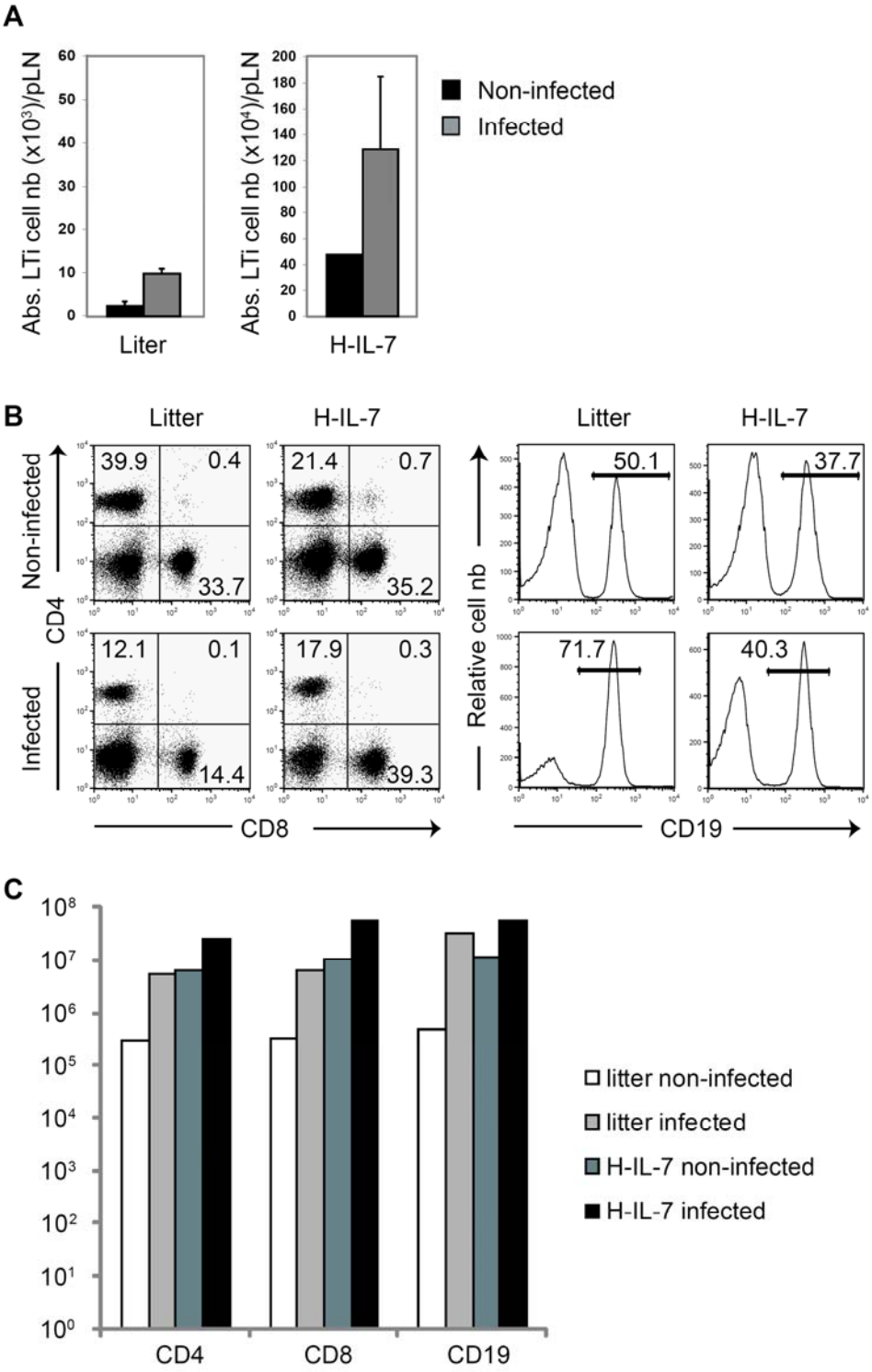
