Characterization of the function of thymic stromal lymphopoietin in lymphopoiesis and lymph node organogenesis

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Stéphane Chappaz

Annecy le Vieux, Frankreich

Basel, 2010

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
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Basel, den 16 September 2008

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Abbreviations

Ab	Antibody
AD	Atopic dermatitis
Ag	Antigen
APC	Antigen presenting cell
BAFF	B-cell activating factor belonging to the TNF family
BAL	Bronchoalveolar lavage
BCR	B cell receptor
B-LPD	B-cell lymphoproliferative disorder
BM	Bone marrow
CRTH2	Prostaglandin D2 receptor
DC	Dendritic cell
DN	Double Negative
DNA	Deoxyribonucleic acid
DP	Double positive
EST	Expressed sequence tags
FB	follicular B
FcγR	Fc receptor γ chain
Flt3L	fms-related tyrosine kinase 3 Ligand
FoxP3	Forkhead box P3
FRC	fibroblastic reticular cells
FTOC	Fetal thymus organ culture
HBE	Human bronchial epithelial
HC	heavy chain
HDM	House dust mite
HEV	high endothelial venule
HSC	Hematopoietic stem cell
HVEM	HSV entry mediator
ICAM-1	Intercellular adhesion molecule-1
Id2	Inhibitor of DNA binding
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
K	Keratin
KGF	keratinocyte growth factor
LC	light chain
LN	lymph node
LTi	Lymphoid tissue inducer
MAdCAM-1	Mucosal Vascular addressin cell adhesion molecule-1
MC	Mast cell
MDC	Macrophage-derived chemokine
MHC	Major histocompatibility complex
MW	molecular weight
MZ	marginal zone
NCBI	National center for biotechnology information
NK	Natural killer
OX40L	OX40 Ligand
OVA	Ovalbumin
RAG	recombination activating genes
RNA	Ribonucleic acid
RORγ	RAR-related orphan receptor γ

RXR	Retinoid X receptor
SCF	Stem cell factor
SP	Single positive
T1	transitional 1
T2	transitional 2
TARC	Thymus and activation-regulated chemokine
TCR	T cell receptor
Tdt	terminal deoxynucleotidyl-transferase
TEC	thymic epithelial cells
TLR	toll-like receptor
TNF	Tumor necrosis factor
TRAF6	TNF Receptor associated Factor 6
TRANCE	Tumor necrosis factor-related activation induced cytokine
Treg	regulatory T cell
TSLP	Thymic stromal lymphopoietin
VCAM-1	Vascular cell adhesion molecule-1

Acknowledgments

While the analogy might not last, making a Ph-D is pretty much like making a lymph node. It is all about finding the right place, sticking to the spot, interacting with others, being ready for feedbacks and hoping for maturation…

I am grateful to Daniela Finke in the lab of whom, I have learned enormously about immunology and about science. I would like to thank her for the continuous support and for the independence she has offered me along the way. Daniela has truly been an excellent supervisor, ready to provide critiques, help and encouragements whenever needed. I owe Daniela a dept of gratitude for the abuse of her dissecting skills, and for repeatedly asking her to perform single-blind experiments for me.

I am also indebted to Ton Rolink. I owe him several dozen of hours of FACS-sorting, some millions of purified cells and a non-quantifiable amount of answers. I would like to thank him for: his support early on, some very fruitful discussions in the staircase, his willingness to share his knowledge about immunology and for being my faculty responsible.

I would like to thank Rod Ceredig for being always available to discuss scientific issues and whose scrutiny of ongoing experiments and on manuscripts has been of immense help.

I am grateful to Jan Andersson for sharing practical experience, knowledge and home keys and for providing me with some vertiginous historical perspectives on immunology.

The teams of Daniela, Klaus and Ton have given our shared lab meetings quite a challenging atmosphere. I would like to thank the people who participated to these lab meetings as a whole, because many of their suggestions were converted into fertile experiments.

I would like to thank Klaus Karjailanen, Gioacchino Natoli, Marcus Thelen and Michele Goodhardt, without the encouragements of whom; I likely would have never reached Basel.

I want to express my gratitude to people with whom I worked on a daily basis. The work at the bench has been so enjoyable also because they were around. Thanks to Sandrine Schmutz for countless discussions, for sharing her experience about immunofluorescence and for spontaneously inviting me to her wedding. Thank you to Caroline Bornmann for always being available for all sorts of matter, for coping with the french connexion with such a good mood, for making my life a lot easier during flat-hunting times and for letting me drive her car.

I would like to thank the people of the animal facility without who the work presented here would not have been possible. Especially, I would like to thank Angelika Offinger for always being available, fast and precise and Sabine Eckervogt for her patience and scrupulous work with the animals.

Thanks to Jörg Kirberg and Andrew Farr for always being willing to provide us with reagents and mouse strains and to Lukas Flück for performing crucial molecular experiments. I would like to thank Werner Krenger, Thomas Barthlott for emergency FACS sorting and Jason Gill and Pascal Lorentz for help with confocal microscopy. Thanks to Dominic Van Essen for critical comments on manuscripts and for the mythical Brombacherstrasse discussions.

I would like to thank Melanie Schmid, Dorit Hässler and Nicole Salvisberg, for dealing with administrative reality always with the same amazing efficiency.

Time has come to thank many people with who I shared not only lunches, but also bets, scientific frustrations, political discussions, journal clubs and some hilarious moments. Thanks to Lee Kim Swee, Corinne Engdahl, Angèle Bénard, Roxane Tussiwand, Nabil Bosco and Giuseppina Capoferri.

I am also gratefull to Jean Pieters for having accepted to be co-referee of my thesis committee and to Gerd Pluschke for holding the position of chairman.

A. Summary

Interleukin (IL)-7 is a cytokine, which is crucial for the development of the murine immune system. It is required for lymphopoiesis and for the development of peripheral lymph nodes (LN). IL-$7^{-/-}$mice have impaired B and T cell lymphopoiesis, decreased numbers of peripheral B and T cells and are devoid of γδ T cells. IL-7 signals through a receptor composed of the common γ (γ_c) and the IL-7Rα chain. The latter chain can also pair with the γ_c-like chain called thymic stromal lymphopoietin receptor (TSLPR). Both form the receptor of the cytokine called thymic stromal lymphopoietin (TSLP). .

Originally identified for its capacity to promote B cell development *in vitro*, TSLP was later shown to induce dendritic cell maturation, to trigger allergic diseases and to drive TH2 differentiation. Several evidences suggested that TSLP might play a role in fetal B lymphopoiesis and that fetal but not adult cells were TSLP-responsive. However, the function of TSLP in hematopoiesis and in LN organogenesis *in vivo* remained elusive. In the work presented in the first part of this thesis, I have characterized the function of TSLP in adult lymphopoiesis.

This study shows that TSLP transgene (Tg) expression restored all developing B cell compartments in the bone marrow (BM), DN1 and DN2 thymocytes and thymic architecture, and all peripheral B and αβ and γδ T cell compartments in IL-$7^{-/-}$ mice. The expression of the TSLP Tg increased thymic and splenic cellularities. The analysis of the junctions of the immunoglobulin heavy chain locus showed that the DNA of B cells from IL-$7^{-/-}$ TSLP Tg mice contained N nucleotides, suggesting that adult hematopoietic progenitors are TSLP-responsive. Moreover, BM chimera experiments showed that WT BM precursors differentiated towards B- and T-cell lineages in response to TSLP, further suggesting that adult hematopoietic cells are TSLP-responsive. In this line, we show that TSLP had the capacity to promote the proliferation and the differentiation of DN1 and DN2 thymocytes as well as the differentiation of uncommitted adult BM precursors towards the B and the T cell lineage *in vitro*. Hence, these results altogether showed that TSLP has the capacity to promote long-term adult lymphopoiesis in the absence of IL-7.

Lymph node (LN) development starts during fetal life and crucially relies on the interaction between the hematopoietic lymphoid tissue inducer (LTi) cells and the mesenchymal organizer cells. Both together cluster in a cellular aggregate called LN anlage. This LN anlage is colonized by peripheral lymphocytes after birth, and gives rise to a mature LN organized into B cell follicles and a T-cell zone. Mice deficient for IL-7 or for molecules of the IL-7 signaling pathway lack several LN but the reasons underlying this defect are still not clear. As IL-7 regulates the size of the LTi cell pool, a possibility is that LN development in IL-$7^{-/-}$ mice is

impaired because of insufficient LTi cell number. Alternatively, it was proposed that the lack of colonization of the LN anlage by peripheral lymphocytes might prevent the maintenance of the LN anlage. I show in the second part of this thesis, that TSLP overexpression increased LTi cell number and restored LN development in IL-$7^{-/-}$ and RAG$2^{-/-}$ $\gamma_c^{-/-}$ mice, suggesting that LTi cell number is a critical parameter for LN organogenesis. The LN anlage of RAG$2^{-/-}$ $\gamma_c^{-/-}$ TSLP Tg mice were devoid of peripheral lymphocytes, ruling out that lymphocytes are required for LN maintenance. Thus, the results shown here define organizer and LTi cells as the minimal cellular requirement for LN development and suggest that the lack of LN in mice lacking molecules of the IL-7 pathway is the result of suboptimal LTi cell number. This study further shows that lymphocyte colonization is required for establishing a correct LN architecture and for the differentiation of some mesenchymal populations within the LN microenvironment.

Overall, this study shows that TSLP can substitute IL-7 for murine lymphopoiesis and for LN organogenesis and suggest that the impaired lymphopoiesis and LN organogenesis in IL-$7^{-/-}$ mice is the consequence of limited availability of endogenous TSLP.

B. Introduction

1. Lymphopoiesis

1.1 Introduction

Hematopoietic stem cells (HSCs) are defined by their ability to self-renew and to differentiate towards all blood cell lineages. The generation of B and T lymphocytes relies on the continuous differentiation of progenitors derived from HSC within the bone marrow (BM) and the thymic microenvironments, respectively. Both B and T lymphopoiesis are regulated by a number of differentiation, survival and growth factors that are provided by the resident stromal cells. The factors and cells supporting hematopoiesis constitute what is defined as the "niche". Here are described the major cellular and molecular events taking place during lymphopoiesis in primary lymphoid organs. A particular emphasis is placed on the factors, which contribute to hematopoietic niches and which are required for generation, maturation and survival of developing and mature lymphocytes.

1.2 B cell development

During fetal life, B cell development occurs in the liver while it is restricted to the BM at adulthood. B cells develop according a similar general program in fetal liver (FL) and adult BM, except that fetal B cells do not express the terminal deoxynucleotidyl-transferase (Tdt) [1], which is required for incorporating N nucleotides during heavy chain (HC) rearrangement [2]. Cytokines are amongst the most important factors for B cell development *in vitro* and *in vivo*. For instance, stem cell factor (SCF) and fms-related tyrosine kinase 3 Ligand (Flt3L) play a central role in adult B lymphopoiesis. Mice deficient for the SCF receptor c-Kit [3] and for Flt3L [4] have an early block in B cell development. Similarly, interleukin (IL)-7 is required for adult B lymphopoiesis *in vivo* [5-7]. While adult B cell development crucially relies on IL-7, it seems that fetal and perinatal B lymphopoiesis is less stringently dependent on IL-7. Thus, the BM of newborn IL-$7^{-/-}$ mice contains substantial numbers of $CD19^{+}$ B cells, which gradually disappear with age [5].

The first subset of committed B cells is defined as $CD19^{+}$ $CD117^{+}$ fraction (Figure 1). It contains pro-B cells having Ig genes in germline configuration and pre-B-I cells with rearranged D_HJ_H loci [8]. Pro-B/pre-B-I cells express IL-7Rα [9], proliferate in response to IL-7 *in vitro* [10] and accumulate in the BM of IL-7 Tg animals [11]. In line with these results, IL-$7^{-/-}$ and IL-7R$\alpha^{-/-}$ adult mice lack pro-B/pre-B-I cells in the BM [5-7].

Pro-B/pre-B-I cells develop into large pre-B-II cells, which are $CD19^+$ $CD117^-$ $CD25^+$ [12] and have rearranged at least one $V_HD_HJ_H$ allele [8]. Productive $V_HD_HJ_H$ rearrangement and pairing with the surrogate light chain coded by the *VpreB* and *λ5* genes, are both required for successful pre-BCR expression. Pre-BCR signals are required for the downregulation of the recombination activating genes (*Rag*) and *Tdt* genes [13]. Importantly, assembly of complete pre-BCR is crucial for the expansion of pre-B-II cells while allelic exclusion depends on the presence of μHC but not of the surrogate light chain components [14-17]. Consistently with the fact that large pre-B-II cells express IL-7Rα [9], the size of the $CD19^+$ $CD25^+$ Pre-B-II compartment is increased in IL-7 Tg mice compared to WT animals [11]. After 5-7 divisions, large Pre-B-II cells go back to a resting state.

The subsequent small $CD19^+$ $CD25^+$ pre-B-II cells start to rearrange the light chain (LC) loci with a strong bias for the κ locus compared to the λ locus [8]. The cells that have productively rearranged the LC locus and that express Ig at their surface are called immature B cells and appear as $CD19^+$ $CD25^-$ IgM^{high}. Immature B cells do not express IL-7Rα and do not respond to IL-7. Hence, the crucial role fulfilled by IL-7 for B lymphopoiesis seems to be in sustaining the generation and expansion of early B cell progenitors from pro-B to large pre-B-II.

Immature B cells undergo selection processes within the BM microenvironment in order to remove auto-reactive B cells from the repertoire. Hence, immature B cells recognizing self-Ag with high affinity are either eliminated (clonal deletion) [18], become anergic, or are given the chance to change specificity by rearranging the LC locus [19, 20], a phenomenon known as receptor editing [21].

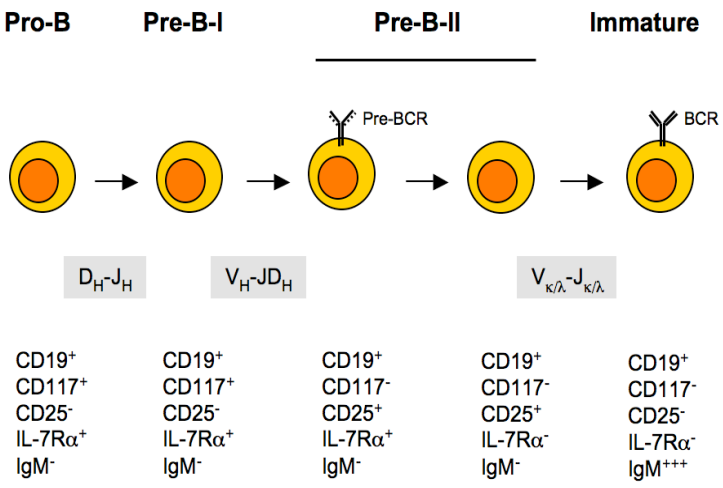

Figure 1. Scheme of B cell development

Adapted from Ceredig and Rolink, Nature Reviews Immunology (2002).

1.3 Peripheral B cells

Immature B cells leave the BM, enter the blood stream and reach the spleen. The newly entered immature B cells are easily detectable in the spleen as they still express CD93. They can be separated into transitional 1 (T1) and transitional 2 (T2) cells. T1 cells are IgM^{high} IgD^{low} $CD21^{-}$ $CD23^{-}$ while T2 B cells are IgM^{high} IgD^{+} $CD21^{+}$ $CD23^{+}$. T1 and T2 B cells are characterized by their propensity to undergo apoptosis upon BCR engagement, rather than to proliferate [22, 23]. B cell maturation is thought to occur in a process during which T1 and T2 B cells give rise to $CD93^{-}$ mature splenic B cells, along pathways that are still debated. Mature splenic B cells can be subdivided into $CD21^{high}$ $CD23^{-}$ marginal zone (MZ) B cells and $CD21^{+}$ $CD23^{+}$ follicular B (FB) cells [24].

In mice, MZ B cells represent 5% of splenic B cells and are sessile cells located in the vicinity of the marginal sinuses of the spleen [25]. MZ B cell generation crucially depends on Notch2 signals, as deletion of the *Notch2* gene in hematopoietic cells specifically prevents the development of MZ B cells [26]. MZ B cells have a limited BCR repertoire and mount mainly T-independent Ab responses against blood-borne viral and bacterial pathogens [25]. MZ B cells differentiate into Ab-secreting cells upon BCR ligation or toll-like receptor (TLR) activation.

FB cells represent the main splenic B cell population and are the cells mounting T-cell dependent Ab responses against protein Ags [27]. Upon concomitant BCR activation and ligation of CD40 by activated T cells, FB cells undergo somatic hypermutation, class switching recombination, and differentiation towards long-lived Ab-secreting plasma cells. FB cells are circulating cells that are found in the spleen, LN, the blood and the BM [27]. Interestingly, increased IL-7 availability promotes the expansion of the FB but not of the MZ compartment [28].

Recently, a crucial factor for peripheral B cell compartments called B-cell activating factor belonging to the TNF family (BAFF) was discovered. BAFF is a survival and maturation factor for T2, mature B and for autoreactive B cells in the spleen. $BAFF^{-/-}$ mice have a block at the T1 stage, and lack T2, MZ and follicular B cells [29]. Conversely, BAFF overexpression leads to B cell hyperplasia and to autoimmune disease [30] through the rescue of self reactive B cells from deletion [31]. These results indicate that both the deletion of auto-reactive B cells and the size of peripheral B cell compartments are dependent on BAFF availability.

B-1 B cells are peripheral B cells which are mainly found in the peritoneal cavity and are phenotypically defined as $CD19^{+}$ $B220^{low}$ IgM^{high} IgD^{low}. B-1 B cells contribute to T-independent responses against bacterial Ag such as phosphorylcholine [27]. B-1 B cells can be separated according expression levels of CD5 into $CD5^{+}$ B-1a and $CD5^{-}$ B-1b B cells. B-1 B cells develop normally in $IL\text{-}7^{-/-}$ [5] and $BAFF^{-/-}$ [29] mice, suggesting that this subset requires different factors for generation and/or homeostasis.

1.4 T cell development

The thymus is continuously seeded by BM precursors, which have entered the blood circulation. These progenitors enter the thymus at the corticomedullary junction. While the phenotype of the early thymic immigrants is still unclear, the identification of subsequent populations is well established. T lymphopoiesis occurs in a stepwise process during which early T cell progenitors cells sequentially rearrange the loci of the *T cell receptor* (TCR) genes. The cells, which have productively recombined *tcr* genes will later undergo positive and negative selection.

Adult T lymphopoiesis relies on the crucial IL-7 and Notch1 signals. IL-$7^{-/-}$ and IL-$7R\alpha^{-/-}$ mice have severely decreased thymocytes numbers [6, 7] and an early block in thymopoiesis [32]. Hematopoietic cells deficient for Notch1 fail to differentiate towards T cells *in vivo* [33] and T cell development *in vitro* can be achieved only in the presence of Notch ligands [34].

The first T cell progenitors are called double negative (DN), as they express neither CD4 nor CD8 (Figure 2). DN thymocytes can be further separated into 4 subsets according their levels of expression of CD25, CD44 and CD117 [9]. DN1 have TCR genes in germline configuration and appear as $CD117^{high}$ $CD44^{+}$ $CD25^{-}$ cells [9]. These cells differentiate to DN2 cells, which have rearranged the Dβ-Jβ locus and are $CD117^{high}$ $CD44^{+}$ $CD25^{+}$. Both DN1 and DN2 thymocytes are absent in IL-$7^{-/-}$ mice [32] and both proliferate in response to IL-7 and Notch signals [35]. In turn, DN2 develop into DN3 cells that are $CD117^{+}$ $CD44^{-}$ $CD25^{+}$ and have rearranged the Vβ-DJβ locus. In the adult, Notch but not IL-7 signals are required for DN3 proliferation [35]. Most TCRβ rearrangements in the DN3 cells are non-productive while those in the subsequent DN4 cells are mainly in frame [36]. This illustrates a process named β-selection, where only DN3 thymocytes which have productively rearranged the TCRβ locus and that can express the pre-TCR at their surface will differentiate towards the $CD117^{-}$ $CD44^{-}$ $CD25^{-}$ DN4 stage. Notch1 signals are required to make the Vβ-DJβ recombination [37] and for expression of the pre-Tα [38]. IL-7 and Notch signals, which are crucial for DN1, DN2 and DN3 thymocytes, are provided by Keratin $(K)8^{+}$ $K5^{-}$ cortical thymic epithelial cells (TEC) [39].

DN4 cells differentiate into $CD4^{+}CD8^{+}$ double positive (DP) thymocytes, which have productively rearranged the TCRα locus. DP cells express a unique TCR at their surface and interact with peptide-MHC complexes presented by cortical TECs [39]. Low avidity interactions will induce DP thymocytes to survive [40], a phenomenon referred as positive selection. On the contrary, DP thymocytes that recognize self peptide-MHC complexes with high affinity will undergo apoptosis. This process, known as negative selection, ensures the deletion of self-reactive T cells from the repertoire [41]. Thymocytes, which have a very low avidity for self peptide-MHC complexes will die by neglect. Positively selected DP thymocytes differentiate

towards $CD4^+$ or $CD8^+$ single positive (SP) cells and migrate from the cortex to the medulla. SP thymocytes undergo another round of negative selection performed by DC and $K8^-$ $K5^+$ medullary TEC. As many of these stromal cells express tissue-specific Ag in a promiscuous fashion, they further purge the T cells that recognize tissue-specific self-Ags from the repertoire. The thymic medulla is also the place where regulatory T cells (Treg) are generated, which can suppress T-mediated immune responses and are required for tolerance to self-Ag. Overall, only 1-3% of thymocytes successfully differentiated and are exported from the thymus [39].

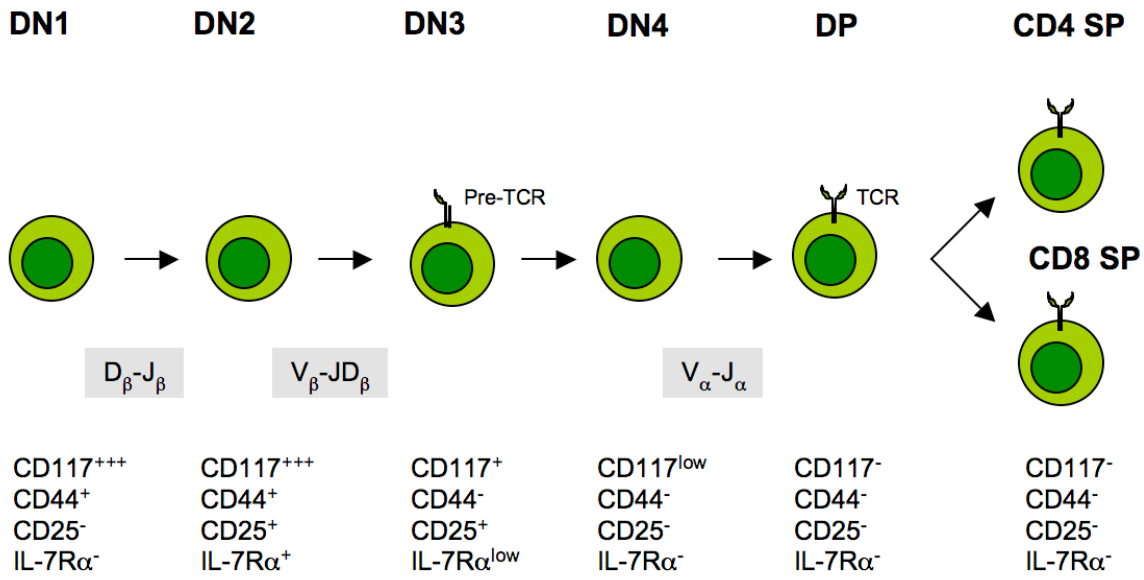

Figure 2. Scheme of T cell development

Adapted from Ceredig and Rolink, Nature Reviews Immunology (2002).

1.5 γδ T cells

γδ T cells are unconventional T cells, which have the capacity to recognize classical and non-classical MHC Ag. γδ T cells develop in the thymus along schemes that are still under debate. They are generated along sequential waves during ontogeny. For example, $V\gamma3^+$, $V\gamma4^+$ and $V\delta1^+$ cells are produced during fetal life, while $V\gamma1^+$ and $V\delta4^+$ develop during adulthood [42]. γδ T cells represent one of the most prominent hematopoietic population within epithelial tissues such as skin, lung and intestinal epithelium [42]. Many γδ T cells have a highly restricted TCR diversity: hence, the skin is mainly populated by $V\gamma3^+$ T cells [43] and the intestinal epithelium harbors mostly $V\gamma5^+$ cells [42]. While their role is not firmly established, γδ T cells might be involved in cellular immunity, tumor surveillance, and epithelial immunoregulation [44].

1.6 Peripheral T cells

Peripheral T cells are mainly composed of $CD4^+$ or $CD8^+$ cells expressing αβ TCR. Both CD4 and CD8 compartments can be further divided into $CD44^{low}$ naïve T cells and $CD44^{high}$ memory T cells [45]. Naïve T cells express also L-selectin (CD62L) which allow them to enter LN via specialized blood vessels called high endothelial venules (HEV). Naïve T cells are almost exclusively found in secondary lymphoid organs. Memory cells are the progeny of T cells that have encountered Ag. The pool of memory cells is composed of $CD62L^-$ T cells, which patrol peripheral tissues and of $CD62L^+$ memory T cells, which home to spleen and LN.

The survival and homeostasis of naïve and memory T cells are controlled by IL-7 [46]. IL-$7^{-/-}$ and IL-7R$\alpha^{-/-}$ mice have decreased number of peripheral T cells [6, 7]. Other γ_c-dependent cytokines, such as IL-15 but also interactions with self-Ag/MHC are factors controlling the size and the homeostasis of peripheral T cell compartments [45].

2. Lymph node organogenesis

2.1 Introduction

Lymph nodes (LN) are highly organized organs, which collect Ag drained from all tissues. They provide specialized microenvironments where adaptive immune responses are mounted. Since adaptive immune responses rely on the activation and expansion of rare Ag-specific cells, LN represent the evolutive solution for maximizing cellular interactions and for mounting rapid Ag-specific response [47]. In agreement with this view, absence of LN prevents the establishment of protective anti-viral response [48]. Besides, LN provide microenvironments where peripheral T cell tolerance takes place [49] and might play a substantial role in lymphocyte homeostasis by producing growth and survival factors [50].

