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Thymic stromal lymphopoietin induces early human B cell proliferation and differentiation

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Abstract

Thymic stromal lymphopoietin (TSLP) is a cytokine that in analogy to IL-7 binds the IL-7Rα chain, but in addition engages a unique TSLP-receptor (TSLPR) chain. The role of TSLP in human B-cell development has not been elucidated yet. Here we show that the TSLPR is expressed most prominently on CD34+ cells from fetal liver, but not fetal bone marrow. TSLP induced the tyrosine-phosphorylation of STAT5 and the proliferation of multi-lineage-committed-progenitor cells (MLCP), pro-B cells and pre-B cells. Compared to IL-7, the levels of proliferation after stimulation of the B-cell progenitors with TSLP were lower. Expression of the BCR on the cell surface of fetal cells was inversely correlated to TSLP or IL-7 responsiveness. Pre-B cells from fetal bone marrow, but not fetal liver, were refractory to TSLP or IL-7 stimulation. When employing an in vitro B-cell differentiation culture system starting from CD34+CD38− multipotent hematopoietic stem cells, IL-7 induced a short wave of precursor cell expansion but did not result in long-term survival of mature B-cells, whereas TSLP was capable of significantly increasing both the proportion and the absolute numbers of human mature BCR+ B-cells. Overall, we provide evidence that TSLP supports human B-cell differentiation from fetal hematopoietic progenitors.
Introduction

Human immunodeficiencies have uncovered the key role of cytokine signaling pathways in lymphocyte development. Patients suffering from X-linked severe combined immunodeficiency (X-SCID), which is caused by a loss of function mutation in the common γ (Cγ) chain, or patients with defective interleukin-7-receptor-alpha (IL-7Rα) chain expression, are accompanied by severely diminished T-lymphopoiesis but apparently normal B-cell numbers [1, 2]. This has raised the suggestion that in humans, B-cell development is interleukin-7 (IL-7)–independent. In contrast, in mice IL-7 is a major cytokine involved in B-cell development, since both IL-7 deficient and IL-7Rα-deficient mice showed a profound block at an early stage of B-cell development, i.e. at the pro-B cell to pre-B cell transition [1, 3-5]. Interestingly, fetal and early postnatal B lymphopoiesis in the mouse have been described to be largely driven by IL-7-independent mechanisms [3, 5, 6]. It has been proposed that thymic stromal lymphopoietin (TSLP) acts as a primary regulator of IL-7-independent fetal B-lymphopoiesis [7, 8]. This, together with the fact that B-cell development in SCID patients has only been analyzed in infants, questions the importance of TSLP during fetal human B-cell genesis.

TSLP and IL-7 have overlapping biological activities, although they are not homologous [9]. Both IL-7 and TSLP signal via a heterodimeric receptor containing the IL-7Rα chain [10, 11]. The common γ (Cγ) chain is the second component of the IL-7 receptor [12], whereas TSLP requires an unique TSLP-Receptor (TSLP-R) chain [10, 11]. A common downstream event of both IL-7 and TSLP induced signaling is the tyrosine phosphorylation of the transcription factor STAT5 [13], which then translocates to the nucleus where it initiates transcription of target genes. Most cytokine receptors, including the receptor for IL-7, require Janus Activating Kinases (JAK) to activate members of the STAT transcription family [14]. In mice the TSLP receptor failed to induce tyrosine phosphorylation of any of the four known JAKs [15]. In contrast, the human TSLP receptor has been shown to induce JAK2 tyrosine phosphorylation, revealing differences between mouse and human TSLP signaling [16]. In cell lines it was shown that a Src family kinase inhibitor blocked TSLP-mediated proliferation but not Stat5 activation, suggesting that STAT5 tyrosine phosphorylation alone was not sufficient to induce proliferative signals [13].

TSLP has been shown to be involved in a number of processes in the immune system, including proliferation of T-cells [17-19], development of regulatory T-cells [20], and
activation of human dendritic cells [21, 22]. With respect to B-cells, TSLP is not essential for B-cell development in adult mice as illustrated by observations in TSLP receptor deficient mice [16]. However, TSLP has been implicated during fetal B-cell development, since the B-cell compartment in young IL-7Rα deficient mice, which are unresponsive to both TSLP and IL-7, was even further reduced as compared to IL-7 deficient mice [7, 8]. In line, Cγ deficient mice – which are responsive to TSLP, but not to IL-7 – exhibited residual B-cell development at 4 weeks of age. Once they reach adult age, mice deficient for either Cγ or IL-7 showed a progressive loss of B-cells, which appeared to be mediated by residual T-cells [23]. Collectively, these data support the idea that TSLP and IL-7 have overlapping functions during mouse B-cell ontogeny in fetal and adult life, respectively. As X-SCID patients and humans who suffer from IL-7Rα deficiency have normal numbers of B-cells [2], this implies that IL-7 and TSLP signaling are not crucial for human B-cell development.