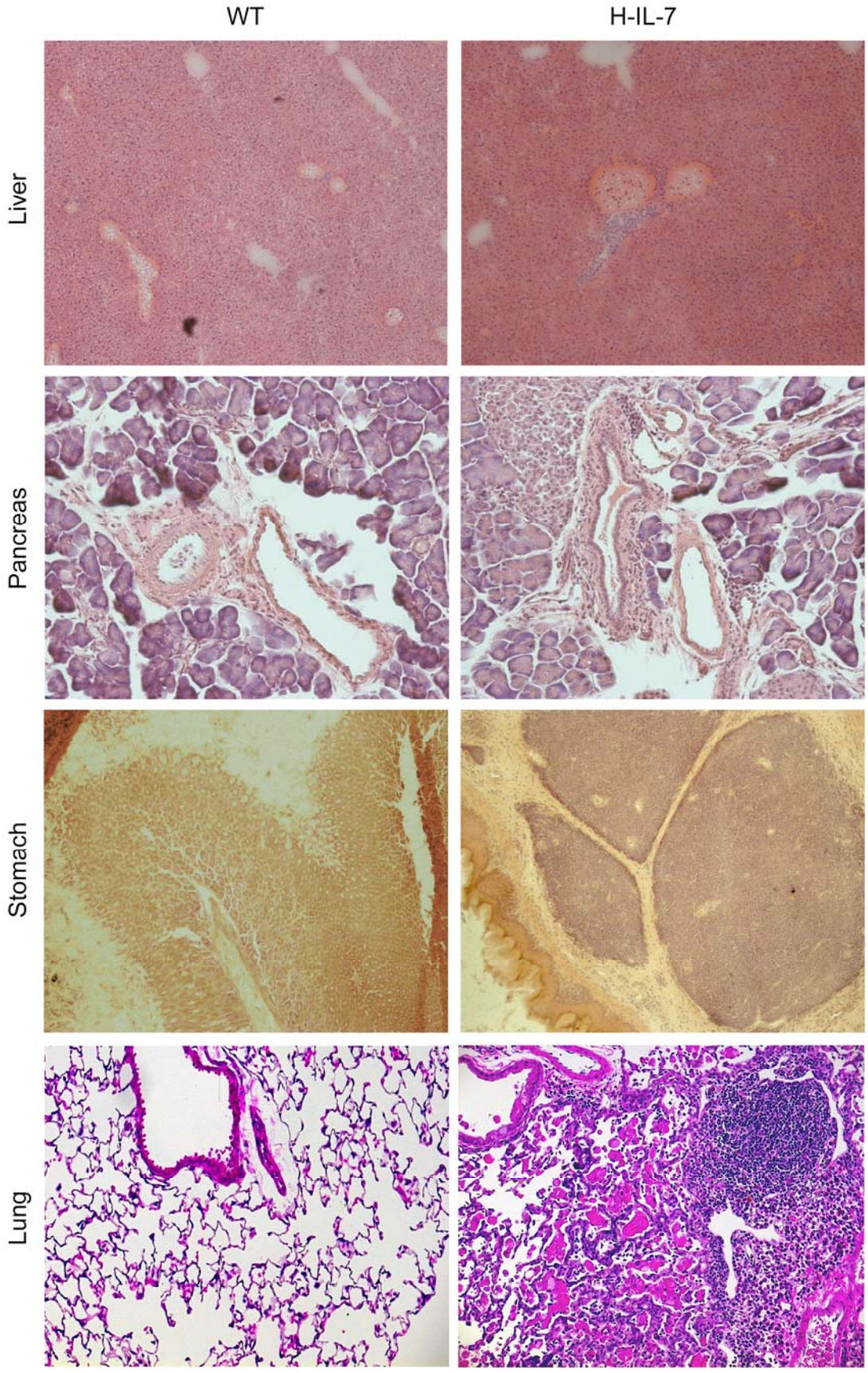