2.2 LN architecture

The main cellular components of LN are hematopoietic, mesenchymal and endothelial cells, the later two forming the stroma together with extracellular matrix and reticular fibers [47]. LN are encapsulated organs composed of cortex and medulla [49] (Figure 3). The medulla contains short-lived plasma cells, few memory T cells and macrophages [49]. It is largely packed with lymph-draining sinuses and blood vessels [49]. The cortex is composed of B cell follicles surrounding the T cell area also named paracortex. B-cell follicles are the sites where humoral responses are mounted while the paracortex is the microenvironment where DC and T cells interact [49]. Most B and T lymphocytes enter LN via HEVs that are localized in the paracortex. T/B compartmentalization is mediated through chemoattractants, which are produced by stromal

cells. Hence, B-cell stroma produces CXCL13 [51], which attracts $CXCR5^+$ B cells while T-cell stroma releases CCL19 and CCL21, which attract $CCR7^+$ T cells [52].

Lymph enters the LN via afferent lymphatic vessels in the space below the capsule, called the subcapsular sinus. From there, it flows through intermediate sinuses within the LN parenchyma towards the medulla, where it eventually exits the LN at the medullary sinus via the efferent lymphatic vessel. It was recently found that LN also contain small channels called conduits, which transport low molecular weight (MW) particles such as chemokines from the subcapsular sinus to the T-cell area and the HEVs [53]. The lymph content is sampled by peculiar DC, which are intimately associated with the conduits [54]. High MW compounds (>80 kD) entering the subcapsular sinus are excluded from the conduits and are directly conducted towards the medullary sinus [53]. The conduits and the HEVs are enwrapped by stromal cells called fibroblastic reticular cells (FRC). FRCs form a 3-dimensional reticular network within the LN [55], which is dynamically remodeled during immune responses [56]. While the FRC network assures T cell motility, networks of follicular dendritic cells allow B cell movements within the follicle [57].

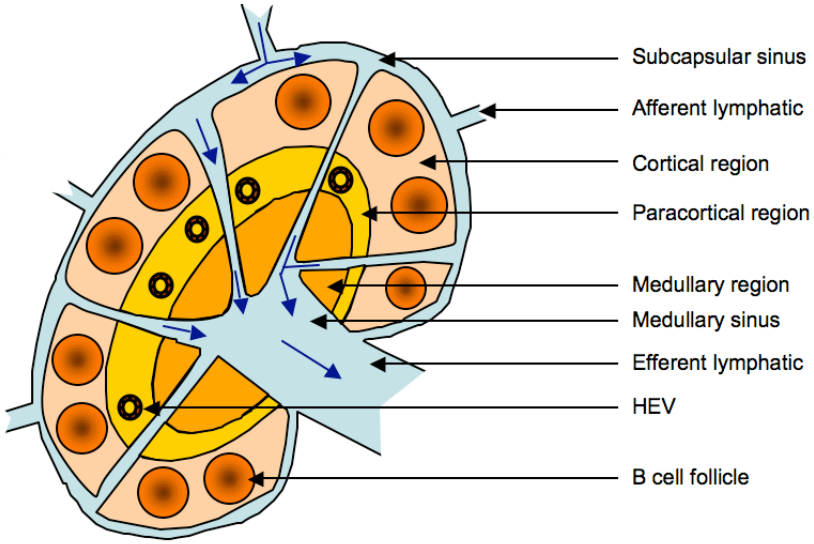

Figure 3. General structure of a lymph node
Blue arrows indicate direction of the lymph flow.
Adapted from Crivellato, Trends in Immunology (2004).

2.3 Role of the Lymphotoxin pathway for LN organogenesis

The emergence of mouse embryonic stem cell technologies opened up the possibility to generate mice with a specific deletion of a gene of interest. Deletion of genes from the tumor necrosis factor (TNF) family shed light on central mechanisms underlying LN organogenesis.

The TNF family includes 4 members [58]: TNF, lymphotoxin (LT)α, LTβ and LIGHT (Figure 4). TNF signals through two receptors TNFR1 and TNFR2 [58]. LTα and LTβ can form the membrane-bound heterotrimer LTα1β2 which signals through the receptor LTβR [59]. Beside LTα1β2, LIGHT is the sole ligand known to bind LTβR [60]. LTα can form the soluble trimer LTα3 that is a ligand for both TNFR1 and TNFR2 [58]. LIGHT and, to a lesser extent, LTα3 bind to the receptor HSV entry mediator (HVEM) [60].

One of the first hints concerning the molecular mechanisms of LN formation emerged from the generation of LTα deficient mice. $LT\alpha^{-/-}$ animals were reported to lack all LN and PP [61, 62]. Approaches neutralizing the LT pathway with LTβ-R-Fc chimeric proteins showed that LN organogenesis occurred in sequential steps during fetal development [63]. In agreement with a central role of the LT pathway in organogenesis, $LT\beta R^{-/-}$ mice were shown to lack LN and PP [64]. However, later studies showed that $LT\beta^{-/-}$ mice lacked PP and peripheral LN except cervical and mesenteric LN [65, 66], suggesting that LTβR ligands other than LTα1β2 play a role in LN organogenesis. In fact, $LIGHT^{-/-}$ $LT\beta^{-/-}$ mice develop mesenteric LN in lower frequency than $LIGHT^{-/-}$ animals showing that LIGHT contributes to some extent to mesenteric LN formation [67].

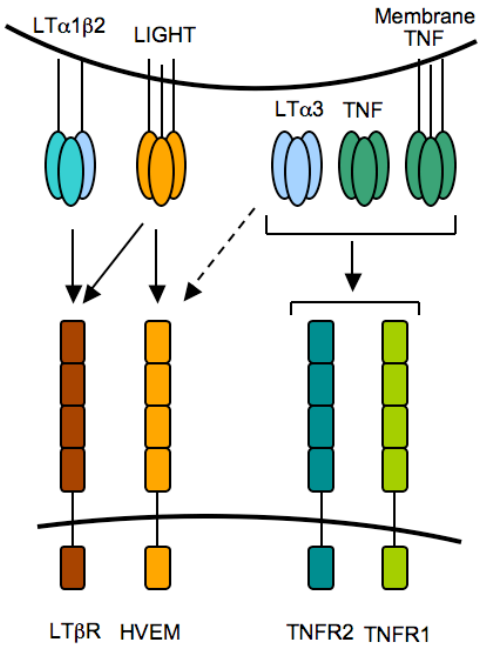

Figure 4. Ligands and receptor for the TNF/Lymphotoxin system
Adapted from Gommerman and Browning, Nature Reviews Immunology (2003).

There is no evidence indicating that TNF participate to LN organogenesis. Hence, animals deficient for TNF [68], TNFR1$^{-/-}$ [69], or TNFR2$^{-/-}$ [70] have normal LN development. TNF$^{-/-}$ LTβ$^{-/-}$ mice develop mesenteric LN [71], further demonstrating that TNF does not participate in mesenteric LN development. However, TNFR1$^{-/-}$ LTβ$^{-/-}$ mice are devoid of mesenteric LN [72], indicating that LTα3 and LTα1β2 cooperate in the formation of these LN.

Overall these results showed that the interaction between LTα1β2 and LTβR is the main molecular pathway involved in the development of peripheral LN and that molecules such as LTα3 and LIGHT might participate and/or cooperate to the formation of mesenteric LN.

2.4 Lymphoid tissue inducer cells

Lymphoid tissue inducer (LTi) cells are hematopoietic cells, which were initially described as CD4^{+} CD3^{-} IL-7Rα^{+} c-kit^{low} in the spleen, LN and peripheral blood of embryos and newborn mice [73]. As these cells expressed high levels of LTα1β2, it was speculated that they might be implicated in LN development [73]. Cells with an identical phenotype were reported to cluster in fetal gut and participate to Peyer's patch (PP) development [74-76]. Furthermore, adoptive transfer of LTi cells restore PP development in CXCR5$^{-/-}$ newborn mice [77], demonstrating that LTi cells have the capacity to induce secondary lymphoid organ development.

Further information about the crucial role of LTi cells in LN organogenesis came from the fact that mice deficient for the transcription factors Id2 [78], Ikaros [79] and RORγ$^{-/-}$ [80], which all lacked LTi cells, were completely devoid of LN and PP. In this line, RORγt, a RORγ isoform expressed in LTi cells during fetal life, is crucial for LTi cell generation [81]. Altogether, these results show that LTi cells are instrumental for LN and PP development.

2.5 Fetal liver progenitors of LTi cells

Two FL progenitors have been independently identified as putative precursors for LTi cells. Sca-1^{low} CD117^{low} IL-7Rα^{+} FL cells were reported to differentiate towards cells with LTi phenotype when injected into newborn mice [82]. Independently, lineage^{-} α4β7^{+} IL-7Rα^{+} FL cells were described for their capacity to differentiate towards LTi cells *in vitro* [83]. Of note, both populations do not exclusively give rise to LTi cells but have the capacity to differentiate towards other hematopoietic lineages. Various approaches revealed that LTi cells are present in LN and PP anlagen, in blood and spleen but not in FL [73, 76, 81]. While these results do not give definitive answers about the direct LTi precursor(s), they altogether suggest that LTi precursors originate from the FL and that they migrate to the periphery where they differentiate into LTi cells.

2.6 Organizer cells

Organizer cells are from mesenchymal origin, express the LTβR and were originally identified as VCAM-1^+ ICAM-1^+ cells in the fetal gut [84]. These cells form clusters along the intestinal gut and form the PP anlage [75]. PP organizer cells express CXCL13, CCL19 and IL-7 [84, 85]. Similarly, LN organizer cells were described in mucosal and peripheral LN anlagen as VCAM-1^+ ICAM-1^+ cells [86, 87]. In addition to LTβR, LN organizer cells express the cytokines TRANCE and produce IL-7 and the chemokines CXCL13, CCL19 and CCL21 [86, 88]. Organizer from inguinal and mesenteric LN differ by their levels of expression of adhesion molecules [86], suggesting that peripheral and mucosal LN develop from phenotypically distinct organizer cells.

2.7 Model for LN development

LN organogenesis crucially relies on the interaction of LTi cells with organizer stromal cells [85, 89]. At 12.5-13.5 days post-coitus (dpc), LTi cells start to cluster at sites of nascent LN anlage [87]. LTi cells engage the LTβ receptor present on organizer cells (Figure 5). Organizer cells cluster the successive day in a LTα-dependent manner [87]. LTβR engagement leads to the upregulation of adhesion molecules such as VCAM-1 and ICAM-1 but also to the production of chemokines such as CXCL13, CCL19 and CCL21 [90]. LTi cells, which migrate *in vitro* in response to these chemokines [84], are recruited to the LN anlage, and provide further LT signals. This positive feedback loop allows the formation of stable clusters of LTi and organizer cells, which will later be colonized by mature lymphocytes.

2.8 Important factors for LN organogenesis

2.8.1 Cytokines

Several cytokines play a substantial role in LN organogenesis. For instance, IL-7 is required for complete LN development as IL-$7^{-/-}$ and IL-$7R\alpha^{-/-}$ mice lack several peripheral LN [91, 92]. The lack of LN is identical in $\gamma_c^{-/-}$ mice and IL-$7R\alpha^{-/-}$ mice [91], suggesting that IL-7 is the main γ_c cytokine for LN formation. The reasons why IL-$7^{-/-}$, $\gamma_c^{-/-}$ and IL-$7R\alpha^{-/-}$ mice have a reduced number of LN remain unclear. IL-7 has two effects on LTi cells. On one side, IL-7 induces the upregulation of LT expressed by LTi cells *in vitro* [84, 87, 91] and *in vivo* [91, 92]. On the other side, IL-7 mediates LTi cell survival *in vitro* and its overexpression leads to the increase in LTi cell number in the spleen and mesenteric LN of newborn mice compared to WT controls [92]. Indeed, mesenteric LN from IL-$7R\alpha^{-/-}$ newborn mice contain decreased number of LTi cells compared to WT, suggesting that either LTi cell recruitment in the LN anlage is

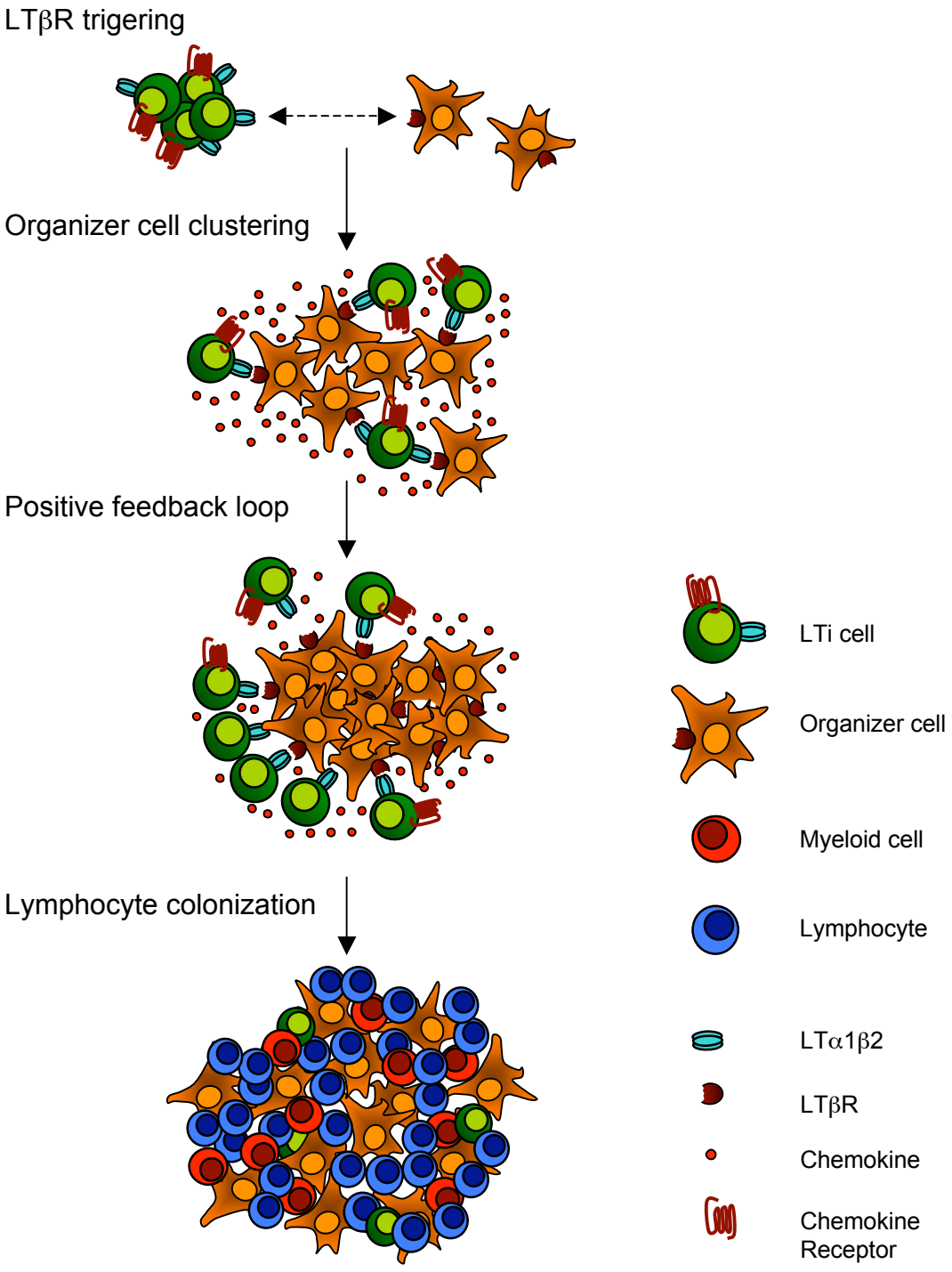

Figure 5. Model for lymph node organogenesis

Adapted from Mebius, Nature Reviews Immunology (2003).

defective in the absence of IL-7Rα signals or/and that IL-7Rα signals are required for maintaining normal LTi cell numbers. Mice double deficient for IL-7Rα and flt3L are devoid of LN [93], suggesting that synergism between cytokines might play a role in LN organogenesis.

Mice deficient for the tumor necrosis factor-related activation induced cytokine (TRANCE) also lack all LN [94]. The percentage of LTi cells among $CD45^+$ cells is decreased in the LN anlage of $TRANCE^{-/-}$ newborn mice compared to LN [94]. These results suggest that either TRANCE is required for LTi generation or for their recruitment to the LN anlage. Interestingly, TRANCE has the capacity to induce LTi cells to upregulate LT *in vitro* [87]. However, the fact that agonistic LTβR Ab injections failed to restore LN development in $TRANCE^{-/-}$ mice [94], indicates that TRANCE activity is not solely related to induction of LT expression. Mice deficient for TRANCE receptor [95] or for a signaling molecule downstream of TRANCE named TNF Receptor associated Factor 6 (TRAF6) [87] do not develop LN. It was shown that in $TRAF6^{-/-}$ embryos, LTi cells fail to cluster at the site of LN anlage [87]. LN formation can be partially rescued by intraembryonic injection of IL-7 [87]. It is still not clear, whether in this experimental setting, IL-7 restores LN development by upregulating LT on LTi cells or/and by increasing LTi cell number.

2.8.2 Chemokines

The chemokine family also plays a central role in LN organogenesis. For instance, mice deficient for the chemokine CXCL13 [96] or its cognate receptor CXCR5 [97], lack several peripheral LN. Furthermore, in mice deficient for lymphoid chemokines or chemokine receptors such as $CXCL13^{-/-}$ plt/plt mice [91] and in $CXCR5^{-/-}$ $CCR7^{-/-}$ mice [98], peripheral and mucosal LN are completely absent. Altogether, these results indicate that the chemokine-mediated recruitment of LTi cells to the LN anlage is a crucial step in LN organogenesis.

2.8.3 NFκB pathways

Engagement of the LTβR activates the non-classical NF-κB pathway, which leads to the translocation of p52/RelB heterodimers into the nucleus. The disruption of the non-classical NF-κB leads to a complete absence in LN. For instance, aly/aly mice, which have a naturally arising mutation in the NF-κB inducing kinase (*nik*) gene, lack all LN and PP [99, 100]. In the same line, $RelB^{-/-}$ mice have a very severe defect in LN development [101, 102], further indicating a crucial role for the non-classical NF-κB in LN organogenesis. These phenotypes likely result from the impairment of organizer cells to produce chemokines in response to LTβR engagement and therefore from the failure to recruit sufficient numbers of LTi cell to the LN anlage.

LTβR engagement also triggers the classical NFκB pathway, which leads to the translocation of p50/RelA heterodimers into the nucleus. p50 deficiency does not affect LN development [103]. RelA deficiency is embryonically lethal because of TNFR1-mediated signals inducing apoptosis of hepatocytes. In contrast, mice double deficient for RelA and TNFR1 are viable [104]. While $TNFR1^{-/-}$ mice have normal LN development [69], $TNFR1^{-/-}$ $RelA^{-/-}$ mice lack all LN, suggesting that RelA plays a crucial role in LN organogenesis. [104]. $TNFR1^{-/-}$ $RelA^{-/-}$ embryos have normal numbers of LTi cells, which express normal levels of LT, indicating that the LN defect is rather due to a deficiency of the mesenchymal compartment. Indeed, p50/RelA heterodimer is known to induce expression of adhesion molecules such as VCAM-1, ICAM-1and MAdCAM-1 [104], and might therefore be required for the generation of functional organizer cells.

3. Characterization of TSLP gene and protein

3.1 Identification and cloning of TSLP

The conditioned medium from the murine medullary thymic cell line Z210R.1 was shown to support the differentiation of FL cells towards the B cell lineage and the growth of the pre-B cell line NAG8/7 [105]. Fractioning this conditioned medium by chromatography revealed that this activity was independent of IL-7 and suggested the existence of a novel cytokine. This cytokine was named thymic stromal lymphopoietin (TSLP) [105]. Murine TSLP was cloned by generating a cDNA library from the Z210R.1 cell line and by screening the supernatant of cells transfected with cloned cDNAs for TSLP activity [106].

Human TSLP was identified computationally from the murine sequence [107] and from searches through sequences of the IL-7 helical cytokine family [108].

3.2 TSLP gene structure

Murine TSLP consists of 140 amino acids (Aa) of which 121 were predicted to correspond to the mature murine TSLP and 19 correspond to signal peptides [106]. TSLP contains 7 cysteine residues and 3 possible sites for N-linked glycosylation addition [106]. There is a putative site of mRNA polyadenylation [106]. Upon expression in mammalian cells, recombinant TSLP protein shows a 23kD major species and an 18kD minor species [106]. As the cDNA sequence prediction gives a MW of 14 kD for TSLP, the observed differences in MW are likely the result of differential N-linked glycosylation events [106].

Human TSLP protein is only 43% identical to the murine protein [107, 108]. The human TSLP sequence is 740 bp long, which encodes a protein of 159 Aa [107, 108]. The mature TSLP

protein and the signal sequence are respectively 131 and 28 Aa long. Human TSLP contains 2 sites for N-linked glycosylation, none of which are conserved between mouse and human TSLP [107, 108]. While human TSLP MW is predicted at 14.9 kD, practically, it resolves at 23 kD, suggesting that it is indeed glycosylated [108]. All of the 6 cysteines present in murine TSLP are conserved in the human TSLP sequence [107]. Disulfide bonds between cysteine pairs are also conserved between man and mouse [107].

3.3 TSLP genomic location and expression pattern

The murine TSLP gene localizes on chromosome 18 [106]. The human TSLP gene localizes on chromosome 5, at a position that is syntenic with murine chromosome 18 [107].

According to Northern blot and RT-PCR analysis, murine TSLP is expressed at high levels in thymus and lungs and at lower levels in spleen, LN, BM but not in PP, brain, liver nor heart [106]. Upon activation with protease allergen, murine basophils were shown to release TSLP [109].

In humans, Northern blot analysis revealed that TSLP mRNA is expressed at higher levels in heart, liver, prostate and testis while it is expressed at lower levels in lung, skeletal muscle, kidney, spleen, ovary, small intestine and colon [107]. TSLP mRNA expression was investigated in human primary cells [110]. The only hematopoietic cells reported to express large amount of TSLP mRNA are mast cells (MC) upon IgεR cross-linking [110]. TSLP was found to be mainly expressed in bronchial and mammary epithelial cells and in keratinocytes [110]. At the protein level, human TSLP was reported to be expressed by the epithelial cells of the Hassall's corpuscules [111, 112], the keratinocytes from chronic and acute AD lesions [110], by epithelial cells from inflamed tonsils [111] and by the epithelium of bronchial biopsies from asthmatic patients [113].

3.4 Regulation of TSLP expression

Since TSLP expression in human biopsies often correlated with allergic diseases, most of the effort to understand the conditions leading to its expression were concentrated on primary human epithelial cells. Hence, it was shown that human airway epithelial cells release the TSLP protein in response to IL-1 and TNF-α and to the TLR ligands PolyI:C and bacterial peptidoglycans [114]. Human bronchial epithelial (HBE) cells upregulate TSLP mRNA and release TSLP protein upon stimulation with inflammatory cytokines such as TNF-α, IL-1β, IL-4/TNF-α and IL-13/TNF-α but also with TLR ligands such as lipoteichoic acid, Poly(CpG), CpG-B and Poly I:C [115, 116]. The NF-κB site in the promoter of the *tslp* gene is crucial for production of TSLP in response to PolyI:C [115] and to IL-1β [116]. Consistent with the fact

that IL-4 and double stranded RNA synergized for TSLP production [115], the infection of HBE cells by rhinovirus in presence of exogenous IL-4 also led to the production of TSLP [115]. Altogether, these results suggest that inflammatory cytokines, TH2 cytokines, microbes and viruses might induce the release of TSLP by airway and bronchial epithelial cells.

There are few studies on TSLP expression by human skin. However, several mouse models have provided insights into the requirements and the conditions under which TSLP is expressed in the skin. Combination of IL-4, IL-5, IL-13 and TNF-α induced the production of TSLP by human skin keratinocytes [117], suggesting that "inflammatory TH2" cells might be able to induce TSLP production in inflamed skin. In mice deficient for Retinoid X receptor (RXR)α and RXR-β specifically in epidermal keratinocytes, TSLP was overexpressed in the skin leading to an AD-like disease [118]. These results show that RXR are required for direct or indirect inhibition of the *tslp* gene expression and for preventing AD-like disease [118]. Skin application of vitamin D3, which is a ligand of vitamin D receptor, induces the expression of TSLP by keratinocytes [119]. Application of vitamin D3 on the skin of $RXR\alpha^{-/-}$ $RXR\beta^{-/-}$ mice does not increase TSLP expression, suggesting that Vitamin D3 signals through the same pathway as RXR [119]. Specific deletion of Notch signaling in the skin results in a defect of skin barrier formation and subsequently the production of TSLP in the skin [120]. Altogether, these results suggest that breakdown in skin homeostasis, epithelial stress or inflammation might induce TSLP production in the skin.

4. TSLPR cloning and characterization

4.1 Cloning of TSLPR

The biological effect of TSLP requires IL-7Rα but not γ_c [121, 122], indicating that an unknown subunit is part of the TSLP receptor. Several approaches were undertaken in order to identify and clone this subunit. Recombinant murine TSLP was iodinated in order to screen cells that expressed TSLP receptor [122]. The cDNA from a murine T cell line, which bound iodinated TSLP was cloned [122]. The cDNA clones were transfected into mammalian cells and the relevant subunit of the TSLP receptor was identified from the cells binding the radiolabelled TSLP. Analysis of the cDNA from these TSLP-binding cells provided the first sequence of this subunit called TSLPR [122]. TSLPR was otherwise identified through search of sequences homologues to the cytoplasmic domain of the erythropoietin receptor in the EST database from NCBI [123]. Different groups identified and cloned human TSLPR gene by searching genomic database for homologies with human γ_c and/or with murine TSLPR sequences [108, 124, 125].

Similarly, rat TSLPR sequence was identified by searching sequences homologous with mouse TSLPR in the EST database [126].