In vitro TSLP promoted murine B-cell development from both fetal liver and bone marrow (BM) progenitors [7, 8, 15]. When focusing on later stages of development, in vitro experiments showed that TSLP also induced proliferation of pro-B cells originating from the fetal liver, whereas pro-B cells originating from adult BM did not respond to TSLP [7]. In contrast, pre-B cells originating from both the fetal liver as well as the adult BM responded to TSLP, resulting in cell proliferation [7]. In IL-7-deficient mice, TSLP can only drive B-cell development from fetal liver but not adult bone marrow precursors [7], unless the availability of TSLP was increased through enforced transgenic expression [24]. This suggests that the B-cell compartment is responsive to TSLP in adult mice. That this may similarly be the case in humans was illustrated in a recent report, in which it was shown that systemic high levels of TSLP produced by keratinocytes under Notch signaling deficient conditions in the skin leads to a large expansion of peripheral pre-B and immature B lymphocytes [25]. It is currently unknown whether TSLP has a direct effect on the early human hematopoietic compartment, including CD34+CD38– hematopoietic stem cells (HSCs) or CD34+CD38– multi-lineage-committed-progenitor cells (MLCPs) [26], which still have the ability to develop into NK-cells [27], dendritic cells (DC) [28], and B-cells [29], but not into T-cells [30]. Furthermore the effects of TSLP on human B-cell committed precursor cells, including CD34+CD19+ pro-B cells and CD34+CD19+ pre-B cells, are currently unexplored.

Here we set out to determine the effects of TSLP on human B-cell development, considering that the respective role of IL-7 and TSLP during fetal human B-cell ontogeny remains
controversial. We present data about the TSLP receptor expression, induction of STAT5 tyrosine phosphorylation and proliferation of human fetal liver and fetal BM HSCs, MLCPs and B-cells at various developmental stages. We observed that the TSLPR chain was expressed mainly on HSC and pro-B cells from fetal liver, but that this not necessarily correlated with the capacity to activate STAT5. TSLP, like IL-7, induced the proliferation of all B-cell subsets, except BCR+ B cells. HSC were refractory of stimulation by TSLP and IL-7. However, when induced to differentiate in vitro, only in the presence of TSLP HSC differentiated into BCR+ B cells, while culture in the presence of IL-7 induced the generation of mainly pro-B and pre-B cells. Collectively, we present evidence that TSLP plays a role in human fetal B-cell development and, different from IL-7, stimulates the generation of mature B-cells.

Results

Expression of IL-7Rα and TSLP-R chains on fetal human precursor B-cells
As TSLP is shown to be selectively active on fetal liver derived murine pro-B cells, we set out to determine the effects of TSLP on human fetal B-cell precursors. First we determined by flow cytometric analysis the expression of IL-7Rα and TSLP-R chains on human fetal liver and fetal bone marrow (BM) cells, from early precursors to mature B-cell populations. Fetal BM HSCs (Lin-CD34+CD38-), multilineage committed progenitors (MLCPs) (CD34+CD38+CD19-), pro-B cells (CD34+CD38+CD19+) and pre-B cells (CD34-CD38+CD19+BCRκ/λ-) were all IL-7Rαlow, whereas BCR+ B cells (CD34-CD38+CD19+BCRκ/λ+) were IL-7Rα- (Fig. 1A). Fetal liver HSCs and BCR+ B cells were IL-7Rαlow, whereas MLCPs, pro-B cells and pre-B cells were IL-7Rα+ (Fig. 1A). We did not detect TSLP-R chain by flow cytometry on any of the fetal BM derived cell subsets, nor on fetal liver derived MLCPs, pre-B cells and BCR+ B cells (Fig. 1B). In conclusion, the IL-7Rα and TSLP-R chains were differentially expressed along B-cell development, with marked differences between similar subsets of fetal liver and fetal BM origin.

