Lymphoid development; a dynamic interplay of timing and dosing
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High levels of thymic stromal lymphopoietin can compensate IL-7 deficiency in human T cell development and increase human CD4^+CD25^{hi} T cell formation

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Abstract

The cytokine thymic stromal lymphopoietin (TSLP) shares the IL-7Rα chain in its receptor complex with interleukin-7 (IL-7). In human T cell development IL-7 plays a crucial role. The limited available in vitro generated data on the function of TSLP in human lymphoid development has left many open questions about the effects of TSLP on human T cell development and its status next to IL-7. Here it is shown that TSLP can activate the earliest human T cell precursors, but the intensity of the TSLPR signaling diminishes with increasing maturation state. Nevertheless, TSLP can fully substitute for IL-7 in human T cell development, when the availability of TSLP and the TSLPR are optimized by adding high doses of TSLP or enforced expression of the TSLPR in the earliest T cell progenitors. In addition to that, we show that in an IL-7 compromised in vivo model for human hematopoietic development (BALB/c Rag2<sup>-/-</sup>γ<sub>c</sub>-/- mice transplanted with human hematopoietic stem cells), enforced expression of TSLP in the hematopoietic cells increases T cell development and maintenance, as well as the generation of CD4<sup>+</sup>CD25<sup>hi</sup> cells.
Introduction

The development and function of both the innate and adaptive immune systems are critically governed by cytokines. Although the members of this family of proteins display high diversity in terms of structure, expression pattern or function, they also exhibit considerable redundancy and pleiotropism. Among cytokines, the interleukin (IL)-7 has been described as a major factor for the control of lymphocyte differentiation, the maintenance of lymphocyte viability and the regulation of lymphocyte homeostasis (1). The receptor for IL-7 consists of two chains, the IL-7 receptor alpha chain (IL-7Rα/CD127) and the common gamma chain (γC/CD132), a signal-transducing component of various cytokine receptors, including receptors for IL-2, IL-4, IL-9, IL-15 and IL-21. Alternatively, CD127 can form a heterodimer with the thymic stromal lymphopoietin receptor (TSLPR) and function as the receptor for the TSLP cytokine (2, 3).

Shared inclusion of CD127 suggests binding of its complementary receptor complex by TSLP or IL-7 may trigger a common signaling pathway and overlapping biological activity. TSLP was first described as a product from the mouse thymic medullary stromal cell line Z210R.1, supporting in vitro development of B cells and acting as a growth factor on thymocytes (4). Similar to IL-7, which can be detected heterogeneously in multiple non-lymphoid and lymphoid tissues, expression of TSLP in vivo is observed throughout the body in human epithelial cells, stromal cells, smooth muscle cells, lung fibroblasts, and mast cells at various locations including thymus, skin, intestine and airways. TSLP has been shown to induce activation and phosphorylation of Akt (5), signal transducer and activator of transcription (Stat)3 and Stat5, similar to IL-7, but fails to activate any of the four known Janus kinases (Jak) (6), while IL-7 is reported to activate Jak3 and consequently Jak1 (7). Conflicting reports exist on the potential of TSLP to induce phosphorylation of the P42/44 mitogen-activated proteine kinase (Erk) (8, 9), which is activated upon IL-7-induced dimerization of CD127 and γC subunits in e.g. CD34+ immature thymocytes (10). Thus, for what is known, TSLP and IL-7 demonstrate considerable overlap in expression and signaling, nevertheless these are not identical.

In vitro studies demonstrated that both TSLP and IL-7 support the growth of murine fetal liver and bone marrow B cell progenitors. However, TSLP demonstrated specificity for distinct B cell progenitor subsets related to differentiation state and origin; TSLP induced proliferation of pro-B cells originating from the fetal liver, but not when originating from
adult BM. In contrast, pre-B cells originating from both the fetal liver as well as the adult BM responded to TSLP, resulting in cell proliferation (11, 12). Additionally, while TSLP supports B cell development to the B220+/IgM+ immature B cell stage, IL-7 promotes development only to the less mature B220+/IgM- stage (6). Equivalent effects were reported for in vitro cultured human precursor B cell subpopulations (Scheeren et al., manuscript submitted).

Genetic ablation of IL-7 expression in mice (13) has shown that IL-7 is indispensable for normal development of T cells in the thymus. From chimeric human-mouse FTOC data it was hypothesized that IL-7 plays a critical role as survival factor and/or as expansion factor for T cell precursors, however IL-7 was not absolutely required for the maturation of these cells (14). Despite the original isolation of TSLP from a thymic stromal cell line, experimental data showing a role for TSLP in T lymphopoiesis are limited. TSLP was shown to support the expansion of murine CD4-CD8- double negative (DN) thymocyte progenitors in medium in vitro but only when combined with IL-1 (15). In fetal thymic organ cultures (FTOC), it was observed that TSLP specifically enhanced the accumulation and proliferation of murine pro/pre-T (DN1, DN2 and DN4) cells (16). We should note that these experiments were carried out in the presence of murine IL-7, endogenously produced by the FTOC thymic stromal cells. A role for TSLP in human T cell development was deduced from experiments addressing the role of IL-7 in this process. When placed in a chimeric human-mouse FTOC, thymic stromal cells constitutively produce murine IL-7 and human progenitor cells such as CD34+ fetal liver cells can enter T cell lineage and differentiate up to the CD3+CD1a+ stage of T cell maturation. The number of human thymocytes arising in such chimeric FTOCs was strongly reduced in presence of a neutralizing monoclonal antibody against both murine and human IL-7, and even further diminished by a monoclonal antibody against CD127 (14). These results suggest that TSLP can partially rescue T cell development in absence of IL-7 signaling. Overall, TSLP has the ability to affect lymphopoiesis in vitro, but it has restricted potency on its own.

In vivo studies of the role of TSLP in lymphoid development mostly support the aforementioned in vitro findings. While TLSPR−/− mice exhibit normal T cell and B cell development, sublethally irradiated TLSPR−/− mice show weaker recovery of lymphocytes than wild-type controls (17). As compared to γc−/− mice, TLSPR−/−γc−/− mice demonstrate lower thymic cellularity and absence of residual early B cell development (17). Additionally, inoculations of TSLP into γc−/− mice induces the accumulation of B cell progenitors in the
bone marrow as well as peripheral CD4+ T cells (17). Increased TSLP availability through transgene expression (with serum levels ~20-fold increased compared to WT and IL-7/- mice) restores lymphoid development and maintenance in IL-7-deficient mice (18), but also improves B cell development in IL-7-sufficient mice (19). Collectively, these results indicate that TSLP can partially compensate for the lack of IL-7 signaling to drive B and T cell development in mice, although TSLP per se is not essential for lymphocyte development. It is still unclear whether similar conclusions also apply to humans, as TSLP- or TSLPR-deficient patients have not been described yet, likely due to the lack of an overt phenotype.