4.2 TSLPR structure

TSLPR belongs to the type I cytokine receptors [107, 123-125, 127]. All of the receptors from this family are type I membrane glycoproteins, with the N terminal region outside of the plasma membrane and with a single hydrophobic transmembrane domain [128]. The extracellular domain, which is also the ligand-binding domain, is the most conserved region throughout the family. It usually contains 4 cysteines, which are conserved among the family, a fibronectin type III module and, just outside of the membrane spanning domain, a conserved Trp-Ser-X-Trp-Ser (WSXWS) domain [128, 129]. A box 1 domain, which is proline rich and important for the binding of the Janus family kinase (Jak) is present in the cytoplasmic domain [129].

Murine TSLPR was predicted to be either 359 [122, 127] or 370 [123] Aa long. The sixth exon contains two potential splice acceptor sites [126], explaining the difference of length in Aa observed between the first studies presenting the cloning of the *tslpr* gene. TSLPR contains 2 potential sites for N-linked glycosylation [122, 123]. While the predicted molecular mass of TSLPR is 37kD, its actual value is approximately 50 kD, indicating that TSLPR undergoes N-linked carbohydrate addition [123]. TSLPR contains a signal peptide at the NH2 terminus and a single membrane-spanning domain [122]. TSLPR shows a fibronectin type III-like domain, but only 2 of the 4 conserved cysteine residues are present [127]. The TSLPR cytoplasmic domain contains a box 1 region [122]. The exons of the murine *tslpr* gene are organized similarly to those of the γ_c chain [126]. Both genes contain 8 exons, of which the first contains the signal peptides, the sixth codes for the transmembrane domain and the last two code for the cytoplasmic domain [126].

The human TSLPR protein contains 371 Aa, and has a predicted molecular mass of 39.7 kD [124, 125]. As for the mouse, the predicted Aa sequence for human TSLPR contained a cytokine receptor module with 2 of the 4 conserved cysteine residues [125], 4 potential N-linked glycosylation sites, a fibronectin type III-like domain, a WSXWS motif, a single transmembrane domain and an intracellular “box 1” motif [124, 125].

4.3 TSLPR genomic location and expression pattern

Tslpr gene maps to the central region of chromosome 5 [122, 126, 127] and the human *tslpr* gene is present on chromosome Xp22.3 and Yp11.3 [124].

Northern blot analysis reveals that murine TSLPR is expressed in thymus, spleen, brain, heart, kidney, skeletal muscle, liver, lung, testis and BM [122, 123, 127]. TSLPR mRNA was also detected in various myeloid cell lines, in Th1 and at high levels in Th2 cell lines [127].

While Northern blot analysis gave contradictory results about human TSLPR expression pattern [124, 125], RT-PCR and real time PCR analysis showed that TSLPR is expressed in activated DC, in monocytes and in some T cell lines [108]. While it is absent from the surface of resting cells, TSLPR protein is upregulated on activated human T cells [130]. Besides, TSLPR is expressed, together with IL-7Rα, by MCs [114].

4.4 TSLP Receptor signaling

The receptor for murine and human TSLP is composed of the IL-7Rα chain and TSLPR [107, 108, 121-123]. Murine TSLP receptor signaling is independent of the γ_c chain [121, 122]. As for IL-7, TSLP-mediated signaling triggers tyrosine phosphorylation of both signal transducers and activators of transcription (STAT)5a and STAT5b proteins [121]. TSLP receptor signaling leads to the translocation of functionally active STAT5 proteins into the nucleus [131]. Importantly, STAT5 activity is required for TSLP receptor signaling, as a dominant negative version of STAT5 prevents TSLP-mediated signal transduction [131]. In contrast to IL-7, TSLP does not induce the phosphorylation of Janus kinase (Jak)1 nor of Jak3 [131]. Indeed, a dominant negative version of Jak1 does not prevent TSLP signaling [131]. While most of the molecular players involved in TSLP receptor signaling are still unknown, SOC-1, which is a negative feedback regulator of the Jak/STAT pathway, and the Tec family kinases might be implicated in the TSLP receptor signaling pathway [131].

Less is known about human TSLP receptor signaling, but TSLP stimulation was shown to induce the phosphorylation of STAT5 [107, 108] and of STAT3 [108] but not of Jak3 [107].

5. TSLP Activity

5.1 TSLP activity on B cells

5.1.1 *In vitro* B cell development

Murine TSLP was first identified for its capacity to sustain the growth of the pre-B cell line NAG8/7 [105]. These results indicated that TSLP might sustain B cell development *in vitro*. TSLP was later shown to support the growth of colonies in whole BM cultures in a similar fashion as IL-7 [106]. This activity was not observed in B220-depleted BM, indicating that TSLP was acting on B-cell precursors and not on BM myeloid cells [106]. In line with this, TSLP was shown to support the differentiation of uncommitted FL and BM precursors towards the B-cell lineage [106, 132].

In contrast to IL-7 that supports the development of FL or BM cells into $B220^+$ IgM^- pre-B cells, TSLP rather promoted the generation of $B220^+$ IgM^+ [121]. Based on these *in vitro* results, TSLP and IL-7 were proposed to differentially affect B cell development: IL-7 would promote the accumulation of pre-B cells while TSLP would rather support immature B cell development [121].

5.1.2 *In vivo* B cell development

Several gain of function approaches clearly identified the capacity of TSLP to sustain B lymphopoiesis. For instance, intra-peritoneal (i.p.) injections of recombinant TSLP in 7 days old mice led to the increase in percentage of pre-B cells in the BM [106]. In a TSLP Tg model, the absolute number of pro-B, pre-B and immature B cells in the BM was increased [133]. While both pro-B/pre-B-I and pre-B-II cells co-express TSLPR and IL-7Rα, pre-B-II cells were identified as the main TSLP-responsive population [133]. These results demonstrate that TSLP has the capacity to favor B lymphopoiesis, but the question remained whether endogenous TSLP is able to promote B cell development under physiological conditions. TSLPR deficiency does not affect neither pro-B, pre-B nor immature B cell number in 17.5 dpc FL [134] and adult BM [135], ruling out that TSLPR-mediated signals are crucial for B lymphopoiesis in fetal and adult life.

At 4 weeks of age, pre-B cells are detectable in the BM of $\gamma_c^{-/-}$ mice while they are absent in IL-7Rα$^{-/-}$ mice, indicating that endogenous TSLP might promote residual B cell development in young $\gamma_c^{-/-}$ mice [136]. The fact that pre-B cells are no longer present in the BM of 8-week old $\gamma_c^{-/-}$ animals, suggests that TSLP promotes B cell development during perinatal but not adult life [136]. Limiting dilution experiments showed that pro-B cells isolated from $RAG2^{-/-}$ FL cells but

not from adult RAG2$^{-/-}$ BM are TSLP-responsive. The difference in TSLP-responsiveness between adult and fetal precursors was interpreted as the explanation for the discrepancy in B cell development between young and adult $\gamma_c^{-/-}$ mice [136]. Cell limiting dilutions experiments showed that CD19^{+} pro-B cells from adult RAG2$^{-/-}$ BM failed to respond to TSLP [137]. On the contrary, CD19^{+} precursors from RAG2$^{-/-}$ mice that carry a Tg coding for the immunoglobulin heavy chain, and that therefore express the pre-BCR, were TSLP-responsive [137]. These results led the authors to speculate that pre-BCR was required for adult BM precursors to be TSLP-responsive.

IL-7R$\alpha^{-/-}$ mice have lower absolute pro-B, pre-B and immature B cell number in the BM than IL-7$^{-/-}$ mice [135]. However, 3 week-old IL-7$^{-/-}$ TSLPR$^{-/-}$ mice have identical number of pro-B and pre-B cells and significantly lower numbers of immature B cells than IL-7$^{-/-}$ controls. These results show that the endogenous TSLP activity may explain the phenotypic differences in immature B but not the pro-B/pre-B compartments of IL-7$^{-/-}$ and IL-7R$\alpha^{-/-}$ mice [135]. In line with this, at 17.5 dpc, the FL of IL-7$^{-/-}$ TSLPR$^{-/-}$ embryos have no further reduction in pro-B and pre-B cell number compared to IL-7$^{-/-}$ fetuses [134].

Chimera experiments showed that HSC isolated from IL-7R$\alpha^{-/-}$ FL could differentiate into B cells while HSCs isolated from adult IL-7R$\alpha^{-/-}$ BM could not [138]. These results suggest that fetal progenitors can generate B cells independently of IL-7 and TSLP and that IL-7Rα signals are differentially required for generating B cells during fetal and adult life.

5.1.3 Peripheral B cell compartments

In a Tg model, high concentration of TSLP in the serum increased the proportion of B220^{+} CD93^{+} immature and follicular B cells in the spleen while the MZ compartment was lacking [133]. While these experiments showed that TSLP has the capacity to alter peripheral B cell compartments, they did not address its putative function in physiological concentrations. Neither the absolute mature B cell number [135], nor the ratio of IgM^{+}/IgD^{+} cells [139] differ between the spleen of TSLPR$^{-/-}$ and WT animals, showing that TSLP does not play a non-redundant role in peripheral B cell homeostasis. However, several lines of evidence suggest that TSLP might contribute to B cell homeostasis. For instance, a residual splenic B220^{+} population present in Jak3$^{-/-}$ mice was absent from Jak3$^{-/-}$ TSLPR$^{-/-}$ mice [139]. Splenic mature B cell number is 10 times lower in IL-7R$\alpha^{-/-}$ mice compared to $\gamma_c^{-/-}$ mice [136], suggesting that endogenous TSLP promotes B cell accumulation independently of IL-7. Indeed, IL-7$^{-/-}$ TSLPR$^{-/-}$ mice have significantly lower numbers of mature B cells in the spleen than IL-7$^{-/-}$ controls [135]. Together these results showed that endogenous TSLP is not crucial for B cell homeostasis but might contribute to it in the absence of IL-7.

5.1.3 TSLP and B-1 cells

In reconstitution experiments, IL-7Rα$^{-/-}$ FL precursors generate 10-fold less B-1 cells in the peritoneum of RAG2$^{-/-}$ γc$^{-/-}$ recipient than γc$^{-/-}$ precursors, suggesting that TSLP might play a role in B-1 cell generation and/or homeostasis [137]. It was reported that adult and fetal BM lineage^{-} B220^{-} CD19^{+} progenitors gave rise to B-1 cells upon transfer *in vivo*. In the presence of TSLP, these progenitors can differentiate *in vitro* towards cells with a B-1 cell-like phenotype and expand without losing their reconstitution potential [140]. Adult TSLP Tg mice have increased percentage of B-1b in the peritoneal cavity in respect to control mice [133]. As B-1 cells do not express IL-7Rα and are therefore unresponsive to TSLP, it was proposed that TSLP was rather acting on the lineage^{-} B220^{-} CD19^{+} B-1 progenitors. Indeed, TSLP Tg expression increases absolute numbers of lineage^{-} B220^{-} CD19^{+} progenitors [133]. Mice that overexpress TSLP from fetal life onwards, have increased number of both B-1a and B-1b cells in the peritoneum 2 weeks after birth but show an exclusive increase in B-1b cells 4 weeks after birth [133]. These results unambiguously show that TSLP has the capacity to generate B-1 B cells and/or to contribute to their homeostasis. However, they did not answer the question whether endogenous TSLP plays a substantial role *in vivo*.

The number of lineage^{-} B220^{-} CD19^{+} cells was normal in the BM of TSLPR$^{-/-}$ newborn mice [135], ruling out an essential function for TSLP in supporting the generation or maintenance of this population. Furthermore, B-1 B cell progenitor cell numbers were not further decreased in IL-7$^{-/-}$ TSLPR$^{-/-}$ compared to IL-7$^{-/-}$ controls [135], showing that endogenous TSLP does not contribute to the generation and/or maintenance of B-1 B cell progenitors. In line with this, γc$^{-/-}$ TSLPR$^{-/-}$ and γc$^{-/-}$ mice display similar numbers of peritoneal B-1 B cells [141], confirming that the B-1 cell pool is not regulated by TSLP.

5.1.4 TSLP and B-cell lymphoproliferative disorder

TSLP stimulates the proliferation and inhibits the apoptosis of murine and human acute lymphoblastic leukemia (pre-B ALL) cells [142] and of the human acute myeloid leukemia (AML)-derived cell line MUTZ-3 [107]. These results indicate that TSLP might sustain the development of both lymphoblastic and myeloid leukemia. This hypothesis is supported by a murine model in which prenatal and perinatal overexpression of TSLP leads to the expansion of B220^{+} CD43^{-} IgM^{-} pre-B cells and eventually to lethal B-cell lymphoproliferative disorder (B-LPD) [120]. TSLP was proposed to promote B-LPD only during the perinatal period because such disease was absent in animals overexpressing TSLP later than 2 weeks after birth [120].

5.2 TSLP activity and dendritic cells

Unexpectedly for a cytokine initially discovered for its ability to promote B cell development *in vitro* [105], TSLP turned out to be a potent maturation factor for human and murine dendritic cells (DC). In fact, upon TSLP stimulation, human DC upregulate MHC class II molecules, the activation markers DC-LAMP, CD40, CD80 and CD86, and produce the CCR4 ligands CCL17 (TARC) and CCL20 (MDC) [108, 110-112, 143, 144]. In the murine system, TSLP induced the upregulation of CD80, CD86 and of MHC class II molecules on DC surface [145-147] and promote CCL17 release [147]. TSLP-activated DC can prime naïve $CD4^+$ T cells from human and murine origin [110, 145], formally showing that TSLP acts as a maturation factor for DC.

5.3 TSLP activity on T cells

5.3.1 Thymopoiesis

Several evidences showed that TSLP could support T cell development *in vitro*. Recombinant TSLP increased the total thymocyte number in fetal thymus organ culture (FTOC) experiments [148]. Moreover, the percentage of DN1 and DN2 cells incorporating BrdU was increased, suggesting that TSLP promoted their proliferation [148]. In line with this, TSLP was shown to support DN thymocyte proliferation in synergy with IL-1β [106] and, in 2 weeks FTOC, the numbers of DN1 and DN2 cells from $TSLPR^{-/-}$ lobes were lower than those of WT controls [148].

Daily injection of recombinant TSLP into WT and $\gamma_c^{-/-}$ mice can transiently increase thymic cellularity [141], demonstrating that TSLP has the capacity to promote T lymphopoiesis. However, thymus cellularity, percentage and number of DN, DP, CD4 SP and CD8 SP cells are normal in $TSLPR^{-/-}$ mice [139, 141]. Detailed analysis of DN cells showed that none of the DN compartments were affected by TSLPR deficiency [149], suggesting that endogenous TSLP does not significantly contribute to adult T cell development. However, thymus cellularity and the number of DN3, DN4, DP, CD4 SP and CD8 SP thymocytes in IL-7Rα$^{-/-}$ mice are significantly lower than those of $IL\text{-}7^{-/-}$ mice [149], suggesting that TSLP might play a role in T lymphopoiesis in absence of IL-7. To clarify this issue, mice deficient for both IL-7 and TSLPR were analyzed [149]. Absolute thymocyte number, DN, DP, CD4 SP and CD8 SP cell number were identical in $IL\text{-}7^{-/-}$ mice and $IL\text{-}7^{-/-}$ $TSLPR^{-/-}$ animals, demonstrating that endogenous TSLP does not substantially participate to T lymphopoiesis [149]. These results suggest that there might exist a yet unidentified alternative ligand for IL-7Rα, or that others pathways might "cross-activate" IL-7Rα.

5.3.2 Regulatory T cells

The first evidences suggesting that TSLP might be involved in Treg generation arose from studies in the human system. Human TSLP-activated DC induce the upregulation of FoxP3 mRNA in $CD4^+$ $CD8^-$ thymocytes. They also promote the differentiation of $CD4^+$ $CD25^+$ T cells from $CD4^+$ $CD8^-$ $CD25^-$ thymocytes [112]. The resulting $CD4^+$ $CD25^+$ T cells share functional features of Treg as they do not proliferate in response to anti-CD3/anti-CD28 stimulation and inhibit $CD4^+$ $CD25^-$ T cell proliferation *in vitro* [112]. Human Hassall's corpuscles were reported to express TSLP and to be associated with activated DC [112]. Based on the fact that TSLP-activated-DC promote Treg development *in vitro*, it was proposed that TSLP, by activating medulla DC, might indirectly induce Treg differentiation in humans. However, as it was shown later for peripheral $CD4^+$ T cells [130], the possibility that TSLP acts directly on CD4 SP thymocytes might have been underestimated.

TSLP was also reported to promote Treg generation in mice. However, in the murine system, TSLP would act rather on T cells than on DC. For instance, FTOC experiments showed that TSLP increases the percentage and absolute cell number of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg [150]. Moreover, TSLP promoted the upregulation of FoxP3 mRNA in purified CD4 SP thymocytes [150]. In line with this, the percentage of $FoxP3^+$ cells within $CD4^+$ $CD25^+$ population was higher in thymocyte cultures in the presence of TSLP [146]. The percentage of DO11.10 thymocytes, which differentiated towards a $CD4^+$ $CD25^+$ $FoxP3^+$ phenotype in presence of OVA-presenting APC, was 2-fold increased in presence of TSLP [146]. In contrast to the human system [112], TSLP-activated $CD11c^+$ DC fail to induce the differentiation of $CD4^+$ $CD8^-$ $CD25^-$ thymocytes into Treg [146]. Together with the fact that TSLP induce FoxP3 expression in purified CD4 SP thymocytes, these results show that murine TSLP promotes Treg development by acting directly on the CD4 SP thymocytes [146, 150].

5.3.3 Peripheral T cell compartments

TSLP-activated human DC promote the expansion of autologous naïve $CD4^+$ T cells in the absence of exogenous Ag, while DC stimulated with other compounds, such as CpG, Poly I:C or CD40L fail to do so [111]. These results suggest that, in humans, TSLP might promote peripheral T cell expansion indirectly by activating DC.

In mice, TSLP injection increases $CD4^+$ T cell number in the spleen of $\gamma_c^{-/-}$ mice indicating that TSLP promotes $CD4^+$ T cell expansion [141]. In addition, TSLP favors the proliferation and the survival of TCR-stimulated $CD4^+$ T cells *in vitro* [141, 145], demonstrating that TSLP acts directly on murine T cells. This was confirmed by the fact that $CD4^+$ T cells isolated from WT mice expanded more than $TSLPR^{-/-}$ $CD4^+$ T cells when injected into sublethally irradiated $\gamma_c^{-/-}$ recipients [141]. However, absolute numbers and percentages of splenic $CD4^+$ and $CD8^+$ T cells

were identical in TSLPR$^{-/-}$ and WT mice [139, 141], showing that TSLP does not have a crucial role neither on CD4$^+$ nor CD8$^+$ T cell homeostasis.

5.3.4 TH2 differentiation

Human TSLP-activated DC were shown to prime naïve CD4$^+$ T cell in allogeneic mixed lymphocyte reactions [110], demonstrating that TSLP can act as a potent DC maturation factor. Furthermore, naïve CD4$^+$ T cells primed with TSLP-activated DC differentiated towards an "inflammatory TH2" phenotype. Indeed, the resulting T cells produced IL-4, IL-5, IL-13 and the pro-inflammatory cytokine TNF-α, but neither IL-10 nor IFN-γ [110]. Naïve CD8 T cells primed with TSLP-activated DC produced IFN-γ, TNF-α, IL-5 and IL-13 but were weakly cytotoxic [143]. DC primed with TSLP upregulate the expression of OX40L at both the mRNA and protein level [144]. This is of importance, because OX40L signals are mandatory for the priming of naïve CD4$^+$ T cells towards the "inflammatory TH2" phenotype [144]. Altogether, these results suggest that TSLP indirectly promotes the differentiation of CD4$^+$ and CD8$^+$ T cells towards the "inflammatory TH2" phenotype by activating DC.

Recent findings showed that TSLPR expression is upregulated upon CD4$^+$ T cell activation [130]. In contrast to resting cells, activated human CD4$^+$ T cells express TSLPR and phosphorylate STAT5 upon TSLP stimulation [130]. As TSLP increases the level of CD25 expression on activated T cells, it allows them to increase their IL-2 responsiveness [130]. Hence, CD4$^+$ T cells activated in the presence of TSLP proliferate better than cells without exogenous cytokine [130]. The fact that TSLPR expression is upregulated upon CD4$^+$ T cell activation [130], suggest that TSLP could also directly affect T cell differentiation.

It seems that TSLP influences murine T cell differentiation by acting on the T cell itself and independently of DC. For instance, purified murine naïve CD4$^+$ T cells activated with plastic bound anti-CD3 in the presence of TSLP differentiated towards IL-4, IL5 and IL13-producing TH2 cells [151]. TSLP-mediated TH2 differentiation was dependent on IL-4 and STAT-6 [151]. In an *in vivo* model of allergy, TSLP was instrumental for the subsequent priming of naïve CD4$^+$ T cells towards the TH2 phenotype in response to cysteine protease allergen [109]. Indeed, inhibition of TSLP activity with neutralizing Ab blocked the TH2 differentiation [109]. The TH2 differentiation was not accompanied by the upregulation of OX40L by DC, suggesting that TSLP promoted TH2 differentiation by acting directly on T cells [109].

5.4 TSLP and allergic diseases

5.4.1 Atopic Dermatitis (AD)

Skin explants from lesions of AD patients contain very high levels of TSLP protein [110]. The Langerhans cells in AD tissues show an activated phenotype and are found in the dermis, while control sections show inactivated Langerhans cells in the epidermis [110]. TSLP-activated Langerhans cells might play a crucial role in the initiation and the maintenance of AD, because they have the capacity to prime naïve $CD4^+$ T towards an "inflammatory TH2" phenotype [110, 152]. The "inflammatory TH2" phenotype results from the TSLP-mediated OX40L upregulation by DC [144]. Most of the AD lesion-infiltrating T cells express the prostaglandin D2 receptor CRTH2 and have a TH2 phenotype [153]. TSLP-activated DC induce the expansion of such $CRTH2^+$ $CD4^+$ T cells [153], suggesting that TSLP mediates the maintenance of detrimental TH2 cells. Hence the current model is that keratinocyte derived-TSLP triggers AD by activating skin-homing DC and Langerhans cells, which in turn prime naïve $CD4^+$ T cells towards an "inflammatory TH2" phenotype and promotes the expansion of pro-allergic $CRTH2^+$ cells.

Several animal models support this scenario of AD pathogenesis. For example, mice overexpressing TSLP in the skin develop an AD-like disease [118, 154] with epidermal hypertrophy, hyperkeratosis, and mononuclear cell infiltrates [154]. Skin sections showed infiltrates of eosinophils, MCs and lymphocytes and the accumulation of Langerhans cells in the dermis, but not in the epidermis [154]. $CD4^+$ T cells from TSLP Tg mice showed a TH2 phenotype, as they expressed IL-4, IL-5 and TNF-α but no INF-γ upon restimulation [154]. Levels of IgE and IgG1 were highly increased in TSLP Tg animals compared to non-Tg littermates [154]. Intradermal injection of TSLP in mice leads to ear thickness and increased serum IgE levels [155]. All of these effects can be diminished by continuous injection of neutralizing anti-OX40L Ab [155], suggesting that OX40L might play a crucial role in TSLP-mediated AD.

Importantly, several murine models showed that B and T cells do not play a crucial role in the development of TSLP-mediated AD. For instance, AD-like disease developed in $TCR\beta^{-/-}$ mice overexpressing TSLP in the skin, as shown by massive infiltration by eosinophils and MCs [154]. Induction of TSLP in the skin by application of Vitamin D3 led to an AD-like disease in $RAG^{-/-}$ mice [119], demonstrating that T and B cell presence is not required for the initiation and development of AD.

5.4.2 Allergic asthma

In lung biopsies from asthmatic patients there was an increase in mRNA and protein of TSLP, CCL17 and CCL22 compared to non-asthmatic controls [113]. In this study, the main producers of these molecules were epithelial cells [113]. It was proposed that TSLP produced in lung epithelial cells might play a crucial role in asthma pathogenesis by triggering TH2 differentiation and recruitment of TH2 effectors [113]. In line with this hypothesis, mice overexpressing TSLP in the lungs developed an asthma-like disease characterized by abnormal cellular infiltrates in the lung [147]. Lung infiltrates were composed of eosinophils and $CD4^+$ T cells, most of which had a TH2 phenotype and express CCR4 [147]. TSLP Tg mice had increased IgE levels in the serum compared to non-Tg littermates and showed a severe remodeling of airways [147]. These results suggest that TSLP might play an important role in triggering and/or sustaining allergic asthma.

In an OVA-induced allergic asthma model, intranasal challenge with OVA increases TSLP mRNA in the lungs and TSLP concentrations in the bronchoalveolar lavage (BAL) of WT mice [147]. Upon OVA challenge, WT controls show perivascular inflammation, cell hyperplasia, infiltration of eosinophils and neutrophils, high levels of IL-5 and IL-13 in BAL and high levels of IgE in the serum [145, 147]. On the contrary, OVA-challenged $TSLPR^{-/-}$ mice do not develop symptoms of asthma [145, 147], showing that endogenous levels of TSLP play indeed a crucial role in asthma pathology. Consistently, the treatment with a TSLPR-Fc fusion protein of OVA-challenged WT mice limited the development of allergic asthma [145].

In a murine model of lung inflammation, nasal sensitization with TSLP induces recruitment of lymphocytes and eosinophils to the lung, increased levels of the Th2 cytokines IL-4, IL-5, IL-13 in the BALs, and increased levels of serum IgE [155]. All of these effects were prevented by continuous injection of neutralizing anti-OX40L Ab [155]. In a non-human primate model, an asthma-like disease was induced by sensitization with house dust mite (HDM) Ag. This induced the expression of TSLP by lung cells [155]. Treatment with a blocking anti-OX40L Ab reduced the concentration of IL-5 and IL-13 cytokines in the BALs and the titer of specific anti-HDM IgG [155].

5.4.3 TSLP and mast cells

There are several lines of evidence suggesting that TSLP activates MCs, which in turn exacerbate inflammation and allergic responses. For instance, combination of IL-1, TNF and TSLP stimulates human MCs, which in turn produced the TH2 cytokines IL-5, IL-13, IL-6 and the chemokines CXCL8 and CCL1 [114]. Furthermore, in co-culture experiments with biopsies from lesions of AD patients, MCs release IL-13 in a TSLP-dependent fashion [114].