Figure 7. L. major infection increases LTi cell number. (A) Litter controls and H-IL-7 infected mice had higher absolute numbers of LTi cells in the popliteal LNs compared to non-infected mice. **(B)** The percentage of B cells in litter controls was increased upon L. major infection. The ratio between $CD4^+$ and $CD8^+$ cells remained constant. **(C)** Absolute numbers of $CD4^+$, $CD8^+$ and $CD19^+$ were increased upon L.major infection.



Supplementary figure 1. In H-IL-7 mice, TLOs were found in various non-lymphoid organs.

4. GENERAL DISCUSSION

Using a mouse model where an IL-7 transgene is expressed systemically, we have previously shown that increased availability of IL-7 induces the development of ectopic LNs and additional PPs (1). Furthermore, we showed that increased IL-7 regulated the number of LTi cells and induced higher expression of LT$\alpha\beta$ by LTi cells. In H-IL-7 mice, the effect of IL-7 overexpression was already detectable in the E14.5 embryo thereby increasing the number of LTi progenitors in the FL. We demonstrated that *in vitro*, IL-7 had a survival effect on $\alpha_4\beta_7^+$ FL progenitors and LTi cells. When we backcrossed H-IL-7 mice to ROR$\gamma^{-/-}$ mice lacking LTi cells or LT$\alpha^{-/-}$ mice, the development of PPs and ectopic LNs was blocked, speaking in favour of a LTi-dependent mechanism for ectopic follicle development.

4.1. Characterization of adult LTi cells in H-IL-7 mice

Fetal LTi cells express LT$\alpha\beta$, IL-7Rα, the chemokine receptors CXCR5 and CCR7, the adhesion molecules α4β1, α4β7 and ICAM-1. In this thesis work, I have characterized a population of CD4$^+$lin$^-$ cells in the spleen, LNs and PPs of adult H-IL-7 mice sharing the phenotype with fetal LTi cells, including the expression of RORγt. This is in agreement with previous studies describing LTi-like cells in the spleen of adult mice (21). In addition, LTi-like cells were found in the intestine of adult WT mice (35), where they were proposed to have a role in maintenance of mucosal immunity by organizing CPs and ILFs (35). Accordingly, RORγ^+lin$^-$ cells in ILFs were shown to be responsible for inducing a T cell-independent Ig class switch towards IgA (37). This study further demonstrated that the signalling through Toll-like receptors (TLRs) was crucial for the RORγ^+lin$^-$ cell-driven induction of IgA switching *in vitro*. However, the precise mechanism was unsolved, and it remains to be investigated if TLR ligands act on RORγ^+lin$^-$ cells directly or alternatively, have an effect on the stroma compartments, which in turn support RORγ^+lin$^-$ cells survival and function.

Previous studies by Lane and colleagues have proposed that LTi-like cells play a role during splenic immune responses (98). LTi cells were described as accessory cells for priming T cells via the expression of CD30L and OX40L. In our mouse model, however, adult LTi cells express neither CD30L, nor OX40L. This discrepancy

might be explained by the fact that in the study of Lane and co-workers, the expression of these molecules was detected upon overnight culture, whereas I exclusively analysed freshly isolated cells. In my study, a 30 fold increase in $CD4^+lin^-$ cell number was observed in H-IL-7 compared to WT mice suggesting that the increased availability of IL-7 had an effect on the generation and/or maintenance of $CD4^+lin^-$ cells in adult mice. I could further show that adult $CD4^+lin^-$ cells were able to induce lymphoid tissue formation upon transfer into $CXCR5^{-/-}$ mice lacking PPs, and were therefore *bona fide* LTi cells. As for transfer of neonatal LTi cells, PP-like follicles found in the small intestine of reconstituted $CXCR5^{-/-}$ mice were smaller than normal PPs in WT mice. In $CXCR5^{-/-}$ mice, B cells can colonize lymphoid tissue anlagen, but are unable to form mature follicles (99). This explains why PPs in $CXCR5^{-/-}$ mice reconstituted with LTi cells were smaller, and were lacking clearly segregated B cell follicles. It is likely that the co-transfer of WT BM would further improve the restoration of PP with mature B cell follicle formation in $CXCR5^{-/-}$ mice.