Much of recent interest has focused on its expression by epithelial cells of Hassall’s corpuscles in the thymic medulla, of mucosa-associated lymphoid tissues, by keratinocytes of skin atopic dermatitis lesions, and by airway epithelial cells from asthmatic patients. At these anatomical sites TSLP became apparent as an important factor in the generation of regulatory T (Treg cells), T cell homeostasis and the development of T helper type 2 cells in various inflammatory allergic diseases (reviewed in (20)), respectively. Watanabe et al. were the first to note that epithelial cells within Hassall’s corpuscles are producing TSLP, which leads to the activation of thymic CD11c+ dendritic cells to express high levels of CD80 and CD86 and induce CD4+CD8-CD25- thymocytes to differentiate in vitro into functional CD4+CD25+Foxp3+ Treg cells (21). The role of TSLP and IL-7 in the development of murine Foxp3+ Treg cells remains unclear, with contradicting reports in IL-7Rα-/- mice (22, 23). In vitro, exogenous addition of TSLP to E17 murine fetal thymus FTOC for two days induces the expression of Foxp3 by murine thymocytes as well as the generation of CD4+CD25+ T cells, although the functional activity of these cells was not demonstrated (24). Since TSLPR-deficient mice do not exhibit any differences in the generation of Treg cells (8), the role of TSLP in this process – if any – seems again redundant to IL-7.

Overall, despite poor homology between mouse and human TSLP and TSLPR, which share only 43% and 39% amino-acid identity respectively, human and mouse TSLP exert rather similar biological functions. Until recently it was conceived that an important species difference was the direct action of TSLP on murine T cells versus only indirect action of TSLP on human T cells via DCs (20). However, recent studies have reported on direct effects of TSLP on human activated T cells (25, 26). Direct effects of TSLP on human T cell development have not been demonstrated yet, and important questions remain on which T cell precursors are responsive to TSLP and whether TSLP can substitute IL-7 during human T cell ontogeny. We performed experiments to address these questions by
assessing the role of TSLP in IL-7-deprived systems both in vitro and in vivo. We found that early T cell progenitors are the most responsive to TSLP and that TSLP can fully rescue T cell development in the absence of IL-7, but only when the levels of TSLPR expression and signaling are optimal. In addition, we used an experimental in vivo model of development and function of the human immune system (“human immune system” BALB/c Rag2−/−γc−/−mice) to show that enforced expression of human TSLP by human cells leads to enhanced accumulation of T cells, including thymic CD4+CD25hi cells.

Materials and Methods

Cell lines, constructs and production of viral supernatants.
The murine bone marrow stromal OP9-control and OP9-huDL1 cell lines were previously described (27, 28). Cells were maintained in MEMα (Invitrogen) supplemented with 20% FCS (Hyclone, Logan UT, USA). The cDNA sequence encoding human BCL-2, BCL-XL, TSLP and TSLPR were inserted into the multiple cloning site of the LZRS vector upstream of an internal ribosomal entry site and enhanced green (GFP) fluorescent protein (29). Control vectors were empty LZRS IRES-GFP. Retroviral supernatants were produced as described (30) using the 293T-based Phoenix packaging cell line (31).

Isolation of human hematopoietic progenitors.
Human hematopoietic progenitors were isolated from post-natal thymus (PNT) obtained from surgical specimens removed from children up to 3 years of age undergoing open heart surgery. Human hematopoietic progenitors were also isolated from fetal liver (FL) and fetal thymus (FT) tissue samples obtained from elective abortions, with gestational age ranging from 14 to 18 weeks. The use of these human tissues was approved by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam (AMC-UvA) and was contingent on informed consent.

Human hematopoietic progenitors were purified as follows. The PNT tissue was mechanically disrupted and pressed through a stainless steel mesh to obtain a single-cell suspension, which was left overnight at 4°C. The thymocytes were isolated the next day from a Lymphoprep (Axis Shield) density gradient. This population was split into CD34− and CD34+ cells by immuno-magnetic cell sorting, using direct CD34 human progenitor cell isolation kit (Miltenyi Biotec) and collecting both the CD34+ enriched population and the largely CD34− flowthrough. The thymocytes were stained with mAb against CD34, CD1a,
CD3, CD4, CD8, CD56, and BDCA2. The CD34+CD1a-CD56-BDCA2-CD3 (further referred to as CD34+CD1a) and the CD34+CD1a+CD56-BDCA2-CD3 (further referred to as CD34+CD1a+) population were sorted from the CD34+ MACS enriched population. The CD4+CD8 CD34+CD1a+CD56-BDCA2-CD3 (further referred to as CD4+ immature single positive - CD4ISP) and CD4+CD8+CD34+CD1a+CD56-BDCA2-CD3 (further referred to as CD4+CD8+ double positive - CD4-8DP) populations were sorted from the flowthrough. The sorting was performed with a FACS Aria cell sorter (BD Bioscience), to purity always \( \geq 99\% \). In the case of FT samples, staining and sorting was done without CD34 MACS enrichment.

For FL samples, magnetic enrichment of CD34+ cells was performed by using the Indirect CD34 Human Progenitor Cell Isolation Kit (Miltenyi Biotech), after preparation of single cell suspension and isolation of mononuclear cells by density gradient centrifugation over Lymphoprep (Axis Shield). The SCID-repopulating progenitor cell population was further purified by sorting CD34+CD38- cells with an ARIA cell sorter (BD Bioscience) to purity always \( \geq 99\% \).

**Transduction of human hematopoietic progenitors.**

Human hematopoietic progenitors were transduced with retroviral vectors before OP9 co-cultures or inoculation into recipient animals. The CD34+CD1a- PNT progenitors were cultured overnight in IMDM (Invitrogen) supplemented with Yssel’s medium (32), 5% normal human serum (NHS), 20ng/mL human stem cell factor (huSCF; PeproTech), and 20ng/mL human interleukin-7 (huIL-7; PeproTech). The following day, cells were incubated for 6 to 8 hours with virus supernatant in fibronectin-coated plates (30 µg/mL; Takara Biomedicals). The identical procedure was used with FL CD34+CD38- cells, except that the medium was supplemented with 20ng/mL human thrombopoietin (huTPO; PeproTech).

**Co-cultures of human progenitor and OP9 cells.**

The in vitro development of human T cells was assessed by co-culturing 5-30x10^4 PNT CD34+CD1a- or CD34+CD1a+ progenitor cells with 5x10^4 OP9-huDL1 cells in MEMa (Invitrogen) with 20% FCS (Hyclone) and variable amounts huIL-7 (PeproTech) or TSLP (R&D). To all conditions neutralizing goat anti-murine IL-7 antibody (R&D) was added at 10 µg/mL (The \( \text{ND}_{50} \) for this anti-mouse IL-7 antibody was determined to be approximately 0.5 - 1.5 µg/mL in the presence of 1.5 ng/mL of rmIL-7, using human peripheral blood mononuclear cells). The co-cultures were supplemented every 2 to 3 days with fresh medium.
and cytokines, and progenitor cells were transferred to fresh stromal cells every 4 to 5 days of culture (27).