Mouse models showed that MCs can trigger epithelial cells to produce TSLP. Hence, induction of rhinitis with OVA leads to the accumulation of TSLP protein in the nasal epithelium in a MC-dependent fashion [156]. Importantly, mice with MCs deficient for FcγR (and therefore for IgE receptor) do not upregulate TSLP in the epithelium in response to OVA challenge [156]. These results suggest that MC regulate TSLP expression in nasal epithelium and might play a pivotal role in allergic rhinitis pathogenesis. In this line, neutralizing anti-TSLP Ab decreases pathological symptoms of OVA-induced rhinitis [156]. Hence MCs induce the expression of TSLP by epithelial cells and respond to it, suggesting that MC might be at the center of a viscous cycle promoting inflammatory and allergic reactions.

Altogether, these studies on AD, asthma and rhinitis indicate that TSLP is likely to play a central role in the pathogenesis of allergic diseases, and that TSLP or some of its downstream effectors such as OX40L represent promising therapeutic targets.

C. Aim of the study

While TSLP was identified as a cytokine sustaining B cell development *in vitro*, its function on hematopoiesis *in vivo* remained elusive. Our first aim was to characterize the function of TSLP in adult lymphopoiesis. Several studies suggested that IL-7 and TSLP had overlapping activity *in vitro*. Hence, to study the function of TSLP in *vivo*, we have generated mice overexpressing TSLP on an IL-7 deficient background (manuscript 1).

As there are no data available about the function of TSLP in LN organogenesis, our second goal was to characterize the function of TSLP in LN formation and to investigate whether it had the capacity to substitute IL-7 in LN development. To answer these questions, we have used IL-$7^{-/-}$ and $RAG^{-/-}$ $\gamma_c^{-/-}$ mice overexpressing TSLP (manuscript 2).

D. Results

1. Manuscript 1:

Increased TSLP availability restores T- and B-cell compartments in adult IL-7-deficient mice

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Increased TSLP availability restores T- and B-cell compartments in adult IL-7–deficient mice

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Interleukin 7 (IL-7) plays a crucial role in adult lymphopoiesis, while in fetal life its effect can be partially compensated by TSLP. Whether adult hematopoietic progenitor cells are unresponsive to TSLP or whether TSLP is less available in adult microenvironments is still a matter of debate. Here, we show that increased TSLP availability through transgene (Tg) expression fully restored lymphopoiesis in IL-7–deficient mice: it rescued B-cell development, increased thymic and splenic cellularities, and restored double-negative (DN) thymocytes, αβ and γδ T-cell generation, and all peripheral lymphoid compartments. Analysis of bone marrow chimeras demonstrated that hematopoietic progenitor cells from adult wild-type mice efficiently differentiated toward B- and T-cell lineages in lethally irradiated IL-7 deficient mice provided TSLP Tg was expressed in these mice. In vitro, TSLP promoted the differentiation of uncommitted adult bone marrow progenitors toward B and T lineages and the further differentiation of DN1 and DN2 thymocytes. Altogether, our results show that adult hematopoietic cells are TSLP responsive and that TSLP can sustain long-term adult lymphopoiesis. (Blood. 2007;110:3862-3870)

Introduction

Lymphopoiesis is regulated by a number of cytokines that control the proliferation, differentiation, and survival of hematopoietic progenitor cells. Among these factors, IL-7 plays an essential role in B- and T-cell development.[1-3] IL-7 signals through the IL-7 receptor that is composed of the common γ-chain (γ_c or CD132) and the IL-7Rα (CD127) chain.[4] IL-7Rα can also associate with the thymic stromal lymphopoietin receptor (TSLPR) chain to form the receptor for the cytokine TSLP.[5,6] TSLPR signaling occurs independently of γ_c[5-7] and does not rely on the phosphorylation of any known Janus family kinases but leads to the translocation of functional Stat5 to the nucleus.[7,8]

TSLP was originally cloned from a murine thymic stromal cell line.[9] While displaying only 43% protein identity,[10,11] human and mouse TSLP share similar biologic functions. Both promote homeostatic expansion of $CD4^+$ T cells,[12,13] trigger dendritic cell maturation,[14-16] induce TH2 differentiation,[14-18] and are important factors in triggering inflammatory allergic responses.[14-16,18,19]

Several studies have suggested that TSLP may play a role in fetal rather than adult B-cell development. For instance, at 4 weeks of age, $\gamma_c^{-/-}$ mice, which are responsive to TSLP but not to IL-7, showed residual B lymphopoiesis. In contrast, in 4-week-old IL-7Rα$^{-/-}$ mice, which are unresponsive to both TSLP and IL-7, B-cell development was absent. However, at 12 weeks of age, both mouse strains were devoid of B lymphopoiesis.[20] Moreover, although both adult IL-7$^{-/-}$ and IL-7Rα$^{-/-}$ mice lack γδ T cells, fetal IL-7$^{-/-}$ but not IL-7Rα$^{-/-}$ thymi contained γδ T cells.[3,21] It is currently unknown whether these differences reflect distinctive TSLP responsiveness of fetal versus adult hematopoietic cells, or if they are the result of decreased availability of a biologically active form of TSLP in adult mice.

TSLP was shown to support B-cell development in vitro.[7,9,22] However, there are conflicting results on the role of TSLP for B-cell development in vivo. TSLPR$^{-/-}$ mice had no defect in B-cell development,[12,23] while TSLP Tg expression either promoted[24] or inhibited[25] B lymphopoiesis.

Experimental data showing a role for TSLP in T lymphopoiesis are limited. TSLP could induce a moderate in vitro proliferation of adult double-negative (DN) thymocytes in synergy with IL-1, but failed to sustain the proliferation of fetal thymocytes.[26] While TSLPR$^{-/-}$ mice had no defect in T development, mice lacking both the γ_c and the TSLPR chains showed lower thymic cellularity than $\gamma_c^{-/-}$ mice.[12] Moreover, the injection of recombinant TSLP could transiently increase the number of thymocytes in $\gamma_c^{-/-}$ mice.[12] Although these data suggest that TSLP may be involved in T lymphopoiesis, the developmental stage at which TSLP exerts its biologic function has not been clearly identified.

To understand the function of TSLP on hematopoiesis in vivo, TSLP-transgenic (Tg) mice were generated and backcrossed to an IL-7–deficient background. The TSLP Tg expression was driven by the keratin 14 (K14) promoter that targets gene expression to epithelial cells.[27]

In this study, we show that TSLP Tg expression rescued B-cell development, increased the thymic cellularity, and rescued the thymic architecture in IL-7$^{-/-}$ animals. DN1 and DN2 thymocytes but also γδ T cells developed in response to TSLP. Moreover, adult WT bone marrow (BM) cells differentiated normally into B and T lineages and restored peripheral compartments when adoptively transferred into lethally irradiated IL-7$^{-/-}$ K14-TSLP Tg recipients. In addition, we observed a strong effect of TSLP overexpression on peripheral myeloid cell expansion. In vitro, TSLP promoted

Submitted February 13, 2007; accepted August 11, 2007. Prepublished online as *Blood* First Edition paper, August 16, 2007; DOI 10.1182/blood-2007-02-074245.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

the generation of B and T lineage cells from early lymphoid/myeloid BM progenitors and supported the differentiation of DN1 and DN2 thymocytes. Altogether, we show here that TSLP is a potent cytokine able to support adult B- and T-cell development and to expand both lymphoid and myeloid compartments in peripheral lymphoid organs.

Materials and methods

Mice

All mice were bred and maintained in our animal facility under specific pathogen-free conditions. The animal experiments received the approval of the Cantonal Veterinary Office of the city of Basel, Switzerland.

C57BL/6 mice were purchased from RCC (Itingen, Switzerland). IL-7$^{-/-}$ mice were previously described.[1] RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice on C57BL/6 background were kindly provided by Jörg Kirberg (MPI, Freiburg, Germany).

For generation of transgenic mice, the murine TSLP open reading frame[26] was inserted into the pK14pA construct.[28] The transgene DNA was microinjected into fertilized (C57BL/6 × DBA/2) F2 embryos to generate transgenic founders. Progenies of the founder mice were phenotypically identical. One line was backcrossed with C57BL/6 mice for at least 8 generations. The genotype of TSLP Tg mice was identified by polymerase chain reaction (PCR) from genomic DNA with the following primers: 5′-TGCAAGTACTAGTACGGATGGGGC-3′ from the 5′ coding region and 5′-GGACTTCTTGTGCCATTTCCTGAG-3′ from the 3′ coding region. PCR conditions were 94°C for 2 minutes followed by 34 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds, and finally 72°C for 5 minutes. PCR products were separated on a 1% agarose gel and detection of a 323-bp fragment positively identified TSLP Tg presence.

For BM chimera, IL-7$^{-/-}$ K14-TSLP Tg mice, IL-7$^{-/-}$ littermates, and C57BL/6 WT mice were lethally γ-irradiated (9 Gy) and intravenously injected with 10^7 total BM cells from 8-week-old WT mice (CD45.1$^+$). Six weeks or 6 months after reconstitution, BM chimeric mice were analyzed.

Immunization

NP stands for 4-hydroxy-3-nitrophenyl-acetyl. Ten- to 12-week-old IL-7$^{-/-}$ K14-TSLP, IL-7$^{-/-}$, and C57BL/6 mice were immunized intraperitoneally with 50 μg alum-precipitated NP-ovalbumin (NP-OVA) or intravenously with 100 μg NP-Ficoll. Sera were collected prior to immunization and 10 or 14 days after NP-Ficoll or NP-OVA immunization, respectively.

ELISA

NUNC Immunoplate Maxisorb F96 plates (NUNC, Roskilde, Denmark) were coated with 5 μg/mL NP-BSA at 4°C. Plates were incubated with serial dilutions of sera for 2 hours at room temperature. After washing, alkaline phosphatase–conjugated rat anti–mouse IgM (R6–60.2; PharMingen, San Diego, CA) or goat anti–mouse IgG (Southern Biotechnology Associates, Birmingham, AL) was added to the plates. Plates were developed with FAST pNPP (*p*-nitrophenylphosphate; Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. Serum titers were determined using the end point titer procedure. TSLP enzyme-linked immunosorbent assay (ELISA) was performed with the DuoSet ELISA kit (R&D, Abington, United Kingdom) according to the manufacturer's instructions.

Cell culture

OP9[29] and OP9-DL1 cells[30] were cultured as previously described.[31] Semiconfluent cultures of stromal cells were γ-irradiated (30 Gy) before adding sorted DN1, DN2, or early progenitors for lymphoid and myeloid progenitors (EPLMs). Sorted cells (5×10^3)/well of a 24-well plate were cultured in supplemented IMDM on OP9 or OP9-DL1 cells in the presence of either 100 U/mL IL-7 or 500 U/mL TSLP or without additional cytokine. At day 7, cells were harvested, counted, stained, and analyzed by flow cytometry.

Flow cytometry and cell sorting

FITC-, PE-, PE-Cy7–, APC-, or biotin-conjugated α-CD4 (GK1.5), α-CD8α (53–6.7), α-CD19 (1D3), α-CD21 (CR2/CR1), α-CD23 (B3B4), α-CD25 (7D4), α-CD44 (IM7), α-CD45.1 (A20), α-γδ TCR (GL3), α-Vγ3 TCR (536), and α-NK1.1 (PK 136) Abs were purchased from BD Biosciences (Basel, Switzerland). α-CD3 (145–2C11), α-CD11b (M1/70), α-CD24 (M1/69), α-CD62L (MEL-14), α-B220 (RA3–6B2), α-TER119 (TER-119), and α-TCRβ chain (H57–597) were from Biolegend (San Diego, CA). α-CD45 (30-F11), α-CD71 (R17217), α-CD117 (2B8), and α-Gr-1 (RB6–8C5) Abs were from eBioscience (San Diego, CA). α-Vγ1.1-PE (2.11) and α-Vγ2-PE (UC3–10A6) were obtained from A. Wilson (LICR, Epalinges, Switzerland). α-CD93 (PB493) Ab was produced in our laboratory and labeled with biotin by standard methods. As secondary reagent, streptavidin-PE and streptavidin-PE/Cy7 (Biolegend) were used. Flow cytometry acquisition was performed with a FACSCalibur (BD Biosciences) and data were analyzed using Flowjo software (Tree Star, Eugene, OR). EPLM sorting was done as previously described.[31] Briefly, erythrocyte-depleted BM cells were sorted as B220$^+$ CD19$^-$ CD117$^+$ CD93$^+$ NK1.1$^-$ cells. DN1 and DN2 thymocytes were sorted as CD117^{high} CD25$^-$ CD44$^+$ and CD117^{high} CD25$^+$ CD44$^+$ cells, respectively.[32] Cell sorting was done using a FACS Aria (BD Biosciences) and reanalysis of sorted cells indicated that they were more than 98% pure.

V(D)J rearrangement analysis

B220$^+$ CD19$^+$ double-positive cells (5×10^5) from IL-7$^{-/-}$ K14-TSLP Tg mice (8 weeks old) were fluorescence-activated cell sorting (FACS) sorted, and genomic DNA was isolated by standard protocols. DNA amplification was carried out in 2 rounds of PCR. The first round of PCR amplification contained 5 different 5′ V_H primers recognizing the V_H families V_HJ558, V_H7183, V_HQ52, V_HJ606, V_HS107, V_HX24, and V_HGAM308 together with a nested 3′ J_H4 primer. In the second round, 2 μL of the first PCR product was reamplified with a V_H family–specific 5′ primer and the same nested 3′ J_H4 primer. PCR products were purified on a 1.5% agarose gel by cutting the band with the appropriate size of a V(D)J rearrangement followed by TA cloning. A set of clones was sequenced using the Big Dye Terminator method and the automated DNA sequencer 377 (Applied Biosystems, Weiterstadt, Germany). Sequence analysis was performed with 4Peaks (Apple Computer, http://mekentosj.com/4peaks/), IMGT/JunctionAnalysis (The International Immunogenetics Information System, http://imgt.cines.fr/cgi-bin/IMGTjcta.jv) and IgBLAST (http://www.ncbi.nlm.nih.gov/projects/igblast/).[33]

Immunofluorescence confocal microscopy

Acetone-fixed thymic sections (8 μm) were incubated with polyclonal α-K5 (PRB-160B; Covalence, Princeton, NJ), α-CD3-biot (145–2C11; eBioscience), and α-K8-Cy5 Abs (TROMA-1; Developmental Studies Hybridoma Bank, University of Iowa) followed by incubation with streptavidin–Alexa 488 (Molecular Probes, Leiden, the Netherlands) and goat α-Rabbit–Alexa 555 (Molecular Probes), and finally embedded in Fluorsave (Calbiochem, San Diego, CA). Images were captured on a Zeiss LSM 510 Meta Laser Confocal Scanning Confocal Microscope System (Carl-Zeiss, Fedbach, Switzerland). Overlays of blue (Cy5), red (Alexa 455), and green (Alexa 488) stainings were colored by computer-assisted management of confocal generated data with Zeiss LSM 510 software version 3.2.

Quantitative real-time PCR

RNA extraction was performed with the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) followed by DNase digestion with RQ1 RNase-Free DNase (Promega, Madison, WI). RNA (750 ng) was used to perform the Reverse Transcription with Oligo dT (Promega) and dNTPs (Roche, Rotkreuz, Switzerland) with the Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed with Sensimix (Quantace, Watford, United Kingdom) on a Rotor Gene RG-3000 (Corbett Research, Sydney, Australia). The following primers were used: TSLP FWD: AGGCTACCCTGAAACTGAG, TSLP RVS: GGAGATTGCATGAAGGAATACC, TBP FWD: CGTGAATCTTGGCTGTAAACT, TBP RVS: GTCCGTGGCTCTCTTATTCT. TSLP and TBP primer pairs had

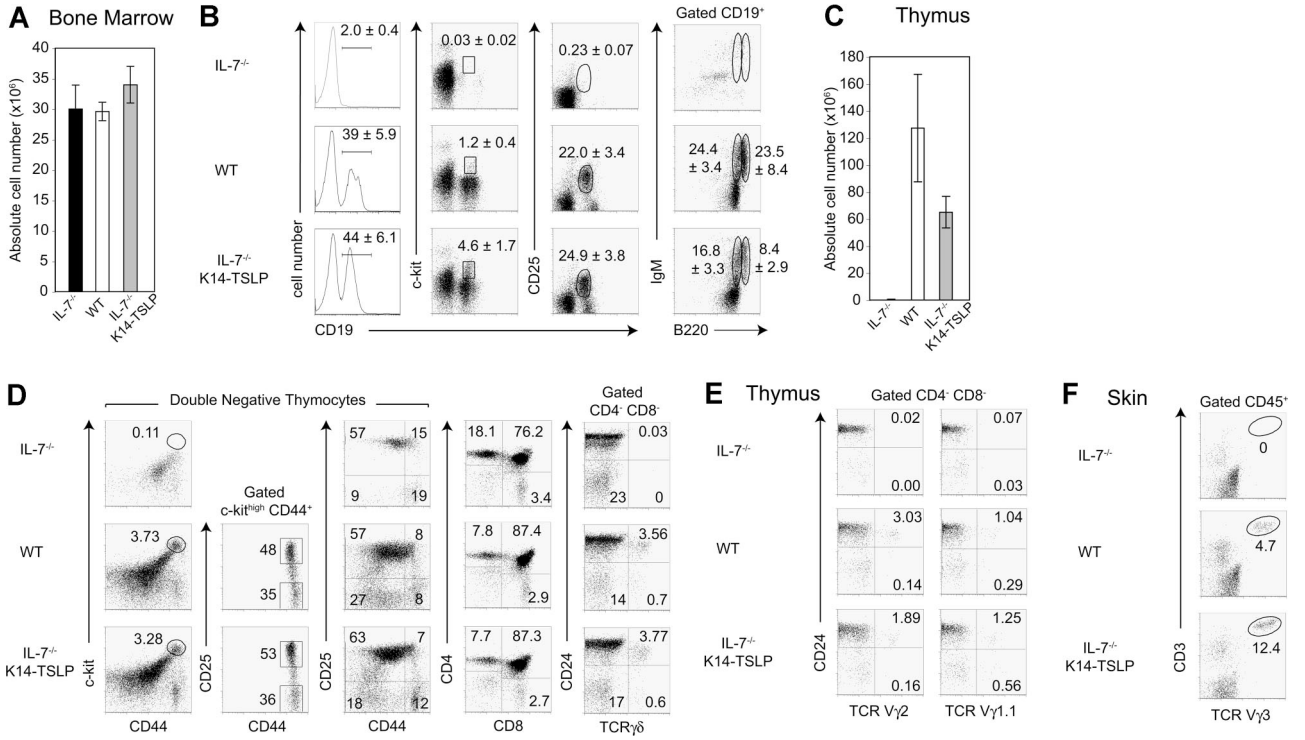

Figure 1. TSLP Tg expression rescues B- and T-cell development in IL-7$^{-/-}$ mice. IL-7$^{-/-}$, WT, and IL-7$^{-/-}$ K14-TSLP Tg mice were analyzed at 12 weeks of age. (A) Absolute cell number per 2 femurs. (B) Regions indicate the pro-B/pre-B-I (CD19$^+$ c-kit$^+$), pre-BII (CD19$^+$ CD25$^+$), mature (CD19$^+$ B220^{high} IgM$^+$), and immature (CD19$^+$ B220$^+$ IgM$^+$) B cells in the BM. Numbers are mean and standard deviation of percentage (n = 5). (C) Absolute thymocyte number and (D) thymocyte profiles of DN1 (c-kit^{high} CD44$^+$ CD25$^-$), DN2 (c-kit^{high} CD44$^+$ CD25$^+$), DN3 (CD44$^-$ CD25$^+$), DN4 (CD44$^-$ CD25$^-$), DP (CD4$^+$ CD8$^+$), and SP (CD4$^+$ CD8$^-$, CD4$^-$CD8$^+$) thymocytes as well as of immature (CD24$^{lo/-}$ γδ$^+$) and mature (CD24$^+$ γδ$^+$) γδ T cells are shown. (E) Pregated on CD4$^-$ CD8$^-$ cells, percentages of Vγ2 and Vγ1.1 T cells in the thymus are shown. (F) Pregated on CD45$^+$ cells, percentages of Vγ3$^+$ CD3$^+$ cells in the skin are shown. Histograms represent the mean and standard deviation from analyzing 5 animals.

identical efficiency. The cycling conditions for both TSLP and TBP amplifications were 10 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C, 15 seconds at 60°C, and 20 seconds at 72°C. The relative expression of TSLP on TBP was calculated with the comparative C_T ($\Delta\Delta C_T$) method.

Isolation of skin-resident lymphocytes

Ears were digested with collagenase IV (Sigma-Aldrich) in HBSS containing 10 mM Hepes, 2.5 mM $CaCl_2$, and 2% FCS for 30 minutes at 37°C. Cell suspensions were filtered, stained, and analyzed by flow cytometry.

Statistical analysis

Statistical significance between the individual groups was analyzed using the unpaired Student *t* test.

Results

The K14-TSLP Tg rescues B- and T-cell development in IL-7$^{-/-}$ mice

As previously reported,[1,2] B lymphopoiesis is dramatically impaired in adult IL-7$^{-/-}$ mice as shown by the absence of CD19$^+$ B-cell progenitors in the BM (Figure 1B). To test if increased TSLP availability could rescue B lymphopoiesis in IL-7$^{-/-}$ mice, K14-TSLP Tg mice were generated and backcrossed to an IL-7$^{-/-}$ background. Absolute numbers of cells in the BM of IL-7$^{-/-}$, WT, and IL-7$^{-/-}$ K14-TSLP Tg mice were comparable (Figure 1A). In the BM of IL-7$^{-/-}$ K14-TSLP Tg mice, the number of CD19$^+$ B cells was restored to WT numbers (Figure 1B). All B progenitor cell subsets were detectable in IL-7$^{-/-}$ mice overexpressing TSLP. The major effect of TSLP was found in the pro-B/pre-B-I (CD19$^+$ c-kit$^+$) cell compartment (4-fold increase in percentage compared with WT controls) and absolute numbers of pro-B/pre-B-I cells were 4-fold increased (S.C. and D.F., unpublished data, December 2006). Pre-B-II (CD19$^+$ CD25$^+$) cells were present at WT percentages in IL-7$^{-/-}$ K14-TSLP Tg mice. Immature (CD19$^+$ B220$^+$ IgM$^+$) and mature (CD19$^+$ B220^{++} IgM$^+$) B cells in IL-7$^{-/-}$ K14-TSLP Tg mice were present in lower percentages than in WT mice. These results show that TSLP Tg expression was sufficient to rescue the block of B-cell development in the BM of IL-7$^{-/-}$ animals. In addition, these results suggest that the major target cells for TSLP were, similar to IL-7,[34] pro-B/pre-B-I cells.

IL-7$^{-/-}$ mice have reduced thymic cellularity and lack γδ T cells.[1,3] The thymus cellularity of IL-7$^{-/-}$ K14-TSLP Tg animals was 60-fold increased compared with IL-7$^{-/-}$ littermates but still remained 2-fold lower than controls (Figure 1C). Contrary to IL-7$^{-/-}$ mice, in which DN1 (c-kit^{high} CD44$^+$ CD25$^-$) and DN2 (c-kit^{high} CD44$^+$ CD25$^+$) thymocytes were absent, IL-7$^{-/-}$ K14-TSLP Tg mice showed normal percentages of the DN1 and DN2 subsets (Figure 1D). Moreover, the percentages of DN3 (CD44$^-$ CD25$^+$), CD4$^+$ SP, CD8$^+$ SP, and double-positive (DP) thymocyte cells in IL-7$^{-/-}$ K14-TSLP Tg mice were in the range of WT controls (Figure 1D). While γδ T-cell development was undetectable in IL-7$^{-/-}$ mice, IL-7$^{-/-}$ K14-TSLP Tg mice had normal percentages of both mature (CD24$^{lo/-}$) and immature (CD24^{high}) thymic γδ$^+$ T cells (Figure 1D). Indeed, Vγ2$^+$ T and Vγ1.1$^+$ T cells were generated in IL-7$^{-/-}$ K14-TSLP Tg mice (Figure 1E). Vγ3$^+$ T cells, which develop during fetal life and are later found exclusively in the adult skin,[35] were present in the skin of IL-7$^{-/-}$ K14-TSLP Tg but not in IL-7$^{-/-}$ mice (Figure 1F).

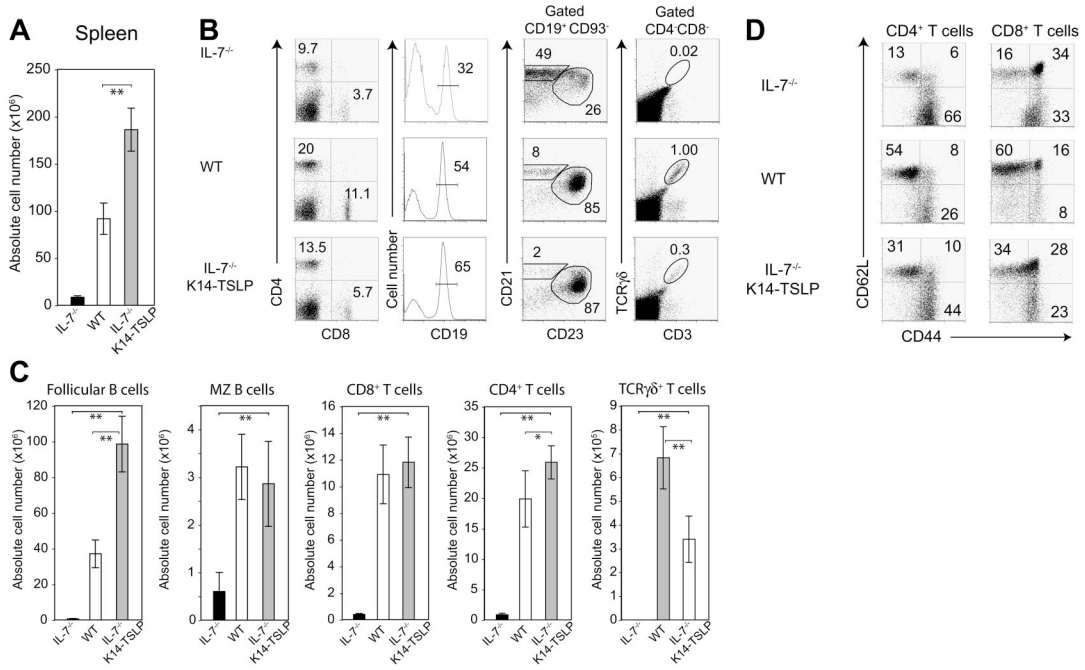

Figure 2. TSLP Tg expression restores splenic lymphocyte compartments in IL-7$^{-/-}$ mice. (A) Absolute splenocyte numbers of IL-7$^{-/-}$, WT, and IL-7$^{-/-}$ K14-TSLP Tg (12-week-old). (B) Percentages and (C) absolute cell number of CD4$^+$, CD8$^+$, and γδ$^+$CD3$^+$ T cells, and CD19$^+$CD93$^-$ mature B cells composed of follicular (CD23$^+$ CD21$^+$) and MZ (CD21^{high} CD23$^-$) B cells are shown. Histograms represent the mean and standard deviation from analyzing 5 animals. *$P < .05$; **$P < .005$ (Student *t* test). (D) Gated on splenic CD4$^+$ and CD8$^+$ T cells, CD62L and CD44 expression is shown.