The data presented here clearly show that the *de novo* formation of PPs in $CXCR5^{-/-}$ mice depends on the number of adult LTi cells transferred. Comparing the efficiency of fetal versus adult LTi cells to induce PP development in $CXCR5^{-/-}$ mice, we found that fetal cells were at least 3 times more efficient than adult LTi cells. Indeed, 5×10^4 adult LTi cells could induce the formation of 16 $VCAM\text{-}1^+$ spots, whereas 2×10^4 fetal LTi cells were already sufficient to induce up to 33 $VCAM\text{-}1^+$ spots (17). The reason for this lower efficiency of the adult LTi cells in not known. It is possible that the homing properties of the LTi cells differ between fetal and adult life due to various expression levels of chemokine receptors or adhesion molecules. Alternatively, the adult intestine is not as accessible as the fetal gut for adoptively transferred cells. It has been published that splenic adult LTi-like cells express the same level of LTβ, TNFα and LIGHT (40). However, the level of chemokine receptor expression has not been tested. Finally, it is possible that the survival rate and requirements for growth factors of LTi cells differ between fetal and adult life.

Altogether, I have identified for the first time that LTi cells exist in SLOs of adult H-IL-7 mice, which share the phenotype and function with fetal LTi cells.

4.2. Effect of IL-7 on WT LTi cells

I could not exclude that due to the increased availability of IL-7, fetal LTi cells persisted throughout adult life. I therefore analyzed normal WT mice and $RAG^{-/-}$ mice for the presence of LTi cells. In WT spleen, a population of LTi cells was barely detectable, whereas in $RAG^{-/-}$ spleen, the number of LTi cells was significantly higher than in WT. One explanation is that due to the lack of IL-7-consuming mature T lymphocytes, the amount of total IL-7 protein was higher in $RAG^{-/-}$ mice, which lead to an increase in the absolute cell number of LTi cells.

In order to test if a pool of LTi cells existed in adult WT mice that could respond to IL-7, I treated WT mice with recombinant IL-7 in combination with anti-IL-7 Ab. Complexes of IL-7 and anti-IL-7 Ab have been shown to possess 50 to 100-fold more biological activity than IL-7 alone (83). Indeed, when mice were treated with IL-7 alone, no effect on the number of LTi cells was observed. However, mice treated with IL-7/anti-IL-7 Ab complexes displayed a significantly higher number of LTi cells. Interestingly, in $IL\text{-}7^{-/-}$ mice the number of LTi cells was also increased upon treatment with IL-7/anti-IL-7 Ab complexes, even more than in WT mice. $IL\text{-}7^{-/-}$ mice lack most of the peripheral LNs (1, 100). LTi cells might therefore accumulate in the spleen, whereas in WT mice, LTi cells are distributed amongst spleen, LNs and the MALT. This could explain why the effect of the treatment was more evident in the spleen of $IL\text{-}7^{-/-}$ than of WT mice. In addition, $IL\text{-}7^{-/-}$ mice have a severe defect in the generation of mature T cells, which could compete with LTi cells for growth and survival factors such as IL-7. Altogether, these data clearly show that there is a pool of LTi cells in adult WT mice that is responsive to IL-7. Furthermore, the data suggest that the amount of IL-7 available *in vivo* regulates the size of the LTi cell pool in adult mice.

From these data, I asked whether IL-7 was implicated in the survival or in the proliferation of adult LTi cells. In order to test this, I cultured sorted adult LTi cells from WT spleen during 8 days either in the presence of IL-7 or in the presence of IL-7Rα blocking Ab. After culture with IL-7Rα blocking Ab, the number of LTi cells was significantly decreased (50% of WT number) suggesting that IL-7 was a survival factor for LTi cells. I tested the proliferation of LTi cells *in vitro* by labelling adult LTi cells with CFSE, and culturing them on stromal cells in the presence or absence of IL-7. Analysing the CFSE expression level of LTi cells 7 days later, I observed one to

two more divisions of the cells cultured in the presence of IL-7. To compare these data with the proliferation *in vivo*, I adoptively transferred CFSE-labelled LTi cells into $RAG^{-/-}\gamma_c^{-/-}$ mice. Seven days later, I observed two or three more divisions in mice treated with IL-7/anti-IL-7 Ab complexes compared to untreated mice. Of note, a significant proliferation of LTi cells occurred already without treatment. In $RAG^{-/-}\gamma_c^{-/-}$ mice, neither T nor B or NK cells are present, which could potentially compete with LTi cells for the consumption of IL-7. This might explain the higher rate of proliferation of adult LTi cells in $RAG^{-/-}\gamma_c^{-/-}$ mice. Altogether, these data suggest that IL-7 has a survival and a proliferative effect on adult LTi cells.