**Generation of HIS (BALB-Rag/γ) mice.**

BALB/c H-2d Rag2−/− γc−/− mice (33) were bred and maintained in individual ventilated cages, and were fed autoclaved food and water. Mice with a human immune system (HIS (BALB-Rag/γ)) were generated with minor modifications as compared to previously described (34, 35). Newborn (<1 week old) Rag2−/− γc−/− mice received sub-lethal (3.5 Gy) total body irradiation with a 137Cs source, and were injected intra-hepatic (i.h.) with 5-10x10⁴ sorted CD34+CD38− human FL cells. The FL cells were previously transduced, and the cell bulk was then inoculated to the newborn recipients. All manipulations of HIS (BALB-Rag/γ) mice were performed under laminar flow. Adult mice at 8-20 weeks of age were sacrificed and analyzed.

**Flow cytometry.**

Cell suspensions were labeled with FITC, PE, PE-Cy5, PerCP-Cy5.5, PE-Cy7, APC, Alexa-647, APC-Cy7 or Alexa-700 coupled anti-human mAb targeting the following cell surface markers: CD3 (SK7), CD4 (SK3), CD8 (SK1), CD11c (B-ly6), CD19 (HIB19), CD34 (8G12), CD38 (HB7), CD45 (2D1) from BD Bioscience (Franklin Lakes NJ, USA), TCRγδ (GL3) from BD Pharmingen (San Diego, CA, USA), BDCA2 (AC144) from Miltenyi Biotech, CD1a (T6-RD1), TCR pan α/β (IP26A) from Beckman Coulter (Fullerton CA, USA), CD127/IL-7Rα (eBioRDR5) and CD25 (BC96) from eBioscience. A rat-anti-human TSLPR antibody (clone LB1.18B3.1E4) was obtained from Schering Plough Biopharma (Palo Alto, CA) and secondary antibody donkey anti-rat IgG2-Alexa488 from Molecular Probes (Leiden, the Netherlands). A second TSLPR antibody, PE-conjugated mouse-anti-human, was acquired from Biolegend (San Diego CA, USA). Dead cells were excluded based on DAPI incorporation. All washings and reagent dilutions were done with PBS containing 2% FCS and 0.02% sodium azide (NaN₃).

Intracellular staining for tyrosine phosphorylated STAT5 was done according to manufacturer’s instructions (BD), using an Alexa-fluor-647-conjugated monoclonal antibody specific for pSTAT5 (Y694) (pSTAT5, clone 47, IgG1, BD Biosciences) or a control antibody on freshly sorted cells, stimulated for 15 minutes with 50ng/mL TSLP (R&D, Abingdon, UK) or IL-7 (Cytheris). For detection of intracellular expression of BCL-2 protein, cells were fixed in cytofix/cytoperm buffer, washed with Perm/Wash buffer (BD Pharmingen), and incubated...
with FITC-conjugated antibody against BCL-2 (DakoCytomation, Glostrup, Denmark). For detection of phosphorylated proteins, cells were fixed in 1% formaldehyde and permeabilized in ice-cold 90% methanol before incubation with rabbit mAb to phospho-p44/42 MAPK (Thr202/Tyr204, Erk1/2) or phospho-Akt (Ser473) (Cell Signaling Technology, Danvers, MA). As secondary antibody, Alexa488-conjugated goat anti–rabbit IgG (Molecular Probes, Leiden, the Netherlands) was used.

All flow cytometry acquisitions were performed with a LSR-II (BD Bioscience) cytometer interfaced to FACS Diva software system.

**Proliferation assays.**
Sorted cells were directly labeled with 2.5 nM carboxyfluorescein diacetate succinimidyl diester (CFSE; Invitrogen) and subsequently cultured in Iscove’s Modified Dulbecco’s Media, supplemented with 1% NHS and Yssel’s medium, for 4-5 days. 1x10^5 cells were used per condition. Cell proliferation was measured by dilution of the CFSE signal by flow cytometric analysis. Alternatively, sorted cells were cultured in Iscove’s Modified Dulbecco’s Media with 10% FCS for 3 days in triplicate in 96-well plate with indicated cytokines, the last 18 hours in the presence of [3H]thymidine (1µCi/well). Cytokines were added at the start of the culture and just before addition of the [3H]thymidine. Proliferative responses are expressed as the mean [3H]thymidine incorporation (cpm) of triplicate wells ± SD.

**Statistical analyses.**
Data were subjected to two-tail unpaired Student’s t-test analysis where indicated in the figure legends. The obtained p values were considered significant when p<0.05.

**Results**

**The TSLPR complex is expressed on all T cell precursor subsets in the fetal thymus, but only the earliest precursor subsets in the post-natal thymus.**
Before evaluating the biological effects of TSLP on human T cell precursors, we first determined TSLPR complex expression on various early thymic subsets. This was done by flow cytometric analysis of the cell surface expression of IL-7Rα and TSLPR chains on human thymocytes. We compared cells originating from both fetal and post-natal thymus for two reasons: (a) we already observed that TSLPR expression and responsiveness to TSLP was strongly dependent on the origin of human B cell precursor subsets (Scheeren et
al, submitted); and (b) studies reported that TSLP has more impact during fetal rather than adult lymphoid development (11, 36, 37).

The TSLPR complex was detected on all fetal thymic subsets, i.e. the most immature CD34^+CD1a^- thymus seeding progenitors (TSP) and early thymic progenitors (ETP), the T cell committed CD34^+CD1a^- pre-T cells, and their progeny of CD4^+ immature single positive (CD4ISP; CD34^+CD1a^-CD4^-CD8^-CD3^-) and CD4^+CD8^+ double-positive (DP; CD34^+CD1a^-CD4^-CD8^-CD3^-) T cell progenitors. IL-7Rα expression appeared slightly reduced in the CD34^- subsets as compared to the CD34^+ subsets (Fig. 1A). Post-natal thymus CD34^-CD1a^- and CD34^-CD1a^- early T cell precursors were similarly positive for the TSLPR. The more mature CD4ISP and CD4^-8DP T cell progenitors in the postnatal thymus appeared mostly negative for the TSLPR except for a very small proportion of the cells. In contrast, the IL-7Rα was present on all analyzed subsets (Fig. 1B). In conclusion, the IL-7Rα and TSLPR chains were consistently expressed along fetal T cell development and along the earliest stages of post-natal T cell development, with a marked reduction in TSLPR expression on the more mature CD4ISP and DP postnatal subsets.