Mouse strains in which thymocyte development is impaired beyond the DN1 stage display an abnormal cortex with cortical thymic epithelial cells (cTECs) coexpressing keratin 5 (K5) and keratin 8 (K8).[36,37] Consistent with this, the majority of the cTECs in IL-7$^{-/-}$ thymi retained an immature K8$^+$ K5$^+$ phenotype and numerous cysts were found in IL-7$^{-/-}$ thymi (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). In contrast, IL-7$^{-/-}$ K14-TSLP and WT thymi were devoid of cysts and showed a normal segregation into K8$^+$ K5$^-$ cTECs and K8$^-$ K5$^+$ mTECs. SP thymocytes, which express high levels of CD3, were localized in the thymic medulla of IL-7$^{-/-}$ K14-TSLP mice. Hence, TSLP overexpression substantially increased thymus cellularity, rescued the generation of αβ and γδ T cells, and corrected the aberrant thymic architecture in IL-7$^{-/-}$ mice.

Peripheral B- and T-cell compartments are normal in IL-7$^{-/-}$ K14-TSLP Tg mice

IL-7$^{-/-}$ mice are lymphopenic[1] and their spleen cell numbers were 10-fold less than WT controls (Figure 2A). TSLP Tg expression in IL-7$^{-/-}$ mice increased splenocyte numbers by an average of 20-fold. The percentages of splenic CD4$^+$ and CD8$^+$ T cells in IL-7$^{-/-}$ K14-TSLP Tg mice were below WT percentages. However, absolute T-cell numbers in IL-7$^{-/-}$ K14-TSLP Tg mice were normal or, for CD4$^+$ T cells, even beyond WT numbers (Figure 2C). To test if the restoration of peripheral T cells was a result of egress of thymic emigrants or peripheral expansion, we tested CD62L and CD44 expression by splenic CD4$^+$ and CD8$^+$ T cells. The percentage of naive (CD62L^{high} CD44^{low}) T cells was clearly higher in IL-7$^{-/-}$ K14-TSLP Tg mice compared with IL-7$^{-/-}$ percentages but was lower than WT percentages (Figure 2D). These results suggest that the restoration of peripheral T cells in IL-7$^{-/-}$ K14-TSLP Tg mice was a result of both restoration of thymic development and peripheral expansion of T cells. γδ T cells were present in the spleen of IL-7$^{-/-}$ K14-TSLP Tg animals (Figure 2B) in numbers corresponding to half that in WT controls (Figure 2C), indicating that TSLP was less efficient than IL-7 in maintaining the peripheral γδ T-cell pool.

In contrast to IL-7$^{-/-}$ mice, the absolute number of marginal zone (MZ) B cells in IL-7$^{-/-}$ K14-TSLP Tg animals was comparable with WT mice (Figure 2C). Follicular B- (FB) cell numbers were even 2.5-fold increased compared with controls (Figure 2C). It has been reported that TSLP promotes the accumulation of myeloid cells in the spleen.[25] In agreement with this, we found a 5- to 6-fold increase in the absolute number of Gr-1$^+$ CD11b$^+$ granulocytes (Figure S2). Altogether, these results indicate that TSLP promoted the accumulation of peripheral T, B, and myeloid cells.

Systemic expression of TSLP in IL-7$^{-/-}$ K14-TSLP Tg animals

The analysis of IL-7$^{-/-}$ K14-TSLP Tg mice revealed that TSLP was able to support lymphopoiesis. We therefore investigated in which organs TSLP was expressed in Tg mice. Quantitative real-time PCR analysis revealed that, consistently with the K14 promoter specificity, TSLP mRNA was highly expressed in the skin (30-fold relative increase compared with WT) and thymus (8-fold) of Tg mice (Figure 3A). Interestingly, a 50-fold increase in TSLP transcripts was observed in the BM of Tg animals compared with WT controls. The amounts of TSLP transcripts in the spleen and mesenteric lymph nodes were not affected by the Tg expression. In the sera of IL-7$^{-/-}$ K14-TSLP Tg animals, an average concentration of 420 pg/mL TSLP was measured, while TSLP concentrations were less than 20 pg/mL in IL-7$^{-/-}$ and WT controls (Figure 3B). Therefore, the rescue of lymphopoiesis and of peripheral lymphocyte compartments might be the result of Tg-mediated increase of TSLP availability in primary lymphoid organs and in the serum.

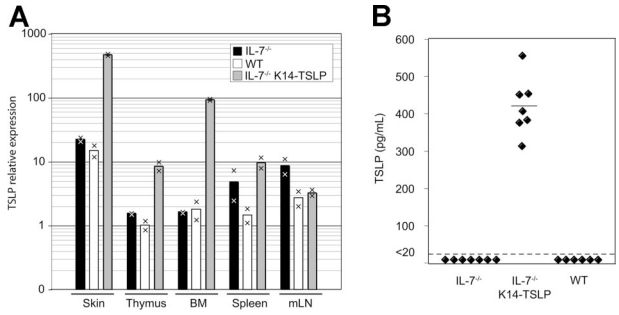

Figure 3. TSLP is detectable in the serum of IL-7$^{-/-}$ K14-TSLP Tg mice. (A) TSLP expression in skin, thymus, BM, et al. Each bar displays the mean of values obtained from cDNA from 2 mice. Results are representative of 3 independent experiments. (B) TSLP concentration in sera from IL-7$^{-/-}$, IL-7$^{-/-}$ K14-TSLP Tg, and WT mice was quantified by ELISA. Each symbol represents the result from an individual mouse. The mean of TSLP concentration is indicated.

In B cells of adult IL-7$^{-/-}$ K14-TSLP Tg mice, the IgH locus contains additional N nucleotides

TSLP was previously shown to support fetal B lymphopoiesis.[20] The rescue of B-cell development in IL-7$^{-/-}$ K14-TSLP Tg mice could therefore be due to the effect of TSLP on fetal liver–derived progenitors. One hallmark of fetal lymphopoiesis is the absence of expression of terminal deoxynucleotidyl transferase (Tdt) in lymphocyte precursors,[38] leading to the generation of B cells that are completely devoid of N nucleotides at the junctions of their rearranged VDJ immunoglobulin heavy (IgH) chain genes.[39] To test whether the B cells generated in IL-7$^{-/-}$ K14-TSLP Tg mice originated from fetal or adult precursors, we examined whether rearranged IgH genes in B cells of 8-week-old IL-7$^{-/-}$ K14-TSLP Tg mice carried N region nucleotide additions. Table 1 shows that of 26 B-cell clone sequences from IL-7$^{-/-}$ K14-TSLP Tg adult spleen, all of them displayed N nucleotide additions. This result indicates that the B lymphopoiesis in adult IL-7$^{-/-}$ K14-TSLP mice was promoted by the action of TSLP on Tdt$^+$ cells and therefore, most likely on adult BM-derived progenitors.

TSLP supports the differentiation of adult BM progenitor cells toward lymphoid lineages

To further assess whether the lymphopoiesis observed in IL-7$^{-/-}$ K14-TSLP Tg mice relied on the differentiation of adult precursors, we generated BM chimeras by adoptively transferring 10^7 total BM cells from adult WT mice (CD45.1$^+$) into lethally irradiated IL-7$^{-/-}$ K14-TSLP Tg, IL-7$^{-/-}$, or WT recipients (CD45.2$^+$). Six weeks after transfer, donor chimerism was higher than 95% in all lymphoid organs of each mouse. Donor BM cell numbers were similar for each of the 3 experimental groups (Figure 4A). Donor-derived B-cell precursors were almost undetectable in the BM of IL-7$^{-/-}$ recipients, whereas all stages of B-cell development were present in both IL-7$^{-/-}$ K14-TSLP Tg mice and WT recipients (Figure 4B). Donor-derived thymocyte numbers were increased 270-fold in IL-7$^{-/-}$ K14-TSLP Tg compared with IL-7$^{-/-}$ recipients but remained 1.4-fold below numbers of WT controls (Figure 4C). Similar percentages of donor-derived DN, CD4$^+$ SP, CD8$^+$ SP, and DP were found in WT and IL-7$^{-/-}$ K14-TSLP mice (Figure 4D). The number of donor-derived splenocytes was substantially increased in IL-7$^{-/-}$ K14-TSLP Tg compared with IL-7$^{-/-}$ hosts (Figure 4E). Normal percentage of peripheral CD4$^+$ T cells, CD8$^+$ T cells, and CD19$^+$ B cells was found in reconstituted IL-7$^{-/-}$ K14-TSLP recipients. In agreement with our previous data, splenic B cells in IL-7$^{-/-}$ recipients were mainly composed of MZ B cells, while those found in IL-7$^{-/-}$ K14-TSLP Tg and WT recipients were mainly FB cells (Figure 4F).

Table 1. Splenic B cells in IL-7$^{-/-}$ K14-TSLP Tg mice display N nucleotide additions at VDJ junctions

Clone	V	P	N1	P	D	P	N2	P	JH4	D element
3	tgtaccgg		—		gattacg		gggc		attactatgctatggactactgg	SP2.2*01
4	tgtgcaaga		—		tggg		gg		tactatgctatggactactgg	DQ52*01
5	tgtgcaaga	tc	ctcg		tatgattac		gga		atggactactgg	SP2.2*01
10	tgtgcaaga		aggg		ttattactacgg		c		ttactatgctatggactactgg	FL16.1*01
12	tgtgcaaga		gg	gg	cctactatagtaac		ccagg		ggactactgg	SP2.x*01
14	tgtgcaaga		cgg		agtaac		—		tacgatgctatggactactgg	SP2.x*01
16	tgtgcaaga		gagaagg		attactacggtagtagct		—		ctatggactactgg	FL16.1*01
19	tgtgcaaga		aacccc		ctacgg		G		actatgctatggactactgg	FL16.2*01
20	tgtgc		cct		ctatga		C		tactatgctatggactactgg	SP2.2*01, SP2.9*01
23	tgtgcgag		ggacaca		gggct		—		ctatgctatggactactgg	ST4
24	tgtgcaaga		tccc		gta		Agaagg		atgctatggactactgg	FL16.1*01, SP2.x,.1,.5,.7,.8
25	tgtgcaaga		cg		ggga		G		atgctatggactactgg	DQ52*01
28	tgtgcgaga		aatttcgcc		tattactacggtagtag		g		actatgctatggactactgg	FL16.1*01
32	tgtgcaaga	t	gggattttag		cggta		g		ttactatgctatggactactgg	FL16.1*01
40	tgtgcaaga		gaga		atgattacgac	g	gg		tactatgctatggactactgg	SP2.2*01
50	tgtgcaaga		a		aactggg	g	gcg		actatgctatggactactgg	DQ52*01
51	tgtgcaaga		—		attactacggtagtag		ctc		actatgctatggactactgg	FL16.1*01
56	tgtgcaa		tgacg		attactacggtagtagc		ggggg		actatgctatggactactgg	FL16.1*01
57	tgtgcaaga		aggaatcg		agttact		tggg		atgctatggactactgg	SP2.12*01
58	tgtgcaaga		gga		tattactacggtagtagc		ctctatggg		tatgctatggactactgg	FL16.1*01
64	tgtgc		c	ga	tctactatggtaac		cacggagggg		tgctatggactactgg	SP2.1*01
65	tgtgcaaga		gcg		tattactacggtag		—		tgctatggactactgg	FL16.1*01
67	tgtgcaaga	tc	ggtgg		gattacgac		agaaggggtc		ttactatgctatggactactgg	SP2.2*01
73	tgtgcaaga		atggaggg		tac		acttct		ctatgctatggactactgg	All, except DQ52
75	tgtgcaaga		gaga		atgattacgac	g	gg		tactatgctatggactactgg	SP2.2*01
80	tgtgcaaga		tggac		tactacggtagtagct		gaaggaggg		atggactactgg	FL16.1*01

Sequences of VHJ558-JH4 rearrangements from 26 individual PCR fragments derived from sorted B220$^+$CD19$^+$ splenic B cells of 8-week-old IL-7$^{-/-}$ K14-TSLP Tg mice are shown.

— indicates no N nucleotide addition.

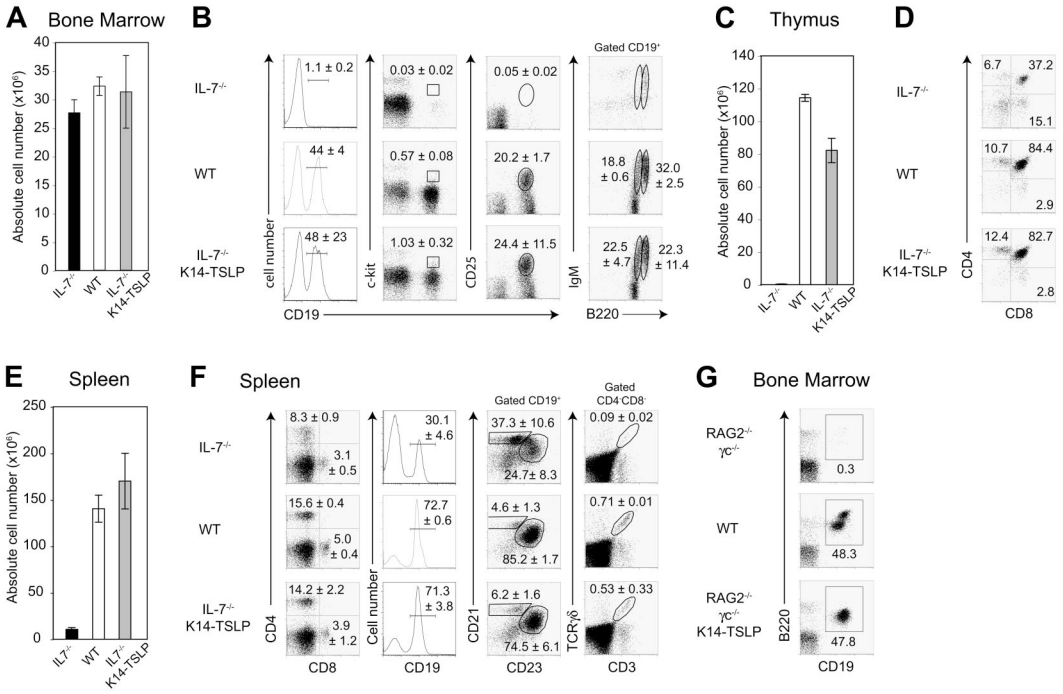

Figure 4. TSLP sustains the differentiation of WT BM progenitors toward the B- and T-cell lineages in vivo. (A) Absolute cell number per 2 femurs 6 weeks after reconstitution of lethally irradiated IL-7$^{-/-}$, WT, and IL-7$^{-/-}$ K14-TSLP Tg recipients with 10^7 BM cells (CD45.1$^+$) is shown. (B) FACS profiles were gated on CD45.1$^+$ donor cells. Regions indicate the pro-B/pre-B-I (CD19$^+$c-kit$^+$), pre-B-II (CD19$^+$CD25$^+$), mature (CD19$^+$B220^{high}IgM$^+$), and immature (CD19$^+$B220$^+$IgM$^+$) B cells. Numbers are mean and standard deviation of percentage (n = 3). (C) Absolute CD45.1$^+$ thymocyte number and (D) percentages of donor-derived SP (CD4$^+$CD8$^-$, CD4$^-$CD8$^+$), DP (CD4$^+$CD8$^+$) thymocytes are shown. (E) Absolute CD45.1$^+$ splenocyte number. Histograms represent the mean and standard deviation from analyzing 3 animals. (F) Regions indicate splenic CD4$^+$, CD8$^+$, and γδ$^+$ T cells, and CD19$^+$ B cells containing follicular (CD23$^+$CD21$^+$) and MZ (CD21^{high} CD23$^-$) B cells. Numbers are mean and standard deviation of percentage (n = 3). (G) RAG2$^{-/-}$ γc$^{-/-}$, WT, and RAG2$^{-/-}$ γc$^{-/-}$ K14-TSLP Tg mice were analyzed at 10 weeks of age. Regions indicate the committed (CD19$^+$B220$^+$) B cells in the BM. Representative FACS analyses of 1 of 3 mice.

Donor-derived cells were able to develop into TCRγδ$^+$ T cells in IL-7$^{-/-}$ K14-TSLP Tg mice. These results collectively show that TSLP could efficiently sustain the differentiation of adult BM progenitors toward both B- and T-cell lineages in vivo. Our data contrast in vitro studies showing that in adult BM only pre-BCR$^+$ progenitors are able to respond to TSLP.[40] RAG2$^{-/-}$ $\gamma_c{}^{-/-}$ mice are unresponsive to IL-7 and hence unable to generate pro-B cells. In contrast, TSLP Tg expression in adult RAG2$^{-/-}\gamma_c{}^{-/-}$ mice clearly promoted pro-B-cell generation in vivo (Figure 4G).

To test whether the K14-TSLP Tg had an additional effect on erythroid and myeloid compartments, we analyzed BM chimeras 6 months after reconstitution. Percentages and absolute numbers of donor-derived granulocytes (Gr-1$^+$ CD11b$^+$) and erythroblasts (Ter119$^+$ CD71$^+$) were similar in the BM of IL-7$^{-/-}$ K14-TSLP Tg and WT recipients (Figure 5A-B). In contrast, a significant increase in granulocyte and erythroblast numbers was found in the spleen of IL-7$^{-/-}$ K14-TSLP Tg mice (Figure 5C,D). Altogether, our results show that adult BM progenitors efficiently differentiated toward B and T lineages in response to TSLP and that TSLP promoted the accumulation of myeloid cells in the spleen.

TSLP promotes B- and early T-cell development from EPLMs in vitro

The in vivo effect of TSLP prompted us to study whether TSLP could promote lineage commitment from a lymphoid/myeloid BM progenitor cell. EPLMs have been identified as B220$^+$ CD117$^+$ CD19$^-$ NK1.1$^-$ BM cells that can give rise to both myeloid and lymphoid lineages.[31] To test if TSLP promoted the development of B and T cells from EPLMs, we plated 5×10^3 FACS-sorted EPLMs on either OP9 or OP9 stromal cells expressing the Notch ligand Delta-like-1 (OP9-DL1) and added either TSLP or IL-7.

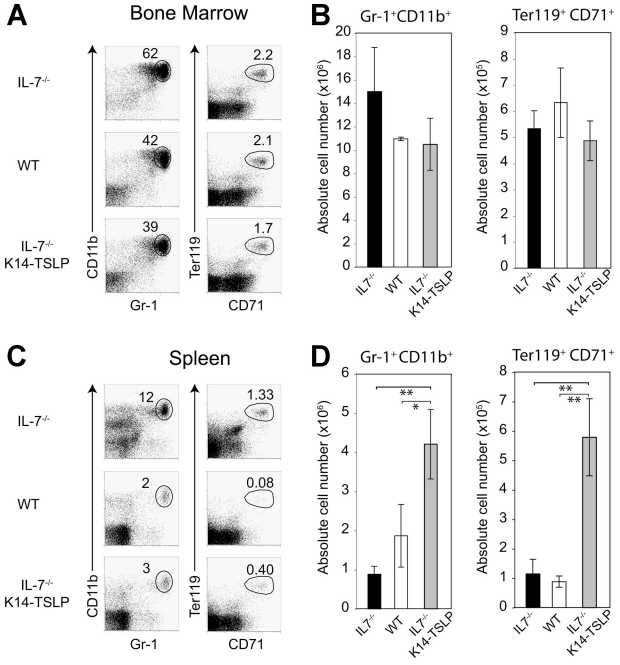

Figure 5. TSLP Tg expression leads to the accumulation of granulocytes and erythroid precursors in the spleen. Six months after reconstitution of lethally irradiated IL-7$^{-/-}$, IL-7$^{-/-}$ K14-TSLP, or WT recipient mice with 1×10^7 BM cells from CD45.1$^+$ mice, recipients were analyzed. All FACS profiles were gated on CD45.1$^+$ donor cells. (A) Granulocytes (Gr-1$^+$ CD11b$^+$) and erythroblasts (Ter119$^+$ CD71$^+$) in the BM are shown. (B) Absolute number of granulocytes and erythroblasts in BM. (C) Granulocytes (Gr-1$^+$ CD11b$^+$) and erythroblasts (Ter119$^+$ CD71$^+$) in the spleen are shown. (D) Absolute number of granulocytes and erythroblasts in spleen. *$P < .05$; **$P < .005$ (Student t test). Histograms represent the mean and standard deviation from analyzing 3 animals.

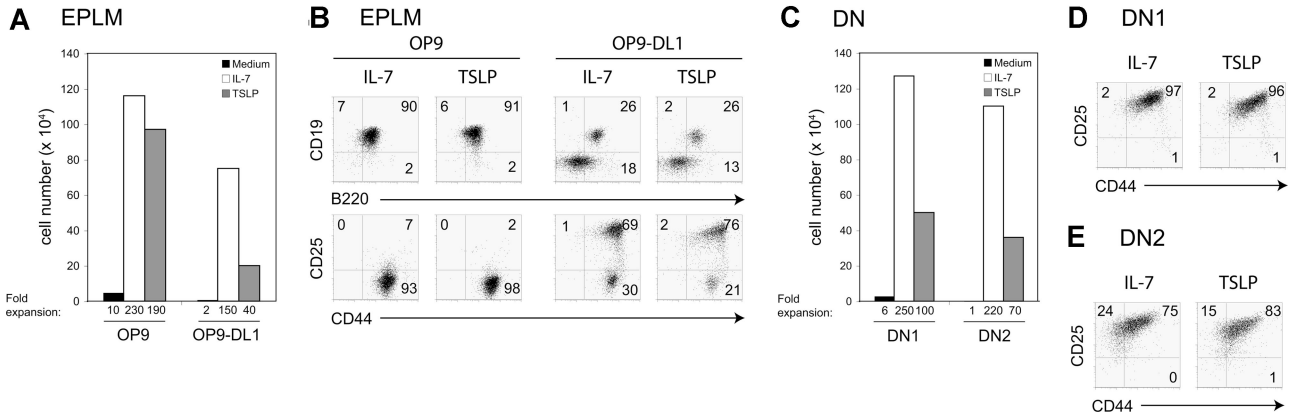

Figure 6. TSLP promotes the expansion and the differentiation of EPLMs, and DN1 and DN2 cells in vitro. (A) Absolute cell numbers harvested after a 7-day culture period of EPLMs ($B220^+$ $CD19^-$ $NK1.1^-$ $CD117^+$ $CD93^+$) on either OP9 or OP9-DL1 cells in the presence of TSLP or IL-7, or without additional cytokine. (B) The percentage of $CD19^+$ $B220^+$ B cells, DN1 ($CD25^-$ $CD44^+$), DN2 ($CD25^+$ $CD44^+$), and DN3 ($CD25^+$ $CD44^-$) T-cell precursors is shown. Data shown here are from 1 representative experiment of 2. (C) Sorted DN1 ($CD25^-$ $CD44^+$ $CD117^{high}$) or DN2 ($CD25^+$ $CD44^+$ $CD117^{high}$) cells were plated on OP9-DL1 in the presence of TSLP or IL-7, or without additional cytokine. Absolute cell numbers were calculated after 7 days of culture. Flow cytometric analysis of (D) DN1 or (E) DN2 cells cultured for 7 days on OP9-DL1 in the presence of TSLP or IL-7. Data shown here are from 1 representative experiment of 2.

EPLMs cultured on OP9 cells without additional cytokine did not substantially expand (Figure 6A). In contrast, cells cultured with IL-7 or TSLP underwent a 230- and a 190-fold expansion, respectively. The majority of EPLMs cultured with either TSLP or IL-7 had differentiated along the B-cell lineage, as 90% coexpressed CD19 and B220 (Figure 6B). When placed on OP9-DL1, EPLMs cultured with IL-7 or TSLP expanded 150- and 40-fold, respectively (Figure 6A). TSLP promoted the differentiation of EPLMs toward the T-cell lineage when cultured on OP9-DL1 cells (Figure 6B). Indeed, after 7 days of coculture, 76% showed a DN2 ($CD25^+$ $CD44^+$) phenotype, whereas 26% had entered the B lineage ($CD19^+$ $B220^+$). Comparably, cocultures supplemented with IL-7 gave rise to 69% DN2 cells and 26% B-committed cells. These results show that TSLP efficiently supports both B- and T-cell differentiation from adult lymphoid/myeloid BM progenitors.

DN1 and DN2 thymocytes are responsive to TSLP

We investigated which T-cell progenitors were directly responsive to TSLP. DN1 (Figure 6C,D) or DN2 (Figure 6C,E) cells were FACS sorted and cultured on OP9-DL1 stromal cells either alone or in the presence of TSLP or IL-7. Sorted DN1 cells cultured for 1 week with IL-7 underwent a 250-fold expansion, whereas cells cultured with TSLP expanded 100-fold (Figure 6C). Most of the cells had progressed to the DN2 stage after a culture period of 7 days (Figure 6D). DN2 cells grown in the presence of IL-7 or TSLP expanded 220- and 70-fold, respectively (Figure 6C). After 7 days in medium supplemented with TSLP, 15% of the originally sorted DN2 cells had a DN3 ($CD25^+$ $CD44^-$) phenotype, whereas 83% retained the DN2 phenotype (Figure 6E). Similar results were obtained from culturing DN2 cells with IL-7. Taken together, these results show that TSLP is able to promote the expansion and differentiation of adult DN1 and DN2 thymocytes in vitro.