4.3. Origin of adult LTi cells

Whether LTi cells found in adult mice are derived from FL or from other progenitors present in adult mice remained an open question. In adult mice, the BM harbours hematopoietic progenitor cells, which could give rise to adult LTi cells (36, 37). For example, common lymphoid progenitors (CLPs) express CD4 and c-Kit but are negative for all other lineage markers (101). A CLP-like progenitor cell has been described in the FL to give rise to fetal LTi cells (23). To address this question, we adoptively transferred FL or adult BM cells into IL-$7R\alpha^{-/-}$ mice. From previous studies we knew already that CD4-expressing LTi cells were absent from the FL. It was possible, however, that in the BM there were already pre-existing $CD4^+$ LTi cells. Since we intended to determine the frequency of LTi precursor cells in the adult BM, we depleted the BM for CD4- (and CD8-) expressing cells. The same number of FL cells was 10 times more efficient than BM to give rise to LTi cells. After treatment of mice with IL-7/anti-IL-7 Ab complexes, the number of LTi cells was significantly increased. These data demonstrate that firstly, the adult BM harbours precursors of adult LTi cells. Secondly, IL-7 supported the *bona fide* generation of LTi cells derived from the adult BM. Thirdly, FL cells were able to give rise to LTi cells in an adult environment. Finally, the BM was far less efficient than FL in generating LTi cells in adult mice.

4.4. Adult LTi cells and TLO development

H-IL-7 mice display an increased availability of IL-7. By quantitative real-time PCR, we found significantly higher levels of IL-7 mRNA in the salivary gland of H-IL-7 mice compared to WT mice (data not shown). However, at the protein level, we were unable to detect IL-7 either in the blood (data not shown) or in cell suspension of the salivary gland. Histology of the spleen revealed an increased expression of IL-7 protein by VCAM-$1^{+}CD45^{-}$ cells of the red pulp of H-IL-7 compared to WT mice.

A correlation between increased level of IL-7 and the presence of TLOs has been shown in mouse models for autoimmune diseases such as RA (88), colitis (89, 90), multiple sclerosis (91) and diabetes (92). In our mouse model, diffuse or organized TLOs developed in various non-lymphoid organs such as salivary gland and lung. Interestingly, LTi cells were detectable in these TLOs. In order to test if LTi cells were required for the development of TLOs, I crossed H-IL-7 mice with $ROR\gamma^{-/-}$ mice. These mice did not develop ectopic LNs or PPs, but the number of TLOs found in the salivary gland was not affected. These data show that the development of TLOs occurred independently of LTi cells. This confirms previous data obtained from a mouse model where the ectopic expression of CCL21 in the thyroid gland induced the development of TLOs. In these mice, the deletion of the Id2 gene, which is required for LTi cell generation, did not affect TLO formation demonstrating that the development of TLOs was independent of LTi cells (65). To test if LTβR signalling pathway was important for TLO development, I crossed H-IL-7 mice with $LT\alpha^{-/-}$ mice. In the salivary gland of H-IL-7 $LT\alpha^{-/-}$ mice only diffuse infiltrates around the ducts of the salivary gland were detectable, but no organized TLOs, suggesting that the LTβR pathway was critical for TLO organization. Adult mice harbour a wide variety of circulating mature lymphocytes expressing LTαβ. Amongst splenocytes of adult H-IL-7 mice LTαβ was expressed by B and LTi cells, but was undetectable on naive T cells and DCs. Hence, both B and LTi cells might be potential candidates for providing LT signals required for TLO organization. In order to formally test the effect of the B cells it would be interesting to cross H-IL-7 mice with B cell-deficient $\mu MT^{-/-}$ mice.