A  Fetal thymus

![Histograms showing receptor expression](image)

**Receptor Expression**
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To evaluate whether the observed TSLPR receptor complexes expressed on the various subsets were able to induce cell activation, we first determined tyrosine phosphorylation of STAT5 (pSTAT5), which is a known downstream target of TSLP signaling (38). We performed a phospho-flow cytometric analysis for pSTAT5 after ex vivo TSLP stimulation of fetal or post-natal thymocytes. As a comparison we stimulated the cells with IL-7, also known to induce phosphorylation of STAT5 (39). The freshly sorted cells were incubated for 15 minutes with TSLP (50 ng/mL), IL-7 (50 ng/mL) or no cytokine. Specific fetal thymus (Fig. 2A) and postnatal thymus (Fig. 2B) derived subsets responded in a comparable fashion to a given cytokine. pSTAT5 was observed in all subsets upon IL-7 stimulation, the CD34⁺CD1a⁻ TSP / ETP, CD34⁺CD1a⁺ pre-T cells, CD4ISP (CD34⁺CD4⁺CD8⁻) and DP (CD34⁺CD4⁺CD8⁺). Expression of the IL-7Rα chain and TSLPR chain was determined after sorting or electronic gating on the different subsets of fetal thymus (A) and postnatal thymus (B). Filled grey histograms represent staining of the corresponding subset with isotype control antibody. Black lines represent the receptor staining. A representative example, of six different fetal thymus and two different postnatal thymus donors, is depicted.

**TSLPR induced signaling: the more mature the T cell progenitor, the less responsive.**

To evaluate whether the observed TSLPR receptor complexes expressed on the various subsets were able to induce cell activation, we first determined tyrosine phosphorylation of STAT5 (pSTAT5), which is a known downstream target of TSLP signaling (38). We performed a phospho-flow cytometric analysis for pSTAT5 after ex vivo TSLP stimulation of fetal or post-natal thymocytes. As a comparison we stimulated the cells with IL-7, also known to induce phosphorylation of STAT5 (39). The freshly sorted cells were incubated for 15 minutes with TSLP (50 ng/mL), IL-7 (50 ng/mL) or no cytokine. Specific fetal thymus (Fig. 2A) and postnatal thymus (Fig. 2B) derived subsets responded in a comparable fashion to a given cytokine. pSTAT5 was observed in all subsets upon IL-7 stimulation, the CD34⁺CD1a⁻ cells being the most responsive cell population, lessening over CD34⁺CD1a⁺ and 4ISP to DP cells which were the least responsive. Responsiveness to TSLP by fetal and post-natal thymocytes followed this trend, with markedly higher pSTAT5 levels in the more
mature compartments, and minimal to absent levels in the DP subset (fold MFI change compared to control staining for fetal thymus: CD34⁺CD1a⁻: 1.54±0.16, CD34⁺CD1a⁺: 1.18±0.13, CD4ISP: 1.0±0.14, DP: 1.17±0.19 and for post-natal thymus: CD34⁺CD1a⁻: 5.47±2.27, CD34⁺CD1a⁺: 1.33±0.2, CD4ISP: 1.47±0.43, DP: 0.98±0.33). Thus, although TSLPR expression was detected on all fetal thymus subsets with comparable intensity (Fig 1A), pSTAT5 activation was only clearly increased in the CD34⁺CD1a⁻ and to a very limited extent in the CD34⁺CD1a⁺ subset (Fig. 2A) and we can conclude that TSLPR expression on fetal thymic subsets does not strictly correlate with responsiveness to TSLP, as determined by tyrosine phosphorylation of STAT5. TSLPR expression on
post-natal thymus subsets did correspond to pSTAT5 upregulation in the presence of TSLP (Fig. 1B and 2B).

IL-7 stimulation of human immature CD34+, but not CD34−, thymocytes is described to lead to additional phosphorylation of ERK1/2 and AKT as a result of the dimerization of the IL-7Rα and γc subunits (10). Since both STAT5 and PI3K pathways are reported to be involved in T cell development (40), we investigated whether TSLP was similarly capable of activating these pathways. Although murine peripheral CD8+ mature T cells were shown to concomitantly induce activation of the PI3K/Akt and STAT pathways after TSLP stimulation (5), there are no reports on TSLP induced serine phosphorylation of AKT in human or murine developing T cells. We evaluated the ability of TSLP to induce AKT phosphorylation in human T cell precursors with the same experimental set up as for pSTAT5. Upon TSLP addition, we observed phosphorylation of AKT in a mild but comparable fashion to what is described for IL-7: post-natal thymus CD34+ progenitors were able to activate AKT (fold increase in MFI compared to isotype staining: 1.53±0.31) , whereas the CD34− population showed minimal to no phosphorylation (fold increase in MFI: 1.16±0.1) (Suppl fig. 1A). The CD34+ population was similarly capable of upregulating pERK after TSLP stimulation (fold increase MFI: 1.75±0.11) (Suppl fig. 1B).

It is postulated that the primary function of IL-7 in early lymphoid development is to upregulate Bel-2 via STAT5 (41). However, STAT5 activation does not always lead to
upregulation of Bcl-2. We wondered whether TSLPR triggering, in line with its capacity to induce STAT5 phosphorylation, could increase survival of the most early progenitors by means of upregulating the anti-apoptotic factor Bcl-2. It has been shown that TSLP can replace other cytokines as an anti-apoptotic factor when supplied to a culture of MUTZ-3 cells, which are consistently and absolutely dependent on cytokines for their survival (9) and that TSLP stimulation of murine mature CD8+ T cells leads to upregulation of Bcl-2 (5), but no data on human (developing) T cells have been reported yet. Sorted CD34+CD1a- and CD34+CD1a+ cells were incubated in medium containing no cytokines, 50 ng/mL IL-7 or 50 ng/mL TSLP for 24 hours, after which they were assayed for BCL-2 expression by flow cytometry (Fig. 2C). Both TSLP and IL-7 were able to upregulate BCL-2 expression in the two progenitor subsets, although TSLP at lower levels than IL-7. Taken together, with the exception of fetal thymus CD34- subsets, TSLPR signaling corresponds to the level of TSLPR expression observed on the thymic subsets. Overall, the intensity of the TSLPR signaling diminishes with increasing maturation state.

**Stimulation with TSLP induces proliferation of the earliest T cell progenitors only**

It was shown in murine FTOC assays that the proliferation of pro/pre-T cells is enhanced in the presence of TSLP (16). To further investigate the outcome of TSLPR triggering in developing human T cells, we examined the proliferation of the thymic subsets in cultures containing TSLP. We purified human CD34+CD1a- TSP/ETP and CD34+CD1a+ pre-T populations derived from fetal or post-natal thymus and we used the carboxyfluorescein diacetate succinimidyl diester (CFSE) fluorescent cell staining dye to monitor cell division. After 4-5 day culture in the presence of 10 ng/mL IL-7, homogenous cell division was observed with both the fetal and post-natal CD34+CD1a- and CD34+CD1a+ thymic populations, whereas 10 ng/mL TSLP led to limited proliferation of a small cell fraction of the CD1a- population, as compared to cultures without cytokines (Fig. 3). More mature CD4ISP and DP subsets were lost in the culture over time in the absence or presence of these low doses of cytokine (not shown). Culturing sorted postnatal thymus CD34+CD1a- and CD34+CD1a+ cells for 3 days in the presence of no cytokines or 10 ng/mL TSLP or IL-7, and subsequently assessing their proliferation by measuring [3H]-thymidine uptake, demonstrated the same results with a significant absence of proliferation in the more mature CD1a+ thymic cell subset (Suppl Fig. 2). Thus, even though STAT5, AKT and ERK are all phosphorylated by TSLPR triggering in CD34+CD1a+ progenitors, this appears insufficient to result in the induction of proliferation.
TSLP can compensate for the absence of IL-7 in first week of T cell differentiation starting from CD34\(^+\)CD1a\(^-\), but cannot rescue full in vitro T cell development when IL-7 is lacking.