$IL\text{-}7^{-/-}$ K14-TSLP Tg mice efficiently mount Ab responses to T-independent and T-dependent Ags

The functionality of B and T cells developing in $IL\text{-}7^{-/-}$ K14-TSLP Tg mice was assessed by immunization with the T-independent Ag NP-Ficoll or the T-dependent Ag NP-OVA. Ab titers in preimmune sera from $IL\text{-}7^{-/-}$ K14-TSLP Tg mice and $IL\text{-}7^{-/-}$ mice were higher compared with preimmune sera from WT mice (Figure 7A). Ten days after intravenous immunization with 100 μg NP-Ficoll, $IL\text{-}7^{-/-}$ mice failed to mount a significant anti–NP IgM response. In contrast, $IL\text{-}7^{-/-}$ K14-TSLP Tg animals mounted a clear anti–NP IgM response reflected by a 10-fold increase in specific Ab titer (Figure 7A), an increase similar to that seen in WT controls.

When immunized with 50 μg of the T-dependent Ag NP-OVA, $IL\text{-}7^{-/-}$ K14-TSLP Tg mice showed an increase in anti–NP IgG titers (Figure 7B). These results indicated that both B and T cells generated in $IL\text{-}7^{-/-}$ K14-TSLP Tg mice were functional and could effectively collaborate in mounting an Ab response to a T-dependent Ag. Further investigation will be required to understand the mechanisms underlying the high variation in anti–NP IgG titers observed in $IL\text{-}7^{-/-}$ K14-TSLP Tg immunized mice. Taken together, our results show that B and T cells generated in $IL\text{-}7^{-/-}$ K14-TSLP Tg mice were functional.

Discussion

Mouse models for studying the function of TSLP have left several questions unanswered regarding its role in adult B- and T-cell differentiation and myeloid cell expansion. We show here a so-far-unappreciated capacity of TSLP to promote B- and T-cell

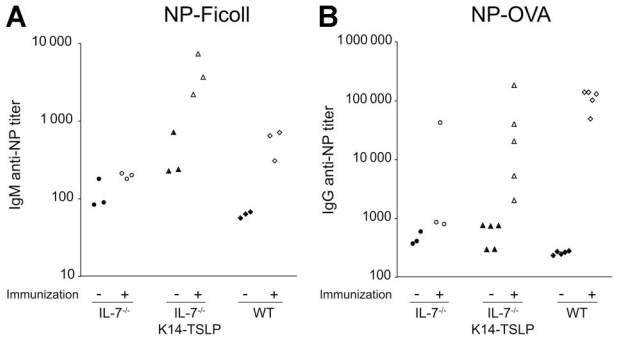

Figure 7. NP-specific Ab titers in the sera of immunized mice. Ten- to 12-week-old $IL\text{-}7^{-/-}$ K14-TSLP Tg, $IL\text{-}7^{-/-}$, and C57Bl/6 mice were immunized and sera were analyzed as indicated. Each symbol represents the result from an individual mouse. Filled symbols represent titers prior to immunization; empty symbols represent titers after immunization. (A) Titers of NP-specific IgM prior and 10 days after intravenous NP-Ficoll immunization. (B) Titers of NP-specific IgG titers prior and 15 days after intraperitoneal NP-OVA immunization.

development in adult mice. BM lymphoid/myeloid progenitors as well as DN thymocytes were responsive to TSLP. In the absence of IL-7, TSLP overexpression was able to restore central and peripheral lymphoid compartments and to amplify myeloid cells in the periphery.

TSLP mRNA is expressed at low levels in primary lymphoid organs[26] and TSLP concentrations are low (< 20 pg/mL) in the serum of WT and IL-7$^{-/-}$ mice[41,42] (Figure 3). These endogenous TSLP levels fail to overcome IL-7 deficiency. Here, we show that TSLP serum concentrations of 420 pg/mL were sufficient to compensate for the lack of IL-7. While even lower levels of TSLP affect B-cell development in a WT background,[24] the minimal concentration that is required locally or in the serum to sustain lymphopoiesis in absence of IL-7 remains to be determined.

TSLP overexpression restored all stages of B-cell differentiation in the BM of IL-7$^{-/-}$ mice (Figure 1B). Adoptively transferred adult WT BM progenitors replenished all B-cell compartments in BM and spleen of IL-7$^{-/-}$ K14-TSLP Tg recipient (Figure 4). Together with the finding that IgH junctions of B cells displayed N regions (Table 1), our data indicate that adult BM progenitors were responsive to TSLP. This is further supported by the fact that TSLP induced the differentiation of adult EPLMs toward CD19$^+$ B cells in vitro (Figure 6). In addition, pro-B cells developed in RAG2$^{-/-}$ $\gamma_c{}^{-/-}$ mice in response to TSLP Tg expression (Figure 4G). These results contrast a previous study showing that in vitro, pre-BCR expression was required for adult B-cell precursors to be TSLP responsive.[40] The differences in our findings might be a result of different requirements for B-cell development in vitro and in vivo.

This study further shows that TSLP promotes the differentiation of adult BM progenitors toward T-cell lineage in vitro (Figure 6A,B) and that DN1 and DN2 cells are directly responsive to TSLP (Figure 6D-E). TSLP Tg expression restored DN1 and DN2 thymocyte compartments in IL-7$^{-/-}$ mice (Figure 1D) and normalized the organization of the thymus (Figure S1). Since DN thymocytes play a crucial role in cTEC maturation,[36,37] our results suggest that the TSLP-driven generation of DN1 and DN2 cells corrected cTEC differentiation in IL-7$^{-/-}$ mice. TSLP Tg expression also rescued the generation of γδ T cells. In IL-7$^{-/-}$ fetal thymus, Vγ3$^+$ T cells are generated but do not persist in the adult.[3] The presence of Vγ3$^+$ T cells in the skin of IL-7$^{-/-}$ K14-TSLP Tg mice shows that TSLP could replace IL-7 in maintaining the dendritic epidermal Vγ3$^+$ T cells.

The effect of TSLP on in vitro and in vivo thymocyte expansion was lower compared with IL-7. This could be due to a less stimulatory activity of TSLP on thymocytes. Indeed, TSLP has a weaker effect on α-CD3 stimulated SP thymocytes than IL-7.[12] Alternatively, it is possible that the frequency of TSLP-responding precursors might be lower than those of IL-7–responding precursors.

In the spleen, however, absolute T-cell numbers were restored and CD4$^+$ T cells were even significantly increased in IL-7$^{-/-}$ K14-TSLP mice compared with WT controls. A substantial percentage of CD4$^+$ and CD8$^+$ T cells in IL-7$^{-/-}$ K14-TSLP Tg mice showed a naive CD62L$^+$ CD44$^-$ phenotype, indicating that they were recent thymic emigrants. On the other hand, 44% of CD4$^+$ and 23% of CD8$^+$ T cells in IL-7$^{-/-}$ K14-TSLP mice showed a CD44^{high} activated phenotype (Figure 2D), suggesting that TSLP-driven peripheral T-cell expansion could compensate for the relatively low thymic output. This result might explain the high variation of NP titer observed in T-dependent Ab response (Figure 7B), as activated T cells might be less efficient in their helper function.

The robust accumulation of FB cells in response to TSLP overexpression confirms previous results in K5-TSLP Tg mice[24] and is reminiscent of the effect of IL-7.[43] In addition, TSLP promoted the accumulation of myeloid precursors in the spleen. Whether this is a direct or indirect in vivo effect of TSLP remains to be clarified. High systemic concentration of TSLP in β-actin–TSLP Tg mice leads to myeloid hyperplasia in the spleen and, surprisingly, impairs lymphopoiesis.[25] In contrast, we find that TSLP can sustain T and B lymphopoiesis. This discrepancy might be a result of significantly lower amounts of TSLP Tg expressed in our mouse model. It will be important to elucidate the mechanism by which high amounts of TSLP inhibit lymphopoiesis.

Altogether, our data demonstrate that TSLP can promote adult B and T lymphopoiesis, restore peripheral lymphocyte compartments, and induce peripheral accumulation of myeloid cells. The compromised lymphopoiesis found in IL-7$^{-/-}$ mice might therefore not be due to the inability of adult hematopoietic cells to respond to TSLP but is rather a consequence of limited TSLP availability.

Acknowledgments

This work was supported by the Swiss National Science Foundation (SNF) grant PPOOA-68855, the Gottfried und Julia Bangerter-Rhyner foundation, and the Swiss life foundation (D.F.). S.C. was supported by a Roche Research Foundation Fellowship. A.G.R. is holder of the chair of Immunology endowed by Hoffman-La Roche Ldt Basel. The AGR laboratory is financially supported by the SNF.

We thank R. Ceredig, D. van Essen, S. Infantino, and H. Acha-Orbea for helpful discussions and comments on the paper; J. Kirberg for providing RAG2$^{-/-}$ $\gamma_c{}^{-/-}$ mice; A. Wilson for the generous Ab gift; and J. Gill for valuable help with confocal microscopy.

Authorship

Contribution: D.F. designed research; S.C. and A.G.R. participated in designing research; S.C. performed research; A.G.F., L.F., and A.G.R participated in performing research; A.G.F. contributed vital reagent; S.C., D.F., and A.G.R. analyzed and interpreted data; S.C. and D.F. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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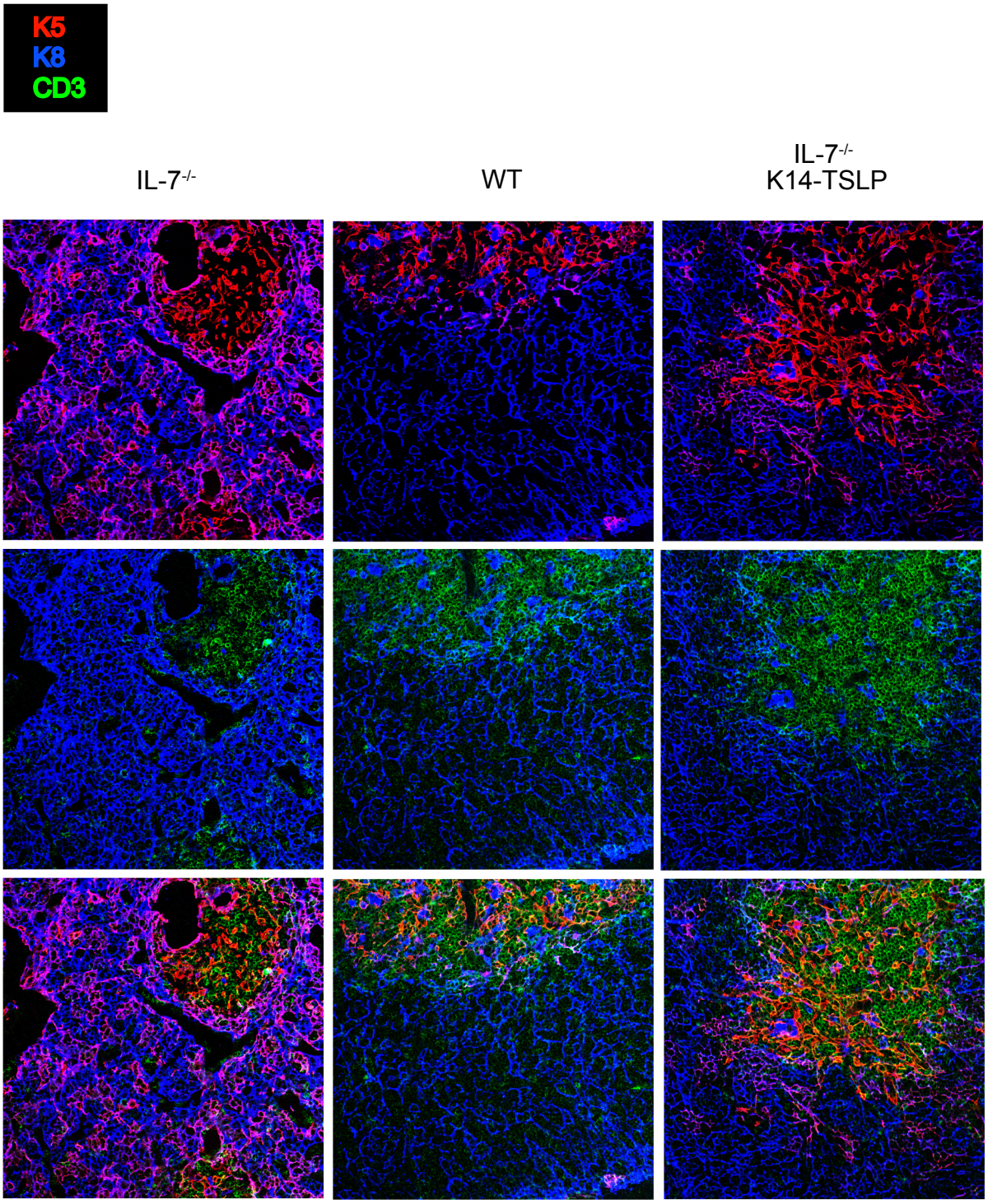
Supplemental Figure 1. TSLP Tg expression restores thymic architecture in IL-7$^{-/-}$ mice.

Immunofluorescence analysis of thymi from IL-7$^{-/-}$ K14-TSLP Tg mice, IL-7$^{-/-}$littermates and WT controls stained for CD3 (green), Keratin 5 (red) and Keratin 8 (blue). Original magnification: x20.

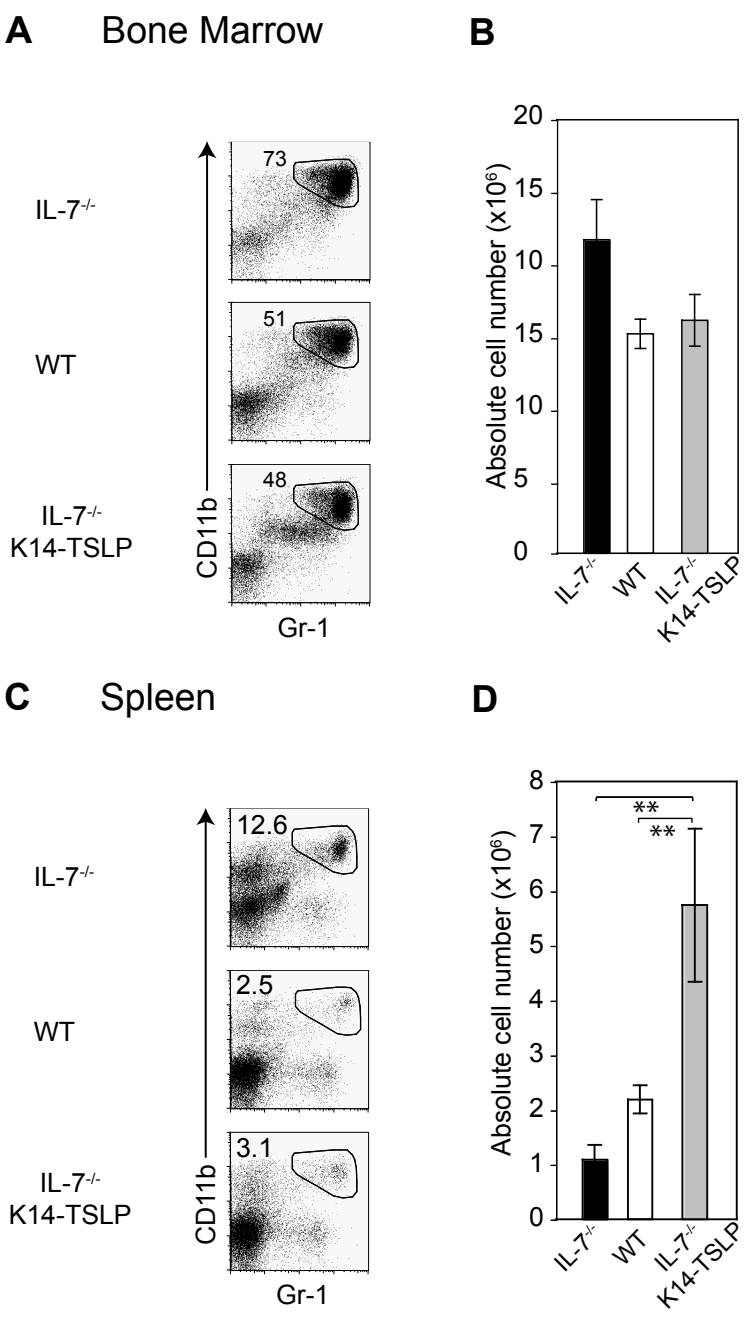
Supplemental Figure 2. TSLP Tg expression induces the accumulation of granulocytes in the spleen.

IL-7$^{-/-}$ K14-TSLP Tg mice, IL-7$^{-/-}$littermates and WT controls were analyzed at 12 weeks of age. (A) Granulocytes (Gr-1^{+} CD11b^{+}) in the BM are shown. (B) Absolute granulocyte numbers in BM. (C) Granulocytes in the spleen are shown. (D) Absolute cell numbers of granulocytes in spleen. (**), $p<0.005$ (Student's t-test). Histograms represent the mean and standard deviation from analyzing 5 animals.

Supplementary Figure S1



Supplementary Figure 2



2. Manuscript 2:

TSLP overexpression restores lymph node development in IL-7$^{-/-}$ and RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice by increasing lymphoid tissue inducer cell number

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Abstract

Lymph node (LN) organogenesis is initiated by the interaction between the hematopoietic lymphoid tissue inducer (LTi) cells and mesenchymal organizer cells. Mice deficient for molecules of the IL-7 signaling pathway have a severe defect in LN development. It is known that IL-7 regulates the size of the LTi cell pool but the reasons underlying the defective LN formation in these mice are unknown. Here, we show that overexpression of thymic stromal lymphopoietin (TSLP) increases LTi cell numbers and restores LN development in IL-7$^{-/-}$ and RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice. LN anlagen from RAG2$^{-/-}$ $\gamma_c^{-/-}$ TSLP Tg are devoid of B, T and NK cells showing that colonization of the LN by peripheral lymphocytes is not required for persistence of LN anlage. In contrast, our results suggest that LTi cell number is a critical parameter for LN organogenesis. Indeed, increased LTi cell number restored organizer cells and LN in RAG2$^{-/-}$ $\gamma_c^{-/-}$ TSLP Tg mice. Altogether these results identify LTi and organizer cells as the minimal cellular players required for LN organogenesis and indicate that the LN defect in IL-7$^{-/-}$ and RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice is the consequence of insufficient LTi cell numbers.

Introduction

The development of LN relies on the crosstalk between hematopoietic $CD4^+$ IL-$7R\alpha^+$ lymphoid tissue inducer (LTi) cells and VCAM-1^+ ICAM-1^+ mesenchymal organizer cells. As early as 12.5-13.5 days post-coitum (dpc), LTi cells start to cluster at sites of nascent LN anlagen [87]. These cells are crucial for LN formation, as $ROR\gamma^{-/-}$ mice, which lack LTi cells, are completely devoid of LN [80]. LTi cells express lymphotoxin (LT) $\alpha1\beta2$ and engage the LTβ receptor present on organizer cells, which subsequently form clusters [87]. The absolute requirement for the LTab/LTbR interaction between LTi and organizer cells is illustrated by the fact that $LT\alpha^{-/-}$, $LT\beta^{-/-}$ and LTβ receptor $(LT\beta R)^{-/-}$ mice lack all peripheral LN [62, 64, 66]. LTβR signaling induces the expression of adhesion molecules and chemokines [90], recruiting more LTi cells to the nascent LN anlage. After birth, the LN anlage is progressively colonized by mature lymphocytes, which later segregate into B cell follicles and a T cell zone [157].

Besides LT, interleukin 7 (IL-7) plays a central role in LN organogenesis. Mice with a defect in the IL-7 signaling pathway, such as $JAK3^{-/-}$, $\gamma_c^{-/-}$, $IL\text{-}7^{-/-}$ and IL-$7R\alpha^{-/-}$ mice, have severe defects in peripheral LN development [91, 92, 158, 159] and have impaired B and T lymphopoiesis [6, 7]. IL-7 signals through the IL-7 receptor, which is composed of the IL-7Rα (CD127) and the common γ (γ_c) chains. γ_c is also a crucial component of the IL-15 receptor that is required for NK cell development [158].

We have previously shown that thymic stromal lymphopoietin (TSLP) is a cytokine that has overlapping biological activity with IL-7 on adult lymphopoiesis *in vivo* [160]. TSLP signals through a unique receptor formed by IL-7Rα together with a γ_c-like chain called TSLPR [122]. TSLP signaling is independent of γ_c and mediates STAT5 phosphorylation [121]. While the physiological levels of TSLP in $IL\text{-}7^{-/-}$ mice are not sufficient to sustain lymphopoiesis, its overexpression is sufficient to restore both B and T cell compartments [160].

The reasons underlying the absence of LN in $IL\text{-}7^{-/-}$ and $\gamma_c^{-/-}$ mice remain elusive. IL-7 availability regulates the size of the pool of LTi cells *in vivo* [92]. Indeed mesenteric LN (mLN) from newborn IL-$7R\alpha^{-/-}$ mice contain decreased LTi cell numbers compared to WT mice [91], suggesting that LN organogenesis might be defective in $IL\text{-}7^{-/-}$ and $\gamma_c^{-/-}$ mice because of low LTi cell numbers. An alternative explanation is that LN organogenesis occurs normally but that lymphopenia prevents the maintenance of the LN anlage after birth [161].

In this study, we have investigated whether overexpression of TSLP could restore LN organogenesis in mice deficient for the IL-7 signaling pathway. Furthermore, we addressed whether LN development in $IL\text{-}7^{-/-}$ and $\gamma_c^{-/-}$ mice results from an early intrinsic defect in LN organogenesis or from the lack of colonization by peripheral lymphocytes.

We show here that IL-7$^{-/-}$ and RAG$^{-/-}$ $\gamma_c^{-/-}$ mice bearing a K14-TSLP transgene had almost normal LN numbers. In RAG$^{-/-}$ $\gamma_c^{-/-}$ TSLP Tg mice, in which B, T and NK cells are absent, LN development was restored, ruling out that peripheral lymphocytes were required for LN development. TSLP Tg expression in IL-7$^{-/-}$ and RAG$^{-/-}$ $\gamma_c^{-/-}$ mice increased LTi cell numbers, restoring organizer cells in RAG$^{-/-}$ $\gamma_c^{-/-}$ mice and rescuing LN formation. Our results further show that some of the mesenchymal and endothelial compartments forming the LN microenvironment are dependent on lymphocyte presence while others are not.

Results and discussion

TSLP Tg expression rescues LN development in IL-7$^{-/-}$ mice.

In IL-7$^{-/-}$ mice LN development is severely impaired [92]. We addressed whether increased TSLP availability could restore LN development in IL-7$^{-/-}$ mice. Adult IL-7$^{-/-}$ K14-TSLP Tg mice and non-Tg littermates were i.p. injected with Chicago Blue and analyzed 7 days later. Except for sacral and deep cervical LN, almost all peripheral LN were present in IL-7$^{-/-}$ mice overexpressing TSLP (Fig1.A-B). Mesenteric LN were found in all animals analyzed regardless of TSLP Tg expression (Fig1.A). These results show that TSLP Tg expression has the capacity to substitute IL-7 for LN organogenesis and extend our previous data on the overlapping activity of IL-7 and TSLP in adult lymphopoiesis [160].

As IL-7Rα is almost exclusively expressed by hematopoietic cells, LN restoration in IL-7$^{-/-}$ K14-TSLP Tg animals was likely mediated through the effect of TSLP on hematopoietic cells. Hence, to gain insight into the mechanism underlying the restoration of LN development in IL-7$^{-/-}$ K14-TSLP mice, we investigated which hematopoietic cells were affected by TSLP Tg expression. TSLP overexpression restored B and T cell compartments in the spleen of 4.5 day old IL-7$^{-/-}$ mice compared to littermate controls (Fig 1.C) with CD19^{+} B220^{+} B cell numbers increased about 100 fold (Fig 1.D). CD4 and CD8 T cell numbers were increased 18 and 20 fold, respectively, while NK cells were less affected. These results show that TSLP overexpression increased the size of B, T and NK cell compartments in the periphery of newborn IL-7$^{-/-}$ mice. In the newborn spleen of IL-7$^{-/-}$ K14-TSLP Tg mice, the percentage of CD4^{+} IL-7Rα^{+} LTi cells was 3-fold increased as compared to non-Tg littermates (Fig1.E). LTi absolute cell numbers were 3.8-fold and 2-fold increased in the spleen and the mLN, respectively (Fig 1.F-G).

As LTi cell function relies on the expression of LT, we analyzed LTα1β2 expression by LTi cells from newborn IL-7$^{-/-}$ K14-TSLP Tg and non-Tg littermates. We found that LTi cells from both origin expressed similar levels of LTα1β2 (Fig 1.H). These result show that TSLP Tg expression increases LTi cell numbers *in vivo,* without altering LTα1β2 expression levels.

To address whether LTi cells were instrumental for the restoration of LN in IL-7$^{-/-}$ K14-TSLP Tg mice, IL-7$^{-/-}$ K14-TSLP Tg mice were backcrossed to ROR$\gamma^{-/-}$ mice. IL-7$^{-/-}$ ROR$\gamma^{-/-}$ K14-TSLP Tg newborn mice were devoid of LTi cells (Suppl. Fig.1) and LN were missing in adults (Table I). Thus, TSLP-mediated LN restoration in IL-7$^{-/-}$ mice was dependent on LTi cells.

TSLP activates LTi cell progenitors in the fetal liver.