There is clear evidence that the colonization of the salivary gland of H-IL-7 mice with LTi cells precedes the infiltration with B cells. Analysis of the salivary glands of

H-IL-7 mice crossed with RAG$^{-/-}$ mice indicated that the recruitment of LTi cells to the salivary gland occurred independently of mature lymphocytes. By immunofluorescence, ubiquitous expression of CXCL12 was observed in the salivary gland of H-IL-7 mice. Similarly, cytokine arrays done with cell suspension from the salivary gland revealed that CXCL12 expression was higher in H-IL-7 compared to WT mice. CXCL12 is expressed in many non-lymphoid organs closely associated with endothelial cells (102). Since LTi cells express the corresponding chemokine receptor CXCR4, CXCL12 may be responsible for the recruitment of the cells to the salivary glands. CXCL13^{+} and CCL21^{+} cells were also detected in the salivary gland of H-IL-7 mice, but in contrast to CXCL12, only in the stroma compartments of TLOs. It is therefore possible that a combination of chemokine-mediated signals navigates LTi cells to developing TLOs. The interaction of LTi cells with stromal cells via LT$\alpha\beta$/LTβR might further stimulate the production of CXCL13 and CCL21, but is not absolutely required for the establishment of TLOs in H-IL-7 mice.

In H-IL-7 mice, some TLOs found in non-lymphoid organs were organized into B and T cell areas. B cells isolated from the salivary gland of H-IL-7 mice had a follicular-like phenotype. In IE-IL-7 tg mice, which express IL-7 under the control of the MHC II promoter, the IL-7-driven increase in B lymphopoiesis affected follicular but not marginal zone B cell numbers (103). This might explain why only follicular B cells were present in TLOs. In the salivary gland of both WT and H-IL-7 mice, some IgA^{+} plasma cells were detectable by flow cytometry. This is in agreement with the fact that in all mucosal tissues, IgA is the prominent Ig isotype produced by B cells. Immunohistochemistry, however, revealed that in TLOs of the salivary gland from H-IL-7 mice, B cells were IgA^{-}. This means that B cells present in TLOs differ from normal plasmablasts found in mucosal tissue.

By transfer of naive or *in vitro*- activated T cells from OT-I tg x RAG-2$^{-/-}$ mice, I could show that only naive T cells migrated to TLOs in the salivary gland of H-IL-7 mice. Naive T cells are known to express CD62L (L-Selectin), which enables them to transiently bind to PNAd^{+} HEV in LNs. The development of HEVs in LNs depends on LTβR signalling (104). In mice which lack HEVs due to blocking LTβR signalling, the number of naive T cells in LNs is largely reduced. TLOs in H-IL-7 mice clearly contained HEVs. It is therefore possible that LT$\alpha\beta$-expressing LTi cells and later B

cells promoted the development of HEVs in TLOs of the salivary gland, thereby facilitating the entry of naive T cells.

In autoimmune diseases, TLOs can harbour GCs with auto-reactive $CD4^+$ T and B cells. For example, in Sjögren's syndrome, the formation of functional GCs is observed in the salivary gland, with the production of auto-Abs (70). In a study about rheumatoid synovitis, three independent factors were defined as critical for the formation of GCs in non-lymphoid organs: CXCL13, CCL21 and LT$\alpha\beta$ (62). The differentiation of FDCs and influx of $CD4^+$ T cells may also support the generation of ectopic GCs. In H-IL-7, CXCL13 and CCL21 were produced in the TLOs of the salivary gland, suggesting a remodelling of the salivary gland with differentiation of lymphoid stroma. B cells and LTi cells expressed LT$\alpha\beta$, and could therefore be responsible for the activation of $LT\beta R^+$ stromal cells followed by the production of CXCL13 and CCL21. It remains to be investigated if in H-IL-7 mice, the engagement of LTβR on stromal cells of the salivary gland (e.g. fibroblast, myofibroblasts, pericytes) results in the transformation into lymphoid stroma or rather differentiation of a lymphoid stromal cell from precursor cells that yet need to be defined.