To extend our findings on the effect of TSLP on human T cell precursors, we wanted to determine whether TSLP was able to sustain development and survival of human lymphoid precursors in an in vitro T cell development assay when IL-7 was lacking. We cultured human CD34\(^+\)CD1a\(^-\) PNT lymphoid progenitors in the presence of mouse bone marrow.
stromal OP9 cells expressing the human NOTCH1 ligand Delta-like1, which are known to support T cell differentiation in the presence of the cytokines Flt3L and IL-7 (27, 28).

To gain insight in the isolated function of TSLP, sorted CD34⁺CD1a⁻ and CD34⁺CD1a⁺ PNT were cultured solely in the presence of either 10 ng/mL IL-7 or TSLP or without any cytokines. All cultures were supplemented with an anti-murine-IL-7 neutralizing antibody, as the murine stromal OP9 cells are described to express limited amounts of mouse IL-7 mRNA (42). We used the expression of CD1a and CD3 to evaluate the degree of T cell commitment induced by TSLP and huIL-7. Expression of CD1a on human thymic progenitors is associated with T cell commitment, and these pre-T cells further differentiate into immature thymocytes (CD1a⁺CD3⁻) and mature thymocytes (CD1a⁻CD3⁺) (43).

In the presence of IL-7 sorted human CD34⁺CD1a⁻ progenitors committed to the T cell lineage (expressing CD1a and/or CD3) and CD3⁺CD1a⁻ mature T cells were generated in the co-culture assay (Fig.4A). In contrast, in the absence of cytokines or in the presence of TSLP, T cell commitment occurred, however sporadic to no mature T cells were generated as all cells in the culture disappeared over time. TSLP gave a minor rescue in cell numbers in the first week, as compared to the condition without cytokines (Fig.4B), but was unable to maintain this proliferation benefit. When starting the cultures from CD34⁺CD1a⁺ pre-T progenitors, IL-7 was similarly capable of supporting full T cell development (Fig.4C), although peak expansion of the bulk culture remained about 5-10 fold lower (Fig.4D) and the absolute number of mature T cells generated was 4 times lower (data not shown), as compared to cultures initiated with CD34⁺CD1a⁺ TSP/ETP progenitors. Conversely, the CD34⁺CD1a⁺ pre-T progenitors quickly disappeared from the culture in absence of cytokine or in presence of TSLP, which appeared to have no beneficial effect.

Thus, TSLP can partially compensate for the absence of IL-7 during the first week of the T cell differentiation assay, with respect to progenitor expansion, but only when starting from CD34⁺CD1a⁻ cells and in a transient manner.

Enforced expression of Bcl-2 in progenitors enables TSLP to partially rescue in vitro T cell development in the absence of IL-7

Within 2-3 weeks of OP9-DL1 PNT co-culture in absence of growth stimulating cytokines, around 60% (starting from CD34⁺CD1a⁻ progenitors) and 90% (starting from CD34⁺CD1a⁺ progenitors) of all human cells incorporated DAPI, as a sign a cell viability loss (Suppl.
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A

starting from CD34+CD1a-

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starting from CD34+CD1a-

- IL-7
- TSLP
- no cytokines

Total Cell No.

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Figure 4. In vitro evaluation of the potential of TSLP to support T cell development. Human CD34+CD1a- and CD34+CD1a+ lymphoid progenitors were purified from post-natal thymocytes and co-cultured with stromal OP9 cells expressing huDL1 ligand of NOTCH. The co-cultures were supplemented with either IL-7, TSLP or no cytokines. The fractions of human cells at different stages in T cell development (expressing CD1a and/or CD3) were followed over time and are shown for cultures starting from CD1a- (A) and CD1a+ (C) progenitors.
The results show one representative experiment out of 3. The graphs at the right show pooled data of global human cell expansion in these co-cultures over time, starting in each condition from 1x10^5 CD34^+CD1a^- (C) or CD34^+CD1a^+ (D) PNT progenitors. Absolute cell numbers of cultures with IL-7 (open squares, striped line), TSLP (closed triangles, full line) or no cytokines (closed circles, dotted line) are shown.

**Fig.3.** The addition of IL-7 to OP9-DL1 PNT co-cultures strongly reduced the fraction of nonviable DAPI^+ cells to less than 10%, which is probably the net result of increased proliferation and survival. TSLP significantly reduces this fraction as well, but only mildly and only when cultures originate from CD34^+CD1a^- progenitors (Suppl. Fig.3). The limited rescue effect of TSLP versus IL-7 reflects its restrained potency to induce proliferation and BCL-2 upregulation compared to IL-7.

We wanted to analyze the capacity of TSLP to support full development to mature T cells in the absence of IL-7, when cell death would no longer be a limiting factor for the analysis. We addressed this issue by genetically engineering human PNT CD34^+CD1a^- precursors for enforced expression of BCL-2. Control transduced or BCL-2 transduced human CD34^+CD1a^- PNT progenitors were co-cultured with stromal OP9-DL1 cells, in the absence of cytokines or the presence of 10 ng/mL TSLP or IL-7 and their development was followed over time. As expected, in the presence of IL-7, there was an accumulation of control GFP^+ CD4^+CD8^+ DP immature T cells and mature T cells (CD3^+CD1a^+) over time (see **Fig. 5A and B** for frequencies in left panels per profile and see **Fig. 5C and D** for absolute cell numbers of generated DP and T cells respectively). However, in the absence of cytokines or in the presence of TSLP, human progenitor cells quickly disappeared from the culture and sporadic to no mature T cells were recovered from co-cultures with control transduced PNT (**Fig. 5B**). In contrast, BCL-2 transduced GFP^+ progenitors showed a limited rescue in total cell numbers and in the generation of CD4^+CD8^+ DP / CD3^+CD1a^+ immature T cells. This rescue of DP was already apparent in absence of cytokines and to a higher extent when TSLP was present in the co-culture. (**Fig. 5A, B and C**). A population of CD3^+CD1a^- BCL-2/GFP^+ mature T cells could only be retrieved in the TSLP culture and accumulated up to 12-fold more than in the cultures without BCL-2 over expression (**Fig. 5D**). Taken together, these results show that over-expression of the anti-apoptotic factor BCL-2 alone can partially rescue accumulation of immature T cell populations but not of mature T cells, whereas enforced BCL-2 expression in conjunction with TSLPR triggering can partially substitute for IL-7. Identical results were obtained when the progenitors were transduced with BCL-XL instead of BCL-2 (data not shown).
### CHAPTER 6

#### A

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The role of TSLP in human T cell development

Increasing TSLPR activation can substitute IL-7 for in vitro development of T cells.