Tyrosine phosphorylation of STAT5 induced by TSLP
To test whether HSCs, MLCPs and early B-cell subsets responded to TSLP, we first determined whether TSLP was able to tyrosine phosphorylate STAT5 (pSTAT5), which is a known downstream target of TSLP signaling [13]. We performed a phospho-flow cytometric analysis for pSTAT5 after ex vivo TSLP stimulation of fetal liver or BM cells.
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For parallel comparison we stimulated the cells with IL-7, which is also known to induce phosphorylation of STAT5 [31]. The cells were incubated for 15 minutes with TSLP (50 ng/mL), IL-7 (50 ng/mL) or no cytokine. In fetal BM derived cells, pSTAT5 was observed in a fraction of HSCs, MLCPs and pro-B cells upon IL-7 stimulation, whereas no pSTAT5 was observed in the pre-B cells and BCR+ B cells (Fig. 2A).

Figure 1. Expression of the receptor for TSLP and IL-7. (A) Fetal liver and fetal bone marrow samples were analyzed by flow cytometry for different subsets of progenitor cells, including hematopoietic stem cells (CD19-CD34-CD38-), MLCPs (CD19-CD34-CD38+), pro-B cells (CD34-CD19'BCRk/λ-), pre-B cells (CD34-CD19'BCRk/λ+) and BCR+ B cells (CD34 CD19'BCRk/λ+) (see Suppl. Fig. 1). Expression of the IL-7Rα chain (A) and TSLP-R chain (B) was determined after electronic gating on the different subsets. Fetal livers and fetal BM of three different donors were analyzed. Data from one representative donor are shown.
In fetal liver derived cells, pSTAT5 was observed in a small fraction of MLCPs and pre-B cells, and a large majority of pro-B cells upon IL-7 stimulation, whereas no pSTAT5 was observed in the HSCs and BCR+ B cells (Fig. 2A). Incubation with TSLP gave almost similar results as IL-7 for fetal BM derived cells, as pSTAT5 was observed in a fraction of HSCs, MLCPs and pro-B cells, while pSTAT5 was not observed in the pre-B cells and BCR+ B cells upon TSLP addition (Fig. 2B). These results were consistently obtained despite detectable TSLP-R expression by flow cytometry on all these cell subsets (Fig. 1B). Stimulation of fetal liver cells with TSLP gave a small but consistent peak of pSTAT5 in the MLCPs and pro-B cells, whereas no pSTAT5 was observed in HSCs, pre-B cells and BCR+ B cells (Fig. 2B).

Figure 2. Induction of STAT5 tyrosine phosphorylation activation by TSLP or IL-7. Induction of pSTAT5 induced by IL-7 or TSLP after electronic gating on HSCs (CD19−CD34+CD38−), MLCPs (CD19−CD34+CD38+),...
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We conclude from the data presented here that TSLP is able to activate STAT5 in fetal BM derived HSCs, MLCPs and pro-B cells and to a minor degree in fetal liver derived MLCPs and pro-B cells.

TSLP induces proliferation of fetal human precursor B-cells

It has been described that TSLP can induce proliferation of early mouse B-cells [7, 8, 15, 24]. This prompted us to determine the effect of TSLP on proliferation of human fetal liver and fetal BM B precursor cells. We sorted MLCPs, pro-B cells and BCR+B cells derived from fetal liver or fetal BM (Suppl. Fig. 1), cultured the cells for 3 days in the presence of TSLP or IL-7 and subsequently assessed their proliferation after [3H]-thymidine uptake (Fig. 3). MLCPs and pro-B cells derived from both fetal BM and fetal liver proliferated when cultured in the presence of either TSLP or IL-7 (Fig. 3A/B/E/H). We extended these finding by culturing fetal liver MLCPs and pro-B cells with increasing amounts of TSLP. Both cell types responded to TSLP in a dose dependent manner (Suppl. Fig. 2A/B). Interestingly, fetal BM and fetal liver pre-B cells differentially responded to TSLP and IL-7, as only the fetal liver pre-B cells proliferated in the presence of TSLP and IL-7, while fetal BM pre-B cells did not (Fig. 3C/G). Furthermore, independent of their original fetal source (BM, liver, or spleen), BCR+B cells were refractory to TSLP or IL-7 stimulation, as no proliferation was induced (Fig. 3D/H and data not shown). Overall, when proliferation was induced by TSLP, it was always significantly lower as compared to IL-7 induced proliferation (Fig. 3). This observation is in line with the lower proliferation rate of mouse pre-B cells in vitro when stimulated with TSLP, as compared to IL-7 [8].