Despite the production of homeostatic chemokines and LT$\alpha\beta$ in the salivary gland of H-IL-7 mice, organized TLOs in these mice did not contain GCs. These data suggest that immune response to foreign Ags or auto-Ags did not occur in these mice. In agreement with this, neither auto-Abs, nor immune complexes were found in H-IL-7 salivary glands or in the serum (data not shown). One plausible explanation is that the frequency of auto-reactive T or B cells in H-IL-7 mice was too low to establish an immune response. Alternatively, auto-Ags in the salivary gland were not accessible to specific lymphocytes. In line with this, in thyroid autoimmune diseases, it was reported that components of the thyroid gland were presented as auto-Ags, and that cytotoxic $CD8^+$ T cells specific for thyroid Ags were critical for triggering GC reactions in non-lymphoid organs, probably through cell lysis and release of auto-Ags (105). Altogether, our data together with previous studies lead to the concept that TLO development alone is not sufficient to trigger autoimmune diseases, and that the recognition of auto-Ag determines the outcome of the disease. It is tempting to speculate that inflammatory cytokines and local release of IL-7 may be the starting point for TLO formation. Whether TLOs are beneficial for clearing a chronic infection, or harmful in case of autoimmune diseases, are still open questions, which remain to

be investigated. For example, blocking the LT pathway might be helpful in order to prevent autoimmune reactions in non-lymphoid organs (106).

The H-IL-7 mouse model can be a useful tool to study if TLOs help clearing infections or may promote the maintenance of chronic immune stimulation in models for autoimmune diseases such as collagen-induced arthritis.

4.5. TLO development during infection

Upon infection with influenza, the development of TLOs was observed in the lungs (63). In patients with chronic post-inflammatory lymph stasis, the neogenesis of local LNs has been reported (107). This would suggest that inflammatory and post-inflammatory signals can trigger *de novo* TLO development. We have previously shown that the injection of NP-CGG/Alum into the footpad of H-IL-7 but not WT mice triggered the development of additional ectopic LNs draining the site of injection (1). I therefore asked if inflammatory signals and IL-7 could have synergistic effects on the formation of TLOs. In order to study this we tested the effect of Leishmania (L.) major infection on TLO development in H-IL-7 mice and litter controls. In H-IL-7 mice, additional LNs were never observed in the popliteal region. Therefore, L.major parasites were injected s.c. into the footpad of mice, and the presence or absence of additional popliteal LNs after infection was analyzed. Interestingly, in around four out of sixteen H-IL-7 infected mice, the presence of ectopic LN could be detected in the popliteal region. These data show that, provided IL-7 was overexpressed, the infection induced the *de novo* formation of additional LNs. In WT and H-IL-7 mice infected with L.major, the number of LTi cells was significantly increased compared to non-infected mice. These data show that adult LTi cells were specifically recruited to or amplified in newly formed lymphoid organs. These data are in agreement with our observations in TLO formation in the salivary of H-IL-7 mice, and shed new light on the role of inflammatory signals for the activation and function of LTi cells.

4.6. Outlook

Using an IL-7 tg mouse model I have shown that IL-7 is mandatory for regulating the size of the adult LTi cell pool. However, precise data about the locations where

adult LTi cells can persist in WT mice are still incomplete. Someone would predict that LTi cell niches are sites where IL-7 and other growth/survival factors are available. In adult mice, large amounts of IL-7 are detectable in the intestine and skin (Thomas Schueler, personal communication). It has indeed been recently shown that intestinal CPs of adult mice harbour RORγ^{+}lin^{-} cells (37) and can therefore be a reservoir for LTi cells in steady-state conditions. It remains to be investigated if LTi cells are found in the skin. I have shown that adult BM cells could differentiate into LTi cells *in vivo*. I was unable to detect LTi cells in the BM of WT or H-IL-7 mice, and therefore the BM may rather contain LTi cell precursors that could give rise to LTi cells if required. LTi cell precursors could also be stored in peripheral organs such as the spleen, the LNs or the gut. The mechanism of differentiation of LTi cell precursors into functional LTi cells either in the BM or peripheral lymphoid organs and intestine needs to be further determined.