In order to understand the mechanism underlying the increase in LTi cell number in IL-7$^{-/-}$ K14-TSLP Tg mice, we investigated whether LTi cells or their fetal liver (FL) precursors were TSLP-responsive by monitoring STAT5 phosphorylation in response to TSLP stimulation *in vitro*. 14.5 dpc lin^{-} $\alpha4\beta7^{+}$ c-kit^{+} FL progenitors expressed TSLPR on their surface (Suppl. Fig.2A) and a substantial fraction phosphorylated STAT5 in response to TSLP (Suppl. Fig.2B). LTi cells expressed low levels of TSLPR (Suppl. Fig.4C) and in contrast to IL-7, they did not phosphorylate STAT5 in response to TSLP (Suppl. Fig.2D). While IL-7 favored the generation of LTi cells from FACS sorted lin^{-} $\alpha4\beta7^{+}$ c-kit^{+} FL cells, we failed to detect a similar activity of TSLP in various *in vitro* settings (data not shown). We therefore investigated whether TSLP can promote the generation of LTi cells from FL progenitors *in vivo*. To this end, we reconstituted lethally irradiated Ly5.2^{+} IL-7$^{-/-}$ K14-TSLP Tg adult and non-Tg littermates with WT 10^6 FL Ly5.1^{+} cells from 12.5 dpc embryos. 10 days after reconstitution, the percentage of LTi cells from donor origin was substantially higher in TSLP Tg recipients than in non-Tg littermates (Suppl. Fig.2E), Consistently, LTi absolute cell numbers were increased approximately 5 fold in the spleen of IL-7$^{-/-}$K14-TSLP Tg recipients compared to non-Tg littermates (Suppl. Fig.2F). These results suggest that increased availability of TSLP increases the number of LTi cells *in vivo* most likely through an effect on FL precursors.

Increase in LTi cell number restores organizer cell and LN development in RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice.

Peripheral lymphocytes were proposed to play a role in the maintenance of the LN anlage during postnatal life [161]. Since we could not rule out that restoration of peripheral lymphocytes partially contributed to the LN restoration in IL-7$^{-/-}$ K14-TSLP Tg mice, we generated RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg mice. The fact that TSLP signals independently of γ_c gave us the opportunity to dissect the relative contribution of peripheral B, T, NK and LTi cells in LN organogenesis in IL-7$^{-/-}$ K14-TSLP Tg mice. By crossing the K14-TSLP Tg mice on a RAG2$^{-/-}$ $\gamma_c^{-/-}$ background, we generated mice devoid of B, T and NK cells but with increased LTi cell numbers. Consistent with our findings in newborn IL-7$^{-/-}$ K14-TSLP mice, the percentage of

$CD4^+$ IL-7Rα^+ LTi in the spleen was 2-fold increased in $RAG2^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg newborn mice compared to non-Tg littermates (Fig2.A). Absolute LTi cell numbers were 2 fold increased in the spleen and mLN of TSLP Tg newborn mice compared to non-Tg littermates (Fig.2B-C). The level of LTα1β2 expressed by LTi cells was similar in the spleen and mLN of newborn $RAG2^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg and of non-Tg littermates (Fig.2D). In 7 day old WT mice, mLN were colonized by $CD45^+$ hematopoietic cells of which the vast majority were $CD4^+$ and $CD8^+$ T cells with some $CD19^+$ $B220^+$ B cells (Fig2.E). In contrast, LN from both $RAG2^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg mice and from non-Tg littermates were devoid of lymphocytes.

Adult $RAG2^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg and non-Tg littermates were analyzed for the presence of LN. As expected, $RAG2^{-/-}$ $\gamma_c^{-/-}$ mice had a severe defect in LN development with the frequency of inguinal, politeal, para-aortic, axillary, pancreatic and hepatic LN below 20% that of WT mice (Fig.2F-G). TSLP overexpression fully restored inguinal, popliteal, axillary, and hepatic LN development and substantially rescued the organogenesis of para-aortic LN (Fig.2F, 2G.II and 2G.IV). The formation of brachial, superficial cervical and deep cervical LN was also clearly enhanced by TSLP Tg expression. Hence, $RAG2^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg animals had almost normal LN numbers. These results show that peripheral B, T and NK lymphocyte compartments are not required for the maintenance of LN anlage. In this line, the depletion of NK cells with NK1.1 antibody (Ab) for the first 2 weeks of life of $RAG2^{-/-}$ mice did not prevent the normal formation of LN (data not shown).

TSLP overexpression increased LTi cell numbers and restored LN development in $RAG2^{-/-}$ $\gamma_c^{-/-}$ mice. Since LTi cells are mandatory for the formation of organizer cell clusters [81, 87], these results suggested that the increase in LTi cell number might have a direct effect on the organizer compartment. To test this hypothesis, we analyzed inguinal LN (iLN) anlage from $RAG2^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg and non-Tg newborn mice. Consistent with the absence of peripheral lymphocytes, iLN from both $RAG2^{-/-}$ $\gamma_c^{-/-}$ and $RAG2^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg animals contained few $CD45^+$ cells (Fig.2H). Within the $CD45^-$ fraction, a homogenous VCAM-1^+ ICAM-1^+ organizer population was present in TSLP Tg animals while it was absent from non-Tg littermates (Fig.2H). These results suggest that the TSLP-mediated increase in LTi cell numbers is instrumental for the restoration of organizer cells in newborn $RAG2^{-/-}$ $\gamma_c^{-/-}$ mice and later for the restoration of LN in adult. These results further indicate that organizer cells are dependent on LTi cell number and suggest that LN development is defective in IL-$7^{-/-}$ and $RAG2^{-/-}$ $\gamma_c^{-/-}$ animals because of suboptimal LTi cell numbers.

Our data are in line with the previously proposed idea [81, 94, 162] that there is a threshold number under which LTi cells fail to effectively “instruct” LN stroma. There are indeed several lines of evidence indicating that low LTi cell numbers fail to induce proper LN development. For

instance, mice that were reported to have decreased LTi cell numbers in their LN anlage, such as TRANCE$^{-/-}$ mice, do not develop LN [94]. In addition, the maintenance of a fetal LN transplanted under the kidney capsule of an adult mouse is dependent on the number of LTi cells [162]. A 15 dpc LN fails to persist unless exogenous LTi cells are added to the graft, whereas 17 dpc LN, containing more LTi cells, persists without LTi addition [162]. Furthermore, mice in which LTi cell recruitment to the LN anlage is impaired are devoid of LN [91, 99].

LTα is required for the generation, the survival and the proliferation of organizer cells [81, 87, 162]. However, the fact that agonist LTβR Ab treatment restores LN development in LTα$^{-/-}$ mice [163] but fails to do so in LTi-deficient mice [81, 94] demonstrates that LTi cells provide additional signals that are independent of LTα. Altogether, our results support the idea that the regulation of LTi cell numbers by cytokines is crucial for LN organogenesis, because LTi cells provide LT-dependent and independent signals, which are crucial for the organizer cell compartment.

Absence of LN in RAG2$^{-/-}$ $\gamma_c^{-/-}$ is independent of lymphopenia.

To test whether the lymphopenia of RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice was responsible for the failure to detect LN, we reconstituted adult sub-lethally irradiated RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP (Ly5.2^{+}) mice and RAG2$^{-/-}$ $\gamma_c^{-/-}$ (Ly5.2^{+}) littermate controls with Ly5.1^{+} WT BM cells. Four weeks after reconstitution, peripheral B, T and NK cells were present in high numbers in the spleen of both Tg and non-Tg recipients, showing the efficient replenishment of peripheral compartments upon BM reconstitution (Suppl. Fig.3A-B). The LN repertoire in RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg and RAG2$^{-/-}$ $\gamma_c^{-/-}$ recipients was identical to that of non-chimeric mice (Suppl. Fig.3C). These results show that the absence of detectable LN in RAG2$^{-/-}$ $\gamma_c^{-/-}$ adult mice was not a result of lymphopenia.

Inguinal LN from RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg mice share features of WT LN.

The LN microenvironment is composed of different stromal populations, which contribute to the LN framework and actively participate to adaptive immunity. For instance, the LN microarchitecture is maintained by a stromal network that can be visualized by staining with an Ab specific for the ER-TR7 antigen [56]. Structures such as capsule, high endothelial venules (HEV), cortex and medulla were readily detectable in iLN from both WT and RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP mice (Figure 3.A-B). ER-TR7 staining clearly resolved B and T cell zones in WT LN while these areas could not be discriminated in LN of RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP mice (Figure 3.A-B). As previously shown in mice devoid of B cells [164], HEV were rather localized below the subcapsular sinus of the LN. While present within the WT B cell follicles, no CR1^{+} networks of

follicular dendritic cells (FDC) were detected in the LN of RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP animals (Figure 3.C-D). As FDC generation is dependent on LT and TNF signals [165], these results indicate that lymphocytes provide crucial LT and TNF signals for the development of FDC network. As expected in WT LN [51, 52], the B and T cell stroma were positively stained for the B-cell attractant CXCL13 and the T-cell attractant CCL19, respectively (Figure 3.E and 3.G). Similarly to what observed in SCID mice [157], staining for CXCL13 in RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP iLN was found along the subcapsular sinus, while CCL19 positive staining was observed in inner parts of the LN (Figures 3.F and 3.H). As for WT, iLN from RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP mice was encapsulated by Lyve-1$^+$ lymphatic endothelium (Figure 3.I and 3.K). The vast majority of CD31$^+$ vascular vessels within the LN from RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP animals were positive for the addressin PNAd, indicating that HEV developed in the absence of peripheral lymphocytes (Figure 3.J and 3.L). Altogether, our results suggest that some mesenchymal and endothelial compartments, which form the LN microenvironment, develop normally in absence of lymphocyte while others critically require lymphocyte presence.

Taken together, our results show that the increased availability of TSLP can restore LN development in IL-7$^{-/-}$ and RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice through increase in LTi cell numbers. Our results shed light on the minimal cellular players required for LN development during ontogeny as they show that the presence of LTi and organizer cells but not of peripheral lymphocytes is critical for LN persistence. They further underscore that there is a threshold under which LTi cells fail to instruct LN stroma. In fact, the TSLP-mediated increase in LTi cell number was sufficient to restore the organizer cell compartment and LN development in RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice. Altogether, our findings support the idea that the crucial role played by IL-7 and γ_c in LN organogenesis is mediated exclusively through the regulation of LTi cell number.

Figure Legends

Figure 1. Increased TSLP availability restores LN development in IL-7$^{-/-}$ mice. (A) Quantification of LN in IL-7$^{-/-}$ and IL-7$^{-/-}$ K14-TSLP Tg littermates. 8 to 11 mice were analyzed per group. (B) Para-aortic region of IL-7$^{-/-}$ (I) and IL-7$^{-/-}$ K14-TSLP Tg (II) littermates. (C) FACS analysis of splenocytes from 4.5 days old IL-7$^{-/-}$ and IL-7$^{-/-}$ K14-TSLP Tg mice. Gates indicate B220^{+} CD19^{+} B cells, NK1.1^{+} NK cells and CD8^{+} and CD4+ T cells. Numbers are mean and standard deviation of percentages. (D) Absolute cell number of B, NK, CD4 and CD8 T cells in 4.5 days old IL-7$^{-/-}$ and IL-7$^{-/-}$ K14-TSLP Tg mice. Histograms represent the mean and standard deviation from analyzing 5 animals per group. (E) Representative FACS plots of IL-7$^{-/-}$ and IL-7$^{-/-}$ K14-TSLP Tg newborn spleens (0.5 days old). Numbers are mean and standard deviation of percentages. Absolute CD4^{+} CD3^{-} IL-7Rα^{+} LTi cell number in spleen (F) and mLN (G) of IL-7$^{-/-}$ (circle) and IL-7$^{-/-}$ K14-TSLP Tg (triangle) 0.5 days old mice. Each symbol represents LTi cell numbers from an individual animal. The mean values are indicated by a bar. (H) LTα1β2 level expression of WT CD19^{+} B cells (shaded), LTi cells from newborn IL-7$^{-/-}$ (plain line) and IL-7$^{-/-}$ K14-TSLP Tg (dotted line) mice in spleen (left) and mLN (right).

Table I. LN restoration in IL-7$^{-/-}$ K14-TSLP Tg mice is LTi cell-dependent. Adult IL-7$^{-/-}$ ROR$\gamma^{-/-}$ K14-TSLP (n=6) mice were analyzed for LN presence together with WT (n=5) controls.

Figure 2. TSLP-mediated increase in LTi cell numbers restores LN development in RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice. (A) FACS profiles gated on CD3^{-} CD19^{-} cells show the percentage of CD4^{+} IL-7Ra^{+} LTi cells found in RAG2$^{-/-}$ $\gamma_c^{-/-}$ and RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg newborn spleens (0.5 days old). Numbers are mean and standard deviation of percentages. Absolute CD4^{+} CD3^{-} IL-7Rα^{+} LTi cell number in spleen (B) and mLN (C) of 0.5 day old RAG2$^{-/-}$ $\gamma_c^{-/-}$ (circle) and RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg (triangle) mice. Each symbol represents LTi cell number for an individual animal. The numbers are the means indicated by the bar. (D) LTα1β2 expression level of LTi cells from LT$\alpha^{-/-}$ (shaded), RAG2$^{-/-}$ $\gamma_c^{-/-}$ (plain line) and RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg (dotted line) newborn mice in spleen (left) and mLN (right). (E) Representative FACS analysis of mesenteric region in 6.5 days old RAG2$^{-/-}$ $\gamma_c^{-/-}$, RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg and WT mice. Histograms show CD45 staining. Gates indicate B220^{+} CD19^{+} B cells, NK1.1^{+} CD122^{+} NK cells and CD8^{+} and CD4^{+} T cells. Numbers are percentages among cells within the lymphocyte gate. 5 animals were analyzed for each group. (F) Quantification of LN present in RAG2$^{-/-}$ $\gamma_c^{-/-}$ and RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg littermates. 9-13 mice were analyzed per group. (G) Para-aortic region of RAG2$^{-/-}$ $\gamma_c^{-/-}$ (I) and RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg (II) littermates. Inguinal region of RAG2$^{-/-}$ $\gamma_c^{-/-}$ (III) and

RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg littermates (IV). (H) Representative FACS analysis of iLN anlage in 2.5 days old RAG2$^{-/-}$ $\gamma_c^{-/-}$ and RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg mice. Gates indicate CD45$^-$ non-hematopoietic cells and, within the CD45$^-$ fraction the VCAM-1$^+$ ICAM-1$^+$ organizer cells. Numbers are percentages among live cells.

Figure 3. Visualization of LN architecture and stromal compartments. Inguinal LN from adult RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg mouse and from WT control were stained for the fibroblastic reticular cell marker ER-TR7 (A-B). CA, capsule; B, B cell zone; T, T cell zone; HEV, High Endothelial Venule. Inguinal LN sections were stained with ER-TR7 together with CR1 (C-D), CXCL13 (E-F), CCL19 (G-H) or Lyve-1 (I and K) or stained for CD31 in combination with PNAd (J and L).

Supplemental Figure 1. Newborn IL-7$^{-/-}$ ROR$\gamma^{-/-}$ K14-TSLP mice lack LTi cells. Representative FACS plot of spleen from 0.5 day old IL-7$^{-/-}$ ROR$\gamma^{+/-}$ K14-TSLP and IL-7$^{-/-}$ ROR$\gamma^{-/-}$ K14-TSLP mice.

Supplemental Figure 2. Lineage$^-$ $\alpha4\beta7^+$ CD117$^+$ FL progenitors but not LTi cells are TSLP-responsive. (A) TSLPR expression level of 14.5 dpc lineage$^-$ $\alpha4\beta7^+$ CD117$^+$ FL progenitors from TSLPR$^{-/-}$ (shaded) and WT mice (plain line). (B) Sorted 14.5 dpc lineage$^-$ $\alpha4\beta7^+$ CD117$^+$ FL progenitors were stimulated *in vitro* with 100 ng/ml of recombinant IL-7 or TSLP or left unstimulated. Levels of STAT5 phosphorylation were assessed by flow cytometry. Numbers indicate the percentage of phospho-STAT5$^+$ cells. (C) TSLPR expression level of newborn splenic CD4$^+$ CD3$^-$ IL-7Rα^+ LTi cells and CD4$^+$ CD3$^+$ T cells from TSLPR$^{-/-}$ (shaded) and WT mice (plain line). (D) Newborn mLN and adult splenic cells were stimulated *in vitro* with 100 ng/ml of recombinant IL-7 or TSLP or left unstimulated. Levels of STAT5 phosphorylation were assessed by flow cytometry analysis. (E-F) Lethally irradiated adult IL-7$^{-/-}$ and IL-7$^{-/-}$ K14-TSLP Tg CD45.2$^+$ mice were reconstituted with 10^6 12.5 dpc CD45.1$^+$ FL cells. (E) Representative cytometry plots of splenocytes from FL chimera 10 days after reconstitution, previously gated on lineage$^-$ CD45.2$^-$ cells. Numbers indicate percentages. (F) Absolute LTi cell numbers in spleen of IL-7$^{-/-}$ and IL-7$^{-/-}$ K14-TSLP Tg recipients (right).

Supplemental Figure 3. LN phenotype in reconstituted RAG2$^{-/-}$ $\gamma_c^{-/-}$ and RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg mice. Sub-lethally irradiated RAG2$^{-/-}$ $\gamma_c^{-/-}$ and RAG2$^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg recipient mice were reconstituted with 10^6 WT CD45.1$^+$ BM cells together with $2x10^5$ regulatory T cells. Four weeks after reconstitution, 8 chimeric mice were analyzed for each group. (A) FACS profiles were gated on CD45.1$^+$ donor cells. Gates show B220$^+$ CD19$^+$ B cells, NK1.1$^+$ CD122$^+$ NK cells, and

$CD4^+$ and $CD8^+$ T cells. Numbers are percentages. (B) Absolute cell number of B, NK, CD4 and CD8 T cells in spleen of $RAG2^{-/-}$ $\gamma_c^{-/-}$ and $RAG2^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg recipients. Histograms represent the mean and standard deviation. (C) Quantification of LN present in $RAG2^{-/-}$ $\gamma_c^{-/-}$ and $RAG2^{-/-}$ $\gamma_c^{-/-}$ K14-TSLP Tg recipients.

Material and methods

Mice

All mice were bred and maintained in our animal facility under specific pathogen-free conditions. The animal experiments received the approval of the Cantonal Veterinary Office of the city of Basel, Switzerland. C57BL/6 mice were purchased from RCC, Itingen, Switzerland. IL-$7^{-/-}$ [7], LT$\alpha^{-/-}$ [62], ROR$\gamma^{-/-}$ [80], TSLPR$^{-/-}$ [141] and K14-TSLP Tg [160] mice were previously described. $RAG2^{-/-}g_c^{-/-}$ mice on C57BL/6 background were kindly provided by Jörg Kirberg, MPI Freiburg, Germany.

For FL chimera, IL-$7^{-/-}$ K14-TSLP Tg mice, IL-$7^{-/-}$ littermates and C57BL/6 WT mice were lethally γ-irradiated (9 Gy) and i.v. injected with 10^6 total FL cells from 12.5 embryos ($CD45.1^+$). 10 days after reconstitution, FL chimera were analyzed for LTi presence. For BM chimera, $RAG2^{-/-}g_c^{-/-}$ K14-TSLP Tg mice and $RAG2^{-/-}g_c^{-/-}$ mice littermates were sub-lethally γ-irradiated (5 Gy) and i.v. injected with 10^6 total BM cells together with 10^5 FACS sorted $CD4^+$ $CD25^+$ regulatory T cells from $CD45.1^+$ animals. 4 weeks after reconstitution, BM chimeric mice were injected with Chicago Blue ink and analyzed.

Flow cytometry and cell sorting

FITC, PE, PE-Cy7, APC or biotin-conjugated α-CD4 (GK1.5), α-CD8α (53-6.7), α-CD19 (1D3), α-NK1.1 (PK 136), α-α4β7 (DATK32), α-ICAM-1(3E2) and α-phospho-STAT5 (47) Abs were purchased from BD Biosciences. α-CD3 (145-2C11), α-B220 (RA3-6B2) Abs were purchased from Biolegend. α-CD45 (30-F11), α-CD117 (2B8), α-CD122 (TM-b1), α-IL-7Rα (A7R34) and α-VCAM-1 (429) Abs were purchased from e-Bioscience (San Diego, CA). As secondary reagent, Streptavidin-PE and Streptavidin-PE/Cy7 (Biolegend) were used. Flow cytometry acquisition was performed with a FACSCalibur™ (BD Biosciences) and data were analyzed using Flowjo software (Tree star). α-TSLPR Ab (AF546) was purchased from R&D systems (Minneapolis).

Erythrocyte-depleted 14.5 dpc FL LTi-progenitors were sorted as lineage (CD3, CD11c, CD19, Ter119, Gr-1, B220, NK1.1)$^-$ α4β7$^+$ $CD117^+$ cells. Cell sorting was done using a FACS Aria™ (BD Biosciences) and re-analysis of sorted cells indicated that they were >98% pure.

For LTi cell number quantification, mLN and spleen from individual 0.5 day old mice were homogenized, filtered and stained. The entire organs were acquired on FACSCalibur™ and $CD4^+$ $CD3^-$ $CD127^+$ LTi absolute cell numbers were obtained from analysis.

For assaying STAT5 phosphorylation, $lineage^-$ $\alpha4\beta7^+$ $CD117^+$ FL cells were FACS-sorted. Alternatively, newborn mLN and adult spleen were homogenized and filtered. Sorted and non-sorted cells were allowed to rest for 2 hours with complete IMDM in round bottom 96-well plate (Falcon). Cells were then stimulated 10 minutes with 100ng/ml of either recombinant TSLP (R&D) or IL-7 (PerproTech) and fixed for 10 minutes with 2% paraformaldehyde (AppliChem) at room temperature. Cells from mLN and spleen were stained for surface epitope for CD45, CD3 and CD4. Permeabilization was done with 90% methanol on ice for 30 min and phospho-STAT5 staining was performed at room temperature for 30 min.

For organizer cells staining, iLN of 2.5 days old mice were digested with 1mg/ml dispase (GIBCO) together with 100µg/ml DNase I (AppliChem) in PBS at 37°C for 20-30 minutes under mild agitation. Cell suspension was then filtered and stained on ice.

Detection of LN

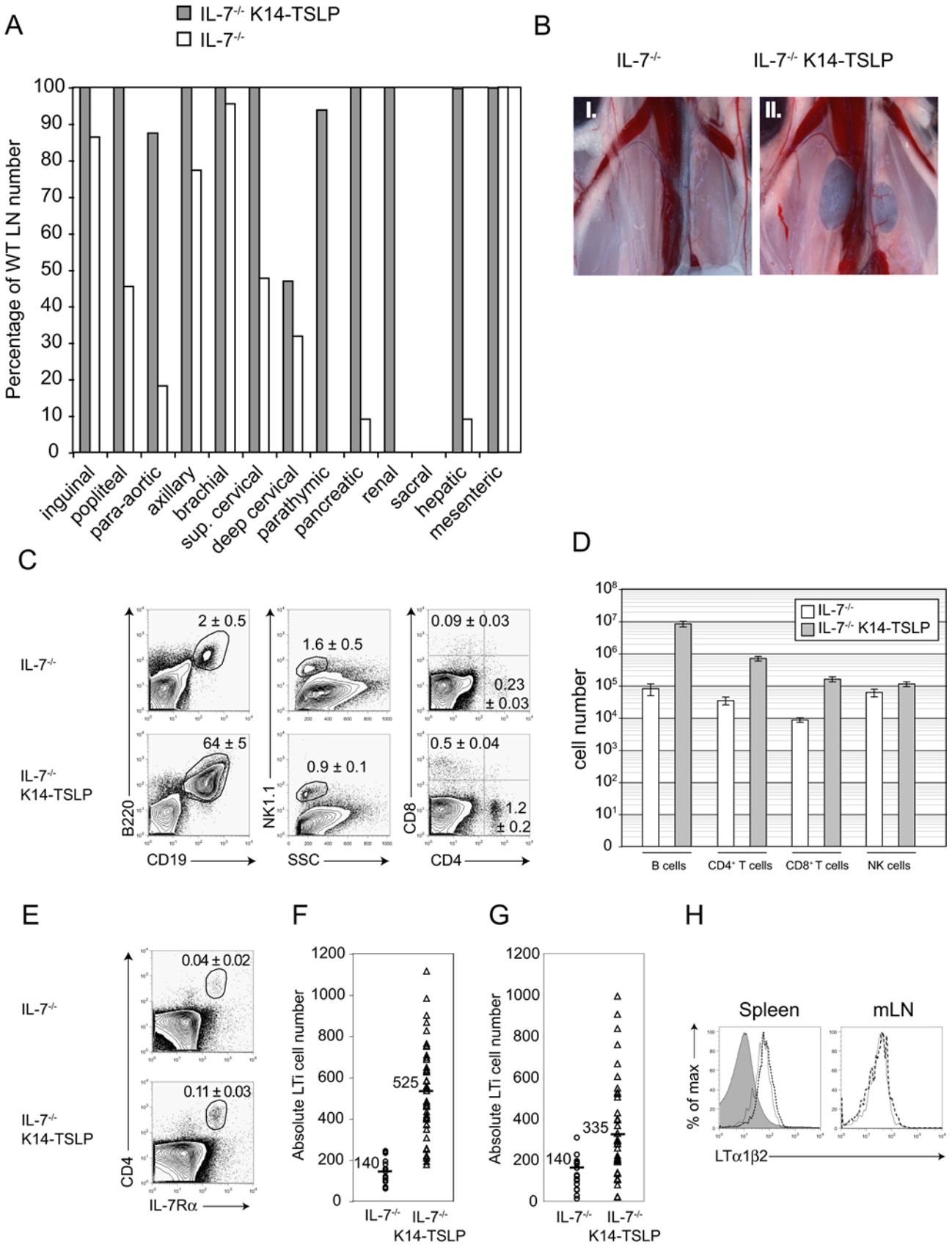
LN enumeration was done a week after peritoneal injection of 100µl of 1% Chicago sky Blue 6B (Sigma) ink in PBS. LN Images were captured with stereoscopic Nikon SMZ1500 microscope coupled with a DS Camera control Unit DS-L1 (Nikon).

Immunofluorescence microscopy

8µm acetone-fixed LN sections were incubated with combinations of ER-TR7 (ER-TR7, AbD Serotec), PNAd (MECA-79, Pharmingen), Lyve-1 (RELIATech), CXCL13 (R&D), CCL19 (R&D), biot-CR1 (Pharmingen) and biot-CD31 (390, e-Biosciences) Abs. ER-TR7 and PNAd were detected with goat α-rat-Cy3 (Jackson ImmunoResearch), CXCL13 and CCL19 with a donkey α-goat Alexa 488 (Molecular probes), Lyve-1 with a goat α-rabbit Alexa 488 (Molecular probes) and CR1 and CD31 with Streptavidin-Alexa-488 (Molecular Probes).