When analyzing HSCs either from fetal liver or fetal BM, no proliferation in response to TSLP or IL-7 as assessed by no [3H]-thymidine uptake could be observed (data not shown). This finding was confirmed by monitoring the absolute cell numbers over time after in vitro culture of fetal liver HSCs in the absence or presence of TSLP or IL-7 (Suppl. Fig. 2C). Over a period of 12 days a slow decrease in the absolute cell number in all culture conditions was observed. In contrast, a 3-4 fold cell number increase was observed when fetal liver MLCPs cells, isolated from the same donor as the HSCs, were cultured in the presence of TSLP and a 5-8 fold increase in the presence of IL-7 (Suppl. Fig. 2D).
Collectively, we conclude that TSLP can induce proliferation of in vitro cultured fetal liver MLCPs, pro-B, pre-B cells and fetal BM MLCPs and pro-B cells, in a dose dependent manner. In contrast, TSLP is unable to induce proliferation of human fetal HSCs, BCR⁺ B cells and fetal BM derived pre-B cells.
TSLP supports differentiation of human precursor B-cells

We extended our findings on the effect of TSLP on fetal precursor cells using a previously described in vitro culture system supporting human B-cell development from HSCs [32]. We co-cultured sorted human fetal liver HSCs with mouse bone marrow stromal OP9 cells in the presence or absence of TSLP or IL-7. Over time, we observed that CD19+ B-cells arising from TSLP-cultured HSCs were increased both in absolute cell number and percentage, as compared to the culture without additional cytokines (Fig. 4A). Addition of IL-7 also resulted in an increase in CD19+ B-cells during the first 3 weeks of culture, but thereafter these numbers declined to even lower levels as compared to medium alone. To gain insight on the B-cell subsets that developed in this in vitro culture system, we phenotypically characterized the cells over time. We could distinguish cells resembling pro-B cells (CD34+CD38+CD19+IgM−IgD−), pre-B cells (CD34+CD38+CD19−IgM−IgD−), immature B-cells (CD34+CD38−CD19+IgM−IgD−) and mature B-cells (CD34−CD38−CD19−IgM−IgD−) (Fig. 4B). We observed that immature and mature B-cells developed in the absence of cytokines as well as in the presence of TSLP, whereas the presence of IL-7 had a negative impact on the accumulation of BCR+ B cells (Fig. 4B). Notably, TSLP enhanced B-cell development, since the absolute numbers of pre-B cells, immature B-cells as well as mature B-cells were increased as compared to the medium control condition (Fig. 4C). In contrast, IL-7 alone had a positive impact on the early stages of B-cell development, by expanding the pool of pro-B and pre-B cells. After 30-40 days B-cell recovery was negligible in all conditions tested, suggesting that the OP9 co-culture conditions are not optimal for long-term support of B-cell survival (Fig. 4C). Altogether, these data confirm and strengthen our findings that TSLP is able to induce expansion of early human precursor B-cells. Given the observation that BCR+ B cells did not proliferate in response to TSLP, this strongly suggests that TSLP affects the early B-cell progenitor stages to promote B-cell differentiation into mature B-cells.

Discussion

Here we show for the first time the capacity of TSLP to induce proliferation of ex vivo isolated and in vitro cultured human early precursor B-cell subpopulations. We present evidence that TSLP has a direct effect on human fetal liver MLCPs, pro-B cells and pre-B cells, but not BCR+ B cells. These findings are in line with studies performed in the mouse [8, 9, 33]. In addition to fetal liver progenitor cells, TSLP induced the proliferation of human fetal bone marrow MLCPs and pro-B cells, but notably not of pre-B cells nor of BCR+ B-
Figure 4. Increased in vitro B-cell development in the presence of TSLP. B-cell development in co-cultures of sorted CD34^+CD38^- fetal liver HSCs and OP9 cells, in presence of TSLP, IL-7 or no cytokine. Results show typical data from one of three experiments. (A) Percentages and absolute cell numbers of total CD19^+ B-cells developing in the co-cultures overtime are shown. (B) B-cell subsets were identified on basis of their expression of CD34, CD38, CD19, IgM and IgD. The dotplots show flow cytometry analysis at d20 of the cocultures, which contain either IL-7, TSLP or no additional cytokines. Numbers represent percentages of gates cells. (C) Pro-B (CD34^+CD38^-CD19^-BCR^-), pre-B (CD34^+CD38^-CD19^-BCR^-), immature B (CD34^-CD38^-CD19^-IgM^-IgD^-) and mature B (CD34^-CD38^-CD19^-IgM^-IgD^-) cell numbers are detailed in separate graphs.
cells. This is consistent with data published in the mouse, in which it was shown that pre-BCR negative precursors responded to TSLP in the liver environment, but not in the bone marrow at the same age [8]. Furthermore, we show that TSLP, but not IL-7, sustained and enhanced the development of human B-cells from HSCs in vitro, which can most likely be attributed to a proliferative effect on the pro- and pre-B cell subpopulations.