As we observed that TSLP was capable of activating transcriptional pathways in CD34^+CD1a^- and CD34^+CD1a^+ T cell progenitors, similar to IL-7, but was still unable to fully substitute for IL-7 during T cell development, we wondered whether this could be a mere result of insufficient intensity of TSLPR triggering. To test this hypothesis, we used two approaches to improve TSLP signaling in human progenitors: (a) increasing dosing of TSLP; and (b) ectopic expression of the human TSLPR in human progenitors. We examined the outcome of OP9-huDL1/PNT co-cultures that were supplemented either with 10 ng/mL IL-7 or with TSLP at concentrations ranging from 5 to 500 ng/mL, with sorted CD34^+CD1a^- or

Figure 5. In vitro evaluation of the potential of TSLP to support T cell development. Human CD34^+CD1a^- lymphoid progenitors were purified from post-natal thymocytes, retrovirally transduced with BCL-2 linked to GFP or an empty control vector expressing GFP, and co-cultured with stromal OP9-DL cells. The co-cultures were supplemented with either IL-7, TSLP or no cytokines. (A) The T cell differentiation of the GFP^+ human cells was followed over time and is shown in the dotplots. For each experimental condition the CD4 versus CD8 profile (A) and CD3 versus CD1a profile (B) is shown. Specific culture conditions are as indicated in the figures. The results show one representative experiment out of 3. (C) Shown is the number of DP cells generated in the cultures starting from 5x10^4 GFP^+ progenitors, measured at d14. (D) Shown is the number of mature T cells (CD3^+CD1a^-) generated in the cultures starting from 5x10^4 GFP^+ progenitors, measured at d18. This figure is representative of three experiments which all showed a partial rescue of mature T cells generated in the presence of TSLP and BCL2. However, due to large interdonor variation with respect to kinetics and extent of proliferation, the experiments could not be pooled to show significant changes by overexpression of BCL-2.

Increasing TSLPR activation can substitute IL-7 for in vitro development of T cells.

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CD34+CD1a+ PNT cells transduced either with a control vector or a vector encoding the human TSLPR chain. Starting from CD34+CD1a+ progenitors, enforced expression of the TSLPR allowed for full T cell development in the presence of TSLP at concentration as low as 5 ng/mL (Fig 6A). In the presence of 500 ng/mL TSLP, TSLPR transduced cells even outcompeted IL-7 induced T cell generation, with a 10-fold increase in mature T cell numbers over 3 weeks of co-culture. In contrast, TSLPR over expression had little effect on T cell generation in cultures starting from CD34+CD1a+ progenitors (Fig. 6B). Strikingly, we observed an increase in TSLPR-GFP+ cells in the cultures containing TSLP starting from CD34+CD1a+ cells (data not shown), suggesting a survival or proliferation benefit mediated by TSLPR without improvement of T cell generation. The data show that when TSLPR signalling is optimized, the full T cell developmental program can be completed.

Fig 6. Improved TSLP usage with enforced expression of the TSLPR chain. The number of human thymocytes in the OP9-DL co-culture was compared between several conditions; in the presence of IL-7, or increasing doses of TSLP, and in the presence or absence of enforced TSLPR expression, as indicated. The bar graphs show a detailed analysis of the human thymopoiesis sub-populations (with immature uncommitted (CD3-CD1a-), committed immature (CD3-CD1a+ and CD3+CD1a+) and mature (CD3+CD1a-) T cell populations) at three timepoints. Cultures were started with sorted CD34+CD1a- (A) and CD34+CD1a+ (B) transduced PNT. This figure is representative of three experiments.
Increased TSLP availability through transgene expression in vivo improves T cell development, peripheral T cell homeostasis and CD4+CD25+ T cell generation.

To study the in vivo role of TSLP in human T cell development and maintenance, we generated “human immune system” (HIS) mice by transplantaing purified human hematopoietic stem cells (HSC) into newborn BALB/c Rag2−/−IL-2Rγc−/− mice (44). The resulting HIS (BALB-Rag/γ) mice are repopulated in a multilineage fashion by human immune lymphoid and myeloid cells in all major primary and secondary lymphoid organs. Circulating, mature human lymphocytes are observed in the blood and secondary lymphoid organs around 6 weeks after HSC transplantation – although in a sub-optimal fashion (34, 35). We have demonstrated that the lymphoid development in these mice takes place in an environment comparable to an IL-7 deficient background, due to poor cross-reactivity of murine IL-7 on human hematopoietic cells (van Lent et al., manuscript submitted).

Before transplantation of the CD34+CD38− sorted fetal liver HSC into the mice, we transduced them with a retroviral vector encoding the human TSLP gene linked to a GFP marker. Control mice were generated with a retroviral vector only expressing GFP. After reconstitution, significant differences could be observed when comparing TSLP-GFP+ cell subsets versus control-GFP+ cell subsets. Although the total number of human CD45+ cells in the thymus was comparable between the two groups of HIS (BALB-Rag/γ) mice, we observed a significantly enhanced proportion of CD3+ cells within the TSLP-GFP+ population in the thymus (Fig. 7A). We observed previously that transduction for huIL-7 enforced expression similarly resulted in a selective advantage for transduced cells with significantly improved thymopoiesis. However this did not result in an accumulation of human T cells in the periphery (van Lent et al., submitted). In contrast, the increased TSLP-GFP+ CD3+ T cell numbers found in the thymus were maintained in the periphery, as shown for the spleen, demonstrating an additional positive effect of TSLP on T cell homeostasis (Fig. 7B). This may reflect the in vitro data suggesting that human TSLP promotes dendritic cell-mediated CD4+ T cell homeostatic expansion (45), as we additionally observed a significant increase in the number of TSLP-GFP+CD11c+ conventional dendritic cells and a trend (p=0.08) towards increased TSLP-GFP+ CD3+CD4+ cell numbers (Fig. 7C). Strikingly, the TSLP-induced accumulation of CD3+ T cells was mostly affecting TCRαβ+ cells, and the number of CD3+TCRγδ cells remained unaltered as compared to control-GFP+ cells (data not shown).

As TSLP was discovered to play a role in the generation of human CD4+CD25hi T cells
Fig 7. Improved CD3+ T cell generation and maintenance in HIS (BALB-Rag/g) mice with enforced expression of huTLSP. (A, B) The number of GFP+ human thymocytes harvested from adult HIS (BALB-Rag/g) mice (20 weeks post-transplantation) produced either with control-transduced or huTSLP-transduced human HSC is shown on the left. The graphs on the right show the frequency (middle) and total number (right) of human CD3+ cells (GFP+ gated) in the thymus and spleen respectively (C) The number of GFP+ CD4+ T cells (left) and the number of GFP+ CD11c+ DC (right) in the spleen are shown. (D) The frequency and number of thymic GFP+ CD3+CD4+CD25hi cells are shown. All number values are indicated as for starting from 1x10^5 TSLP-GFP+ or Ctrl-GFP+ injected cells. * = p<0.05
with a regulatory T cell activity (21), we evaluated the numbers of GFP+ CD4+CD25hi
cells in the two groups of HIS (BALB-Rag/γ) mice. The frequency and number of human
CD4+CD25hi T cells appeared significantly enhanced in the presence of TSLP in the thymus
(Fig. 7D). In the periphery the differences were no longer significant (data not shown). This
is the first in vivo proof of the role of TSLP in human thymic CD4+CD25hi induction.

Discussion

The scarce available in vitro generated data on the function of TSLP in human lymphoid
development has left many open questions about the effects of TSLP on human T cell
development. In this study, we demonstrate the so-far-unappreciated capacity of TSLP to
fully substitute for IL-7 in human T cell development, when the availability of TSLP and
the TSLPR are optimized. In addition to that, we show that in an IL-7 compromised in
vivo model for human hematopoietic development, enforced expression of TSLP in the
hematopoietic cells increases T cell development and maintenance, as well as the generation
of CD4+CD25hi cells.

TSLP has been described as an ‘IL-7-like cytokine’, as TSLP also makes use of CD127 in
its receptor and has similar functions to IL-7, although not all biological actions overlap.
The effect of a particular cytokine on a given cell depends on the cytokine, its extracellular
abundance, the presence and abundance of the matching receptor on the cell surface
and downstream signal cascades activated by receptor binding. TSLP is present in human
serum at comparable levels as IL-7 (46, 47), nevertheless it is conceivable that the levels of
TSLP within the thymus are lower than levels of IL-7. Human TSLP was found selectively
expressed by epithelial cells of Hassall’s corpuscles within the human thymic medulla (8),
while IL-7 is produced in a constitutive manner by a subset of cortical thymic epithelial
cells (TEC) (48), but actual intrathymic levels remain elusive. Otherwise, the differences in
receptor expression on thymic subsets could be determined in a straightforward manner.
As CD132 is widely expressed by lymphoid precursor cells (49), the responsiveness to IL-7
relies on the expression of CD127. For post-natal thymocytes, the intensity of CD127
staining was consistently higher than the TSLPR. This could not be attributed solely to
antibody affinity, as concomitant staining with the anti-CD127 and the TSLPR antibody
revealed a persistent large proportion of the cells which was CD127+ but TSLPR-.
In contrast, as expected all TSLPR+ cells were CD127+ (data not shown). The stoichiometry of the TSLPR and IL-7Rα subunits is reflected in the response to these cytokines; levels of downstream signaling molecules induced by TSLP were confined as compared to IL-7 and subsequent functional read outs as proliferation and survival capacity were markedly reduced. Additionally, not all TSLPR+ cells responded to TSLP with pSTAT5 upregulation; the TSLPR+ fetal thymus DP subset did not respond to TSLP and none of the analyzed subsets responded homogenously to TSLP, although the sorted populations were ≥99% pure. It would be interesting to address in the future whether components required for the TSLP induced signaling cascade are lacking in these cells, or potent suppressors of the signaling pathway are at play. Collectively, the data suggests that the difference in surface receptor expression and the strength of receptor activation together with the (presumed) abundance of its potent brother cytokine IL-7, makes TSLP the redundant cytokine in lymphoid development it is perceived to be. In this study we manipulated various determinants of (in vivo) cytokine responsiveness, by using IL-7 compromised conditions, enhancing cytokine abundance, transgenic expression of downstream signaling molecules BCL-2 and BCL-XL and enforced expression of the TSLPR. In this way we demonstrate that TSLP can have potent and complete IL-7 overlapping effects in human T cell development.

Evaluation of the role of TSLP in human B cell development clarified that only the early immature B cell progenitor stages were responsive to huTSLP (Scheeren et al, manuscript submitted). Within the fetal or postnatal thymus, we clearly observed a gradual loss in TSLP responsiveness with increasing maturation, towards a (almost) non-responsive CD4+CD8+ DP population. This is in agreement with the reported findings that their direct progeny, (non-activated) mature human CD4+ and CD8+ T cells, does neither express the TSLPR nor respond to TSLP (25). Interestingly, the rescue of T cell development in an IL-7 deficient setting was only partially successful by manipulation of cytokine availability and receptor expression when we started from the already T cell committed CD34+CD1a+ T cell stage. Apparently, next to decreased receptor expression, accompanying intracellular changes have occurred in these progenitors during differentiation that make the cells far less responsive to (increased) TSLPR signaling. On the contrary, the IL-7R complex can still activate all factors required for its lymphopoietic function in cultures starting from committed T cell precursors, although the generated cell numbers in the cultures do remain markedly lower compared to the high yield from uncommitted CD1a+ progenitors - this is of course related to strong initial expansion potential of IL-7 on CD1a+ cells.
The precise downstream signaling molecules and inducible genes expression profile activated by TSLP are not fully understood, especially not for humans. However, studies have revealed that human TSLP induces phosphorylation of STAT5 in a distinct mechanism to IL-7. While IL-7R triggering results in Jak1, Jak3 and subsequent IL-7Rα chain tyrosine 499 activation with Stat5 binding and activation (7), TSLP does not rely on the phosphorylation of any known Janus family kinases (6). Additionally, TSLP-mediated Stat5 activation is shown to be insufficient for cell proliferation. The cytoplasmic residue of the TSLPR containing the Y103 phosphorylation site appeared absolutely required for activation of an additional pathway to induce proliferation. It is suggested that this pathway involves a Src family kinase, because addition of a Src family kinase inhibitor blocked TSLP-mediated proliferation (50).

The limited to absent proliferation observed after stimulation of CD34+CD1a+ cells with TSLP in vitro, in contrast to the capacity to induce proliferation in the preceding CD34+CD1a- stage, together with the limited effect after over-expression of the TSLPR in the T cell committed thymic subset, suggests that a still unidentified factor required for TSLP induced proliferation is lost. Determination of levels of Src kinases in the successive thymic subsets may give a clue about its potential role in the observed differences. Apart from that, other unidentified molecules lacking or specifically present in TSLP receptor downstream signaling events, compared to the IL-7 induced cascade, may be involved in T cell development, glucose metabolism, survival mechanisms or other events in these in vitro cultures.