Images were captured on a Zeiss LSM 510 Meta Laser Confocal Scanning Confocal Microscope System (Carl-Zeiss, Fedbach, Switzerland). When required, images were assembled using Adobe Illustrator CS or Adobe Photoshop CS.

Figure 1


A
IL-7-/- K14-TSLP
IL-7-/-
Percentage of WT LN number
100
90
80
70
60
50
40
30
20
10
0
inguinal
popliteal
para-aortic
axillary
brachial
sup. cervical
deep cervical
parathymic
pancreatic
renal
sacral
hepatic
mesenteric
B
IL-7-/-
IL-7-/- K14-TSLP
I.
II.
C
IL-7-/-
IL-7-/- K14-TSLP
2 ± 0.5
64 ± 5
1.6 ± 0.5
0.9 ± 0.1
0.09 ± 0.03
0.23 ± 0.03
0.5 ± 0.04
1.2 ± 0.2
B220
CD19
NK1.1
SSC
CD8
CD4
D
IL-7-/-
IL-7-/- K14-TSLP
cell number
B cells
CD4+ T cells
CD8+ T cells
NK cells
E
IL-7-/-
IL-7-/- K14-TSLP
0.04 ± 0.02
0.11 ± 0.03
CD4
IL-7Rα
F
Absolute LTi cell number
140
525
IL-7-/-
IL-7-/- K14-TSLP
G
Absolute LTi cell number
140
335
IL-7-/-
IL-7-/- K14-TSLP
H
Spleen
mLN
% of max
LTα1β2

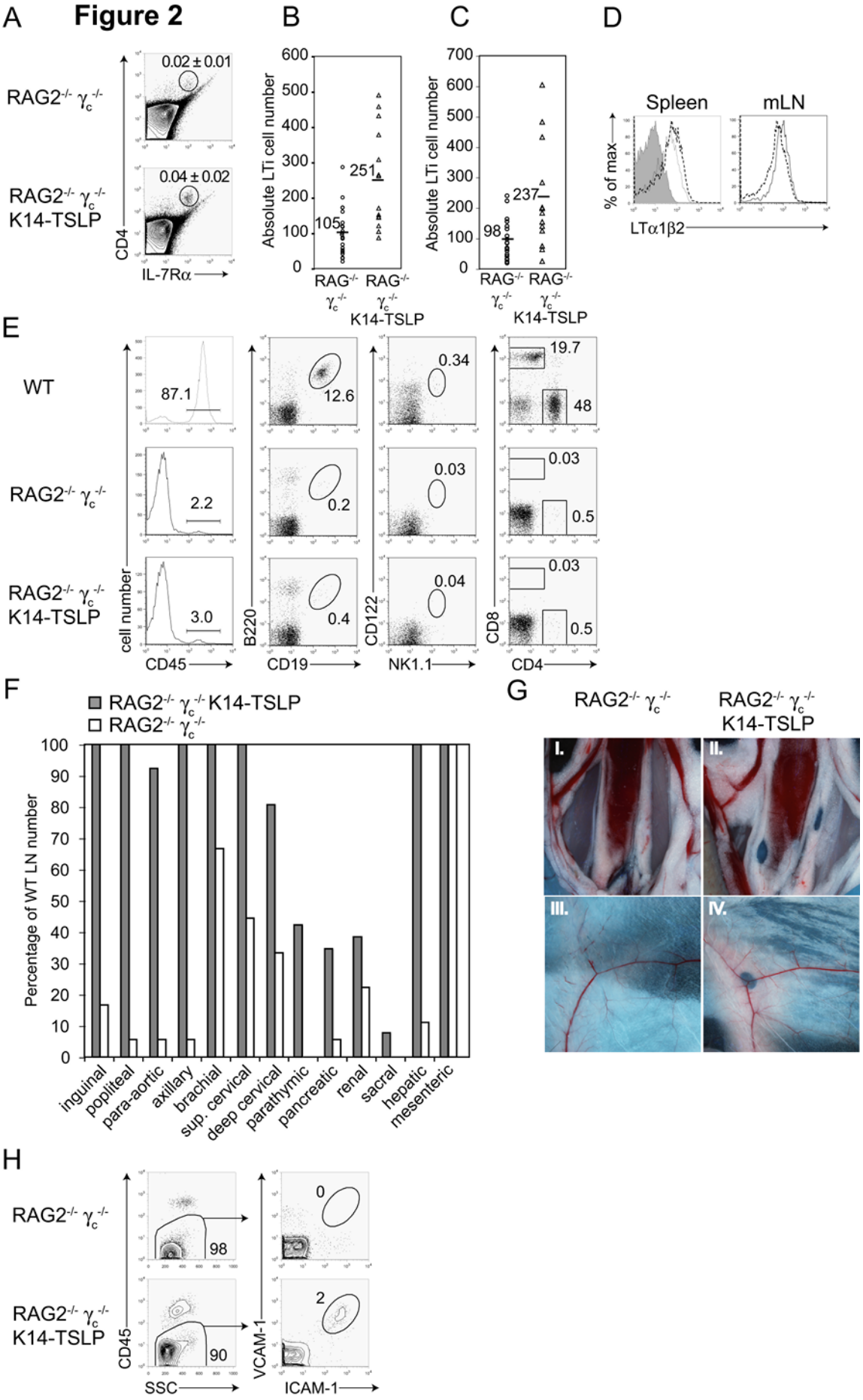
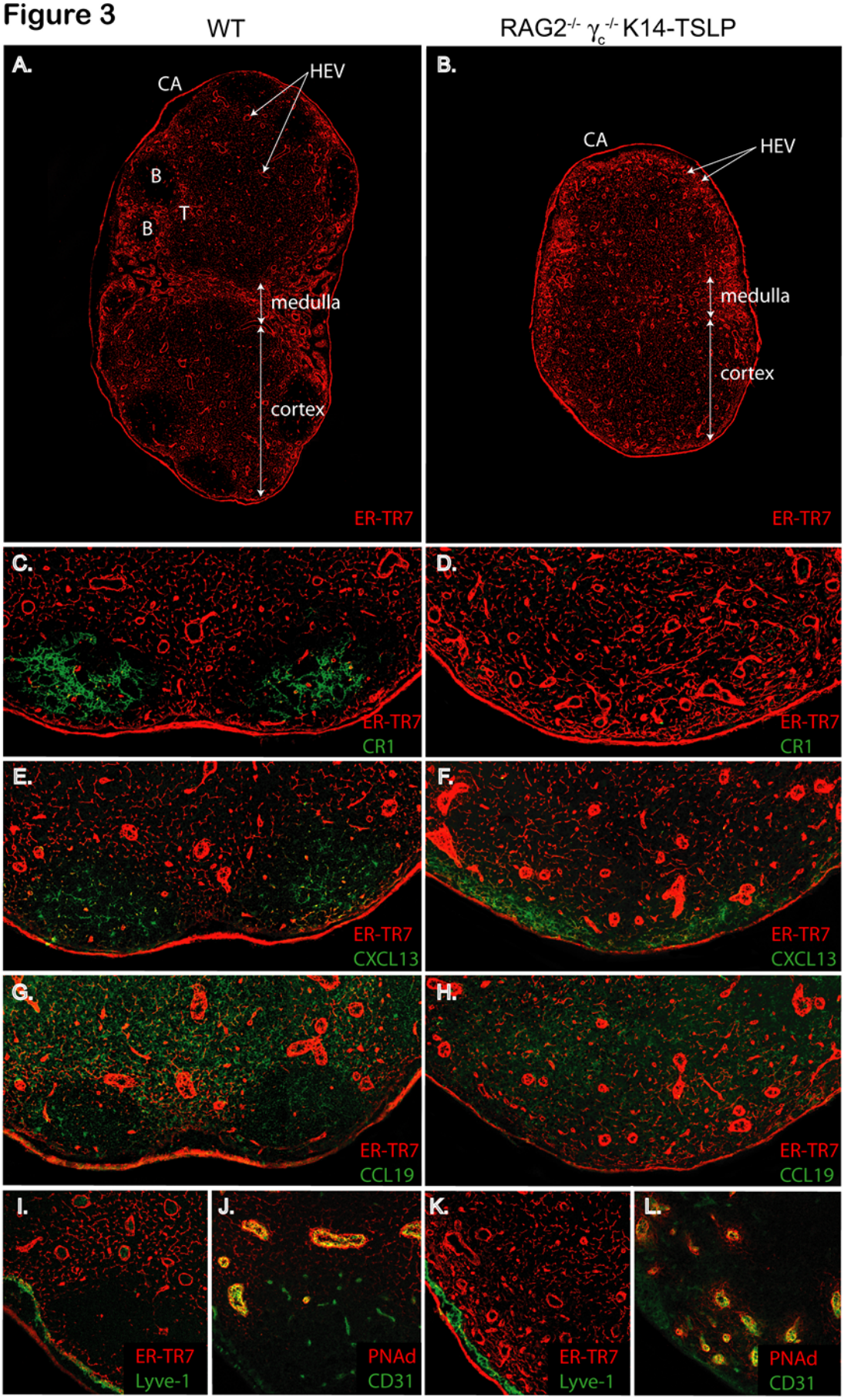
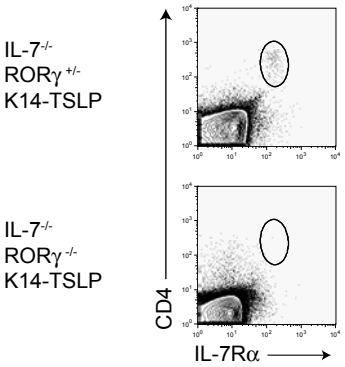


Figure 2
A
B
C
D
RAG2-/- γc-/-
RAG2-/- γc-/- K14-TSLP
0.02 ± 0.01
0.04 ± 0.02
CD4
IL-7Rα
Absolute LTi cell number
105
251
98
237
RAG-/- γc-/-
K14-TSLP
Spleen
mLN
% of max
LTα1β2
E
WT
87.1
12.6
0.34
19.7
48
2.2
0.2
0.03
0.03
0.5
3.0
0.4
0.04
0.03
0.5
cell number
CD45
B220
CD19
CD122
NK1.1
CD8
CD4
F
RAG2-/- γc-/- K14-TSLP
RAG2-/- γc-/-
Percentage of WT LN number
inguinal
popliteal
para-aortic
axillary
brachial
sup. cervical
deep cervical
parathymic
pancreatic
renal
sacral
hepatic
mesenteric
G
RAG2-/- γc-/-
RAG2-/- γc-/- K14-TSLP
I.
II.
III.
IV.
H
RAG2-/- γc-/-
RAG2-/- γc-/- K14-TSLP
98
90
0
2
CD45
SSC
VCAM-1
ICAM-1

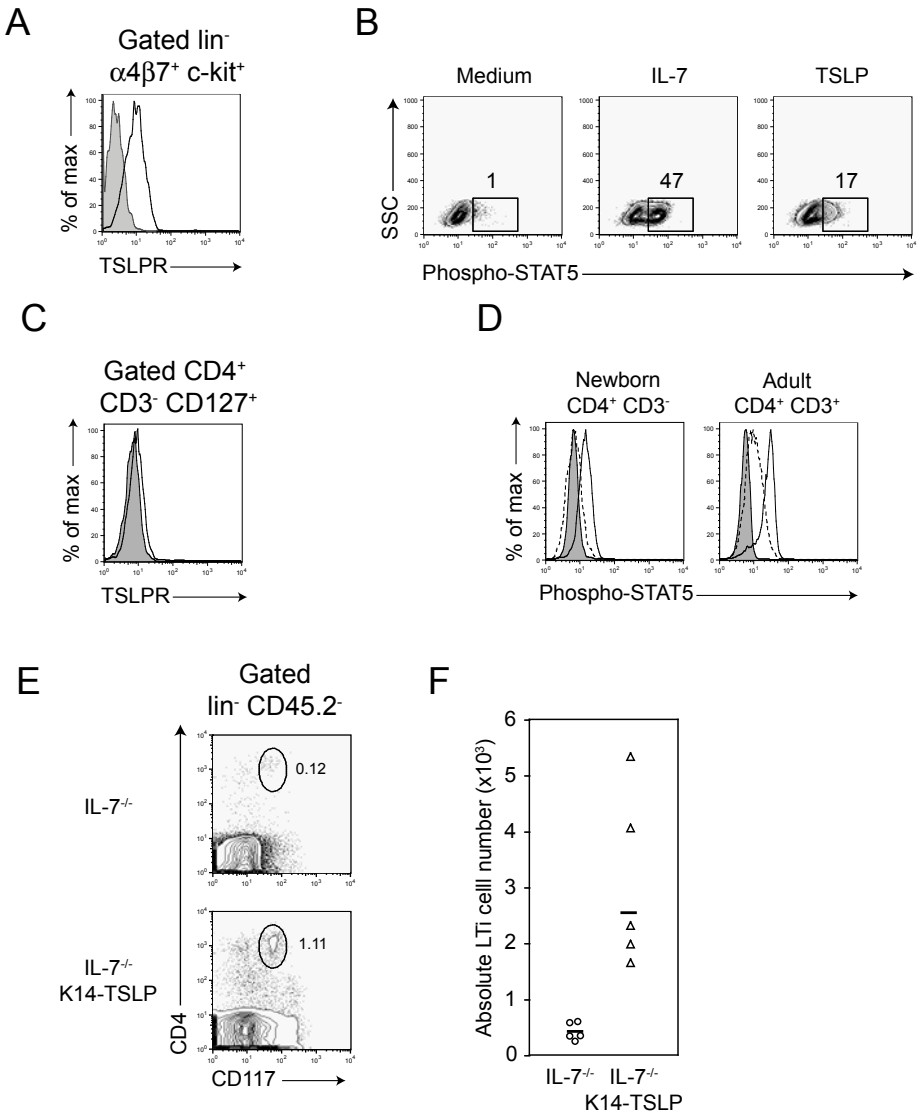
Figure 3


WT
RAG2-/- γc-/- K14-TSLP
A.
CA
HEV
B
T
B
medulla
cortex
ER-TR7
B.
CA
HEV
medulla
cortex
ER-TR7
C.
ER-TR7
CR1
D.
ER-TR7
CR1
E.
ER-TR7
CXCL13
F.
ER-TR7
CXCL13
G.
ER-TR7
CCL19
H.
ER-TR7
CCL19
I.
ER-TR7
Lyve-1
J.
PNAd
CD31
K.
ER-TR7
Lyve-1
L.
PNAd
CD31

Suppl. Figure 1



Suppl. Figure 2



Suppl. Figure 3

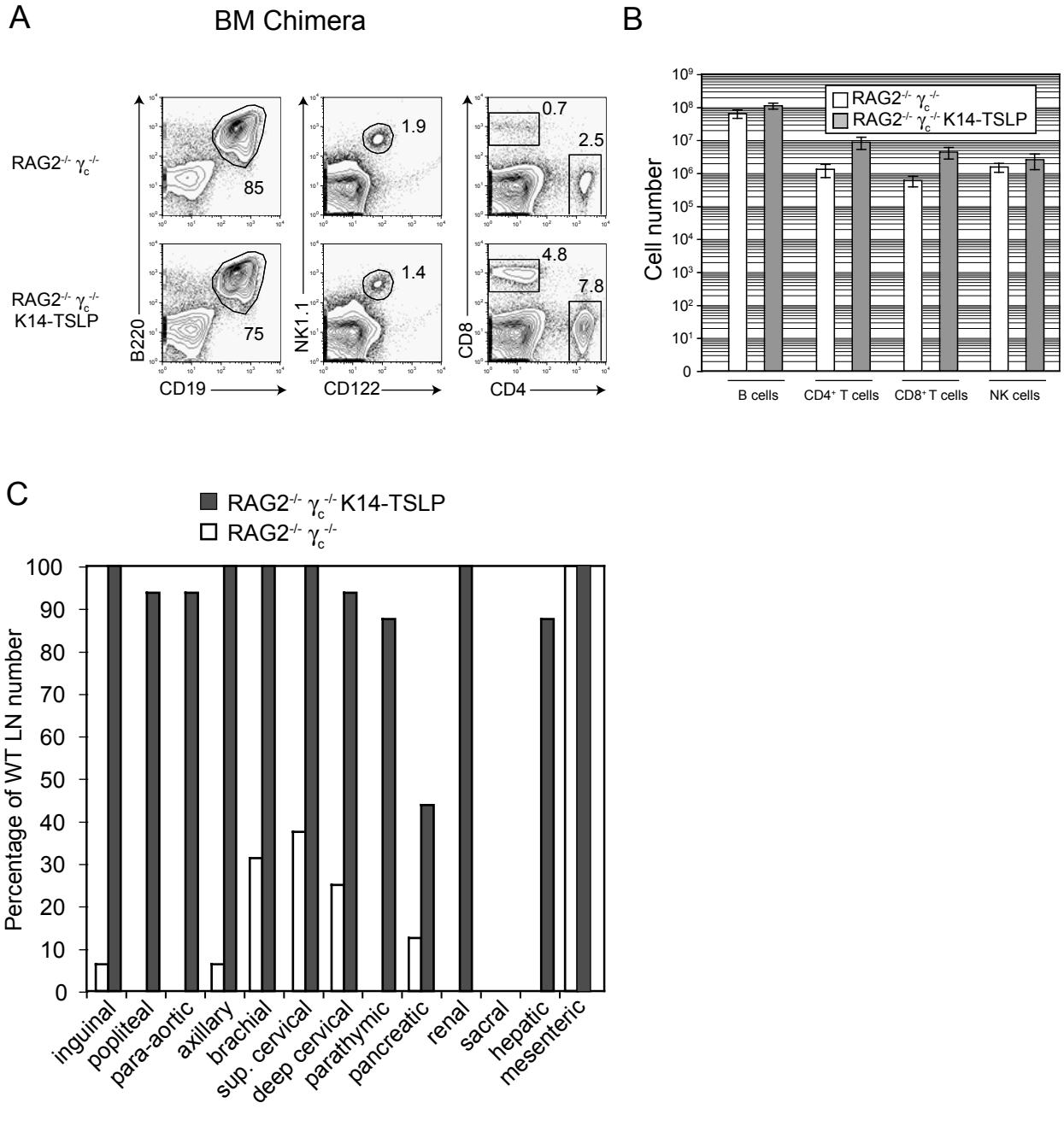


Table I.

	inguinal	popliteal	para-aortic	axillary	brachial	sup. cervical	deep cervical	parathymic	pancreatic	renal	sacral	hepatic	mesenteric
IL-7$^{-/-}$ ROR$\gamma^{-/-}$ K14-TSLP	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/6	0/12	0/6	0/6	0/6
WT	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	5/5	10/10	5/5	5/5	5/5

E. General discussion

IL-7 and TSLP have partially overlapping activity

TSLP is able to sustain B and T cell development *in vitro* [105, 106, 121, 132, 146, 148, 150, 160] and to promote B and T lymphopoiesis *in vivo* [106, 120, 133, 141, 160]. In line with this, we have shown that TSLP can substitute IL-7 for developing and peripheral lymphocyte compartments *in vivo* [160]. We extended this observation to LN organogenesis by showing that TSLP can replace IL-7 for LN development and can increase the size of the LTi cell pool. Altogether these results demonstrate that TSLP and IL-7 have overlapping activities on lymphopoiesis, lymphocyte homeostasis, regulation of LTi cell number and LN development.

TSLP does not share high homology with IL-7, nor TSLPR with γc [106, 123]. Hence, the IL-7Rα/STAT5 axis, which is shared by IL-7 and TSLP, might be the most prominent pathway explaining the redundancy of activity of these cytokines. Anti-apoptotic molecules of the Bcl-2 family, such as Bcl-x_L [166] and Mcl-1 [167] are known targets of this pathway. Overexpression of Bcl-2 Tg expression is able to restore the defect in T lymphopoiesis in IL-7Rα$^{-/-}$ mice [168, 169]. It is therefore possible that TSLP restores T cell development by upregulating the expression of Bcl-2 family members.

TSLP also displays activities, which are not shared with IL-7. For instance, TSLP promotes TH2 differentiation [109, 110, 144, 147, 151] and is perceived by the organism as a "danger" molecule. It is a potent activator of DC [108, 111, 112, 143-147], and is implicated in allergic diseases pathogenesis [110, 113, 118, 145, 147, 154]. Consistent with a role on myeloid cells, we and others have shown that TSLP Tg expression leads to the accumulation of erythroid precursors and granulocytes in the spleen [160, 170]. The understanding of TSLP signaling cascade might help to understand the mechanisms underlying TSLP specific biological activities.

TSLP availability

Several lines of evidences initially suggested that TSLP was promoting fetal rather than adult lymphopoiesis. Lymphopoiesis in newborn IL-7$^{-/-}$ mice is less severely impaired than in adults [5, 32]. The BM and spleen of young $\gamma_c^{-/-}$ animals contain more cells from the B cell lineage than IL-7Rα$^{-/-}$ controls [136] and fetal pro-B cells proliferate in response to TSLP *in vitro* whereas adult progenitors do not [136, 137]. Hence, it has been proposed that fetal but not adult cells were TSLP responsive [136, 137].

In contrast, our results indicate that adult hematopoietic cells are responsive to TSLP. Adult WT BM precursors proliferated and differentiated towards the B and T lineages in response to TSLP *in vitro*. BM chimera experiments showed that TSLP could sustain B and T

cell development from WT adult precursors [160]. The DNA of B cells from IL-7$^{-/-}$ TSLP Tg mice displayed N nucleotides at the rearranged Ig HC locus, suggesting that they were derived from adult precursors [160]. Hence our results rather indicate that TSLP does not play a significant role in adult lymphopoiesis because its availability might be too low.

The analysis of TSLPR$^{-/-}$ or IL-7$^{-/-}$ TSLPR$^{-/-}$ mice later suggested that endogenous levels of TSLP do not play a substantial role in fetal and adult lymphopoiesis [134, 135, 139, 141, 149] nor in LN organogenesis (our unpublished data). Hence, it stems from these results that endogenous TSLP does not contribute to lymphopoiesis and LN organogenesis because TSLP constitutive levels might be too low in lymphoid microenvironments. The promoter of TSLP contains two NF-κB sites [116] and TSLP expression is upregulated under pro-inflammatory conditions [114-117]. Hence, an intriguing possibility is that TSLP might promote lymphopoiesis, lymphoid homeostasis or lymphoid tissue neogenesis under circumstances of stress, physical injuries, microbial or viral infections or inflammation.

Role of peripheral lymphocytes in LN organogenesis

LN are normally found in lymphopenic mice such as severe combined immunodeficient (SCID), RAG1$^{-/-}$ and RAG2$^{-/-}$ mice, showing that peripheral T and B cells are dispensable for LN formation and maintenance. It was possible that, in these mice, LN persisted through an effect of NK cells. However, this possibility was unlikely because the NK compartment, which is normal in IL-7$^{-/-}$ mice [171], is not sufficient to maintain the LN anlage. We show here that peripheral B, T and NK cells are not required for murine LN organogenesis. These results are of importance because they show that lymphopoiesis and the development of LN are independent processes.

By injecting high numbers of adult lymphocytes the first week of life, it was recently shown that LN development could be restored in RAG2$^{-/-}$ $\gamma_c^{-/-}$ mice [161]. These results suggested that in postnatal life, peripheral lymphocytes might play a role in maintaining the LN anlage [161]. In view of our results, we believe that this lymphocyte-mediated LN restoration rather illustrates the capacity for adult lymphocytes to trigger LN organogenesis under certain conditions. In this line, B and CD4$^+$ T cells were shown to be instrumental for the development of lymphoid tissues within the pancreas and the thyroid of mice overexpressing chemokines in these organs [172, 173].

While showing that lymphocytes are not required for LN formation, our results show that lymphocyte presence is instrumental for the full maturation of the LN architecture and for the development of certain stromal populations, such as FDC. Whether, reconstitution with WT BM is sufficient to restore LN architecture, FDC networks and to normalize HEV localization in LN of RAG2$^{-/-}$ $\gamma_c^{-/-}$ TSLP Tg mice, has still to be addressed.

Importance of LTi cell number in LN organogenesis

IL-7 availability regulates the size of the pool of LTi cells [92]. IL-7 overexpression increases LTi cell number and leads to the LTi-dependent formation of ectopic LN [92] whereas IL-7 deficiency leads to defective LN development [92, 160]. Altogether, these results suggest that IL-7 availability controls LN organogenesis by regulating LTi cell number.

In this line, our results show that TSLP Tg expression rescues LN organogenesis in IL-$7^{-/-}$ and $RAG2^{-/-}$ $\gamma_c^{-/-}$ mice and suggest that insufficient LTi cell number is the primary cause for the defective LN development in both strains. The number of LTi cells required for successfully inducing LN formation remains to be determined and is likely to be different for each type of LN. Our results indicate that the cellular requirement for LN formation is limited to LTi and organizer cells, the two populations initially identified as initiating LN organogenesis [87]. However, they do not formally exclude the possibility that other cell types, as in PP organogenesis [174], play a role.

Human patients with γ_c and JAK3 deficiency lack LN [175]. While the cellular and molecular players involved in human LN development are not yet identified [176], it is likely that LN organogenesis occurs according a similar scheme as in the murine system. Hence, our data might help understanding the defective LN formation observed in γ_c and JAK3-deficient patients.

Cross-talk between lymphoid and stroma cells

Our results show that IL-$7^{-/-}$ mice have thymic epithelial cells with an immature phenotype, and that the TSLP Tg expression, likely by restoring T cell development, normalizes thymic architecture [160]. LTi cells were recently shown to promote the maturation of mTEC during fetal life [177]. Hence, thymic microenvironment appears to be shaped by the interaction between lymphoid and mesenchymal cells. Our results also suggest that the LTi cell number has a direct impact on the mesenchymal organizer cells in the LN anlage. They further show that the LN architecture and the localization of HEV are affected by lymphocytes. Consistently, vascular architecture is remodeled upon lymphocyte colonization of the PP anlage [178]. Our data show that the development of mesenchymal FDC compartment in the LN are dependent on lymphocytes. Hence, altogether, our results illustrate the importance of the interaction between cells of the hematopoietic and non-hematopoietic lineages for the normal development of the immune system.

F. References

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