It has been previously reported that TSLP, upon engagement to the heterodimeric IL-7Rα/TSLP-R complex, activates STAT5 by tyrosine phosphorylation [13]. We have made use of this feature to test the responsiveness of human precursor cells to TSLP. Using phospho-flow cytometric analysis, we detected the presence of phosphorylated STAT5 in TSLP stimulated fetal BM HSCs, MLCPs and pro-B cells. No significant tyrosine phosphorylation of STAT5 was observed in the similar precursor subsets present in human fetal liver. It can be ruled out that differential expression of the TSLP receptor underlies this observation, since we showed that fetal liver HSCs and pro-B cells expressed both the TSLP-R and IL-7Rα chains. An alternative explanation might be that fetal liver and bone marrow precursor cells express different levels of STAT5, although this notion has not been validated.

We observed that TSLP was able to induce proliferation of human fetal MLCP cells and pro-B cells independent of their origin, and in fetal liver also of pre-B cells. Activation of STAT5 in fetal bone marrow cells was readily detected, but this was not obvious in fetal liver precursor cells. Consequently, it may be reasonable to assume that TSLP can induce proliferation in a STAT5 independent manner in human. Alternative signaling pathways known to be induced by TSLP have been reported and include the phosphorylation of STAT1 in murine pro-B cells [34] and STAT3 in human and mouse myeloid cells [10, 11, 35]. TSLP did not stimulate the activation of ERK1/2 and p70S6K [36], suggesting that neither the MAPK pathway nor the p70S6K pathway are involved in the signal transduction pathway elicited by TSLP. The present view is that Src type kinases may mediate the proliferative response, based on the finding that the Src family kinase inhibitor PP1 inhibited TSLP-induced proliferation [13]. Other results emanating from our study suggest that expression of the TSLP-R and TSLP induced cell proliferation do not necessarily correlate. This is reinforced by the observation that TSLP, but also IL-7, was unable to induce proliferation of fetal liver HSCs, even though these HSCs express the receptor for TSLP and IL-7. At present it can only be speculated that essential signaling molecules downstream of the receptors engaging TSLP and IL-7 are lacking in fetal liver HSCs, such as Src kinases and non-JAK kinases to activate STATs [34]. Taken together, details of TSLP-R signaling are far
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from being elucidated and it remains a challenge to determine how exactly TSLP induces proliferation in human precursor cells.

The role of TSLP and IL-7 in lymphocyte development has been studied extensively in the mouse [37, 38]. The latest consensus on the role of TSLP and IL-7 in mouse B-cell development is that both cytokines are dispensable for fetal B-cell development, since HSCs isolated from the fetal liver of IL-7R-deficient mice, which are non-responsive to either TSLP or IL-7, can differentiate into mature B-cells [7, 16]. Our data on human B-cell development are in line with these studies, as neither TSLP nor IL-7 had a direct stimulatory effect on the proliferation of fetal HSCs, and moreover human B-cells developed in vitro on OP9 stromal cells without the addition of cytokines. In the absence of IL-7 mediated signaling, a vital role for FLT3L has been implicated to drive mouse fetal B-cell development [7, 39]. The importance of FLT3L has not been directly tested in human, but it might explain why severe combined immune-deficient patients with IL-7R deficiency appear to have normal B-cell numbers [2, 40, 41]. In contrast to the fetal mouse, in the adult mouse it was shown that IL-7, but not TSLP, is required for B-cell development [7, 16]. In support of a unique role of TSLP in embryonic rather than adult B lymphopoiesis, fetal but not adult conventional pro-B cells have been shown to be TSLP responsive [7, 8]. While we show here that pro-B cells from fetal liver as well as fetal bone marrow expanded with TSLP, it will be of interest to determine whether human pro-B cells derived from adult BM are responsive to TSLP.

As mentioned before, TSLP and IL-7 appear to have overlapping, but also unique functions. In this report, we analyzed the role of TSLP and IL-7 in an in vitro culture system using OP9 feeder cells, which has previously been shown to support human B-cell development from HSCs [32]. We observed that TSLP increased the proportion and absolute number of CD19+BCR+B-cells as compared to OP9 control cultures without additional cytokines. In contrast, addition of exogenous IL-7 to the B-cell development culture clearly blocked B-cell differentiation before the stage when IgD or IgM is expressed on the cell surface. This observation is in line with findings in mice, which revealed that IL-7 induced the acetylation of histones, which in fact need to be deacetylated to allow Igλ chain rearrangement [42]. In contrast, we observed that TSLP allowed, and even enhanced, B-cell differentiation into BCR+ B cells, supporting the idea that TSLP does not induce histone acetylation. We further reasoned that this effect of TSLP is