In vivo studies on the role of TSLP in murine lymphoid development have demonstrated the redundancy of TSLP to IL-7. No reports are known that describe exclusive TSLP deficiency in humans, so the contribution TSLP in human lymphoid development remains elusive. The markedly lower potency of equal concentrations of TSLP versus IL-7 on developing T cells suggest a similar redundant function in human T cell development. To dissect the potential in vivo role of TSLP signaling in human T cell development and maintenance we used an experimental model of development and function of the human immune system, namely newborn BALB/c Rag2−/−γc−/− mice transplanted with human hematopoietic stem cells. The exclusive presence in this system of murine IL-7, which is known to be poorly cross-reactive on human T cell progenitors (van Lent et al., submitted), contributes to an environment in which TSLP responsiveness can be efficiently addressed.
In HIS (BALB-Rag/γ) mice which were transplanted with HSC that were partially transduced with the human TSLP gene linked to a GFP marker, we noted an increase in T cell lymphoiesis with significantly increased absolute numbers of GFP\(^+\) CD3\(^+\) T cells generated in the thymus, fitting our generated in vitro data of increased T cell development if high enough levels of TSLP were supplied. The abundance/local available concentration of the cytokine was clearly important, as no increase in T cell numbers was found in the GFP compartment; only the GFP\(^+\) cells that had been able to produce and supply, in an autocrine manner, high enough levels of TSLP were beneficially affected.

In HIS (BALB-Rag/γ) mice, the amount of human thymocytes and peripheral T cell numbers is limited (around 1% of expected numbers in wild-type mice) (34, 35). By using different approaches, such as increased human IL-7 signaling and over-expression of anti-apoptotic factors, we could never significantly improve the peripheral human T cell reconstitution in HIS (BALB-Rag/γ) mice, even when enhanced human thymopoiesis was obtained (van Lent et al., submitted). In mice, a critical role for TSLP in mediating optimal T and B cell lymphopoiesis recovering from lymphopenia has been reported (17). In this study we show that the improved thymopoiesis is reflected in the periphery, with higher absolute T cell numbers in the TSLP-GFP\(^+\) cell pool. In vitro studies on human (mainly CD4\(^+\)) T cell homeostasis have linked T cell proliferation strongly to activation by human TSLP conditioned CD11c\(^+\)DC. So TSLP does not act directly on T cells, but via ‘hTSLP-DC’, which express high levels of CD80, CD86 and CD40. It has been postulated that humans and mice may regulate T cell homeostatic proliferation differently (mice: MHC + IL7, human: MHC + CD28); attributing a role for human IL-7 in T cell survival in steady-state conditions, whereas TSLP-DCs may be involved in T cell survival and homeostatic proliferation in lymphopenic conditions (45). Our data, with the notable absence of improved peripheral homeostasis of human T cells in the presence of high levels of IL-7, versus the increased peripheral T cell reconstitution with enforced expression of TSLP, may support this hypothesis. This said, we should realize that whether the effects on peripheral T cell homeostasis in our HIS mice are a result of direct or indirect stimulation of TSLP remains unclear. Although the absolute cell number of GFP\(^+\) CD11c\(^+\) cells was increased, the positive effect was restrained to the GFP\(^+\) T cell compartment. As the frequency of GFP\(^+\) cells was very limited (5%) the GFP T cells which encountered hTSLP-DCs and proliferated, could potentially not compensate for the large pool of T cells which had not encountered hTSLP-DCs. GFP\(^+\) T cells on the contrary, could have induced their own hTSLP-DCs upon close interaction and paracrine TSLP signaling. Experiments using
Lentiviral vectors, usually guaranteeing both higher transduction efficiency as well as higher GFP recovery, for over-expression of huTSLP are currently underway.

hTSLP-DC are additionally described to be important in in vitro human regulatory T cell (T\textsuperscript{reg}) induction. This study delivered the first in vivo proof of the role of TSLP in human CD4\textsuperscript{+}CD25\textsuperscript{hi} induction. The thymi of our HIS mice transplanted with HSC partially transduced with TSLP-GFP constructs, showed significantly enhanced thymic CD4\textsuperscript{+}CD25\textsuperscript{hi} numbers. Additional FOXP3 stainings and suppression assays with these CD4\textsuperscript{+}CD25\textsuperscript{hi} cells are planned and will demonstrate whether these are true regulatory T cells or not. Also, histological analysis of the HIS mouse thymus, addressing the location of TSLP-producing human cells, CD80\textsuperscript{hi}CD86\textsuperscript{hi}CD11c\textsuperscript{+} DCs and FOXP3\textsuperscript{+} T cells will be helpful to determine the required (spatial) interactions that contribute to human Treg formation.

In conclusion, we here demonstrated a positive effect of enhanced TSLP signaling in human T cell development and long-lasting improved peripheral T cell reconstitution in an in vivo model of human lymphoid development and maintenance. Human TSLP may have therapeutical usefulness in increasing thymopoiesis and the peripheral T cell pool in T cell lymphopenic patients.

**Acknowledgments**

We thank Arjen Bakker for his help in generating the lentiviral TSLP over-expression construct. This work is supported by grants from The “Grand Challenges in Global Health” program (GC4 – “Human Vaccine Consortium”) and the Wijnand M. Pon Foundation. We thank Berend Hooibrink for cell sorting and maintenance of the flow cytometry facility. We acknowledge the Bloemenhove Clinic (Heemstede, The Netherlands) for providing fetal tissues. Dr. M. Hazekamp and staff at the Leiden University Medical Center and the Amsterdam Medical Center are gratefully thanked for providing postnatal thymus tissue. We also thank the staff of the Animal Research Institute Amsterdam for animal care.
References

a critical growth factor in early human T-cell development. In 4239-4245.


Supplementary figure 1. TSLP induces serine phosphorylation of AKT and ERK in CD34+ thymic progenitors

(A) Sorted CD34+CD1a-, CD34+CD1a+, CD4ISP (CD34+CD4+CD8-) and DP (CD34+CD4+CD8+) cells originating from postnatal thymus were stimulated with TSLP or without cytokines and pAKT levels were measured by flow cytometry. Grey histograms represent isotype controls. (B) Sorted CD34+CD1a- and CD34+CD1a+ were stimulated with IL-7 or TSLP and stained for levels of phosphorylated ERK. Grey filled histograms represent isotype control on corresponding subsets, black lines represent pERK levels. Each experiment was repeated three times.
Supplementary Figure 2. Induction of proliferation with TSLP. Proliferation induced of sorted CD34^+CD1a^- and CD34^+CD1a^+ cells originating from postnatal thymus by 10 ng/mL TSLP or IL-7 in a 3 day culture in ratio to cells cultured in medium only, as measured by [^3]H thymidine incorporation. [^3]H incorporation by cells cultured in medium without cytokines is set to 1. Cells from postnatal thymus from two different donors were analyzed.

Supplementary Figure 3. Fractions of non-viable cells in OP9-DL co-cultures in the presence or absence of cytokines. The graphs show pooled data of 7 experiments of the percentage of dead cells over time in PNT - OP9-DL co-cultures, as measured by positivity for 4',6-diamidino-2-phenylindole (DAPI) staining. Fractions of dead cells are shown for cultures starting from CD34^+CD1a^- (A) or CD34^+CD1a^+ (B) sorted progenitors in the presence of IL-7 (open squares, striped line), TSLP (closed triangles, full line) or no cytokines (closed circles, dotted line).