In addition to IL-7, other cytokines may be required for the maintenance of LTi cells during adulthood. For *in vitro* cultures of LTi cells, we used a combination of IL-3, IL-6 and SCF. Besides IL-7, these cytokines contributed to the maintenance of the cells for a few weeks in culture. Whether other cytokines, such as FLT3L, would induce better cell growth and maintenance could be further investigated. FLT3 is expressed by many hematopoietic precursor cells. It is not known if LTi cells express this receptor, but adult LTi-like cells do not expand in response to FLT3L treatment *in vivo* (21). However, FLT3L$^{-/-}$IL-7R$\alpha^{-/-}$ mice are completely devoid of LNs in contrast to IL-7R$\alpha^{-/-}$, where still some LNs are detectable (108), suggesting a role of FLT3L on LTi cells or their precursors.

The transcriptional control of differentiation of $\alpha4\beta7^{+}$ FL cells into LTi cells is not fully understood, but seems to require both Id2 and RORγt. Whether LTi-lineage-specific transcription factors exist that are required for the generation of adult LTi cells remains an open question.

Our data suggest that inflammatory signals stimulate either LTi precursor cell differentiation or proliferation or recruitment of mature LTi cells. Little is known about the homing of LTi cells and in case of inflammation and infection, the stimulation and recruitment of these cells need further investigation. Studies in mice where TLR are specifically deleted in LTi cells (cre/lox) would help to elucidate if TLR expression by LTi cells is essential for their function during infections. It is likely that in addition to

stromal cells, other cell subsets may interact with LTi cells during the process of SLO and TLO development. For example, the role of vascular endothelial cells and underlying pericytes could be object of future investigations.

Finally, it will be important to study, if a cellular equivalent to fetal and adult LTi cells exist in humans.

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6. CURRICULUM VITAE

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French: mother tongue, English and German: Fluent

EDUCATION

University of Basel (CH), Department of Biomedicine

2004-**2008** **PhD in Developmental Immunology** in the lab of Prof. Dr. D. Finke
Characterization of Lymphoid Tissue Inducer Cells and Lymphoid Tissue Development in Adult Interleukin 7 Transgenic Mice

University of Lausanne (CH), Biology and Medicine Faculty

2004 **Diploma in Biology in** the lab of Prof. Dr. H. Acha-Orbea
Employement of Mesenchymal Stem Cells as Carrier Cells for Gene Therapy of Solid Tumors in the Mouse

1998-2004 Studies in Biology
Practicals in Molecular Biology, Immunology, Morphology and Protein Structure and Certificate in Pharmacology and Toxicology

Scientific Gymnasium in Lausanne (CH)

1998 **Federal Maturity, specialisation in physics and maths**

TECHNICAL TOOLS

Cell Biology: cell culture, co-culture, organ culture, ELISA, cell separation with MACS beads, flow cytometry

Biochemistry: immunohistochemistry

Molecular Biology: standard molecular biology techniques: DNA and RNA isolation, PCR, RT-PCR, Real-Time PCR, Northern Blot

Others: fluorescence microscopy, confocal microscopy, transplantations, microarrays, education course to animal experimentation

SCIENTIFIC PUBLICATIONS

D.Meier, C.Bornmann, S.Chappaz, S.Schmutz, L.A.Otten, R.Ceredig, H.Acha-Orbea, and D.Finke. **2007**. Ectopic lymphoid organ development occurs through IL-7-mediated enhanced survival of lymphoid tissue inducer cells. *Immunity* 26(5)

D.Finke and S.Schmutz. **2008**. Interleukin 7-induced lymphoid neogenesis in arthritis: recapitulation of a fetal developmental program? *Swiss weekly* 138(35-36): 500-505

S.Schmutz, N.Bosco, S.Chappaz, O.Boyman, H.Acha-Orbea, R.Ceredig, A.Rolink, and D.Finke. **2008**. Interleukin 7 regulates the peripheral pool of adult RORγ^+ lymphoid tissue inducer cells. (In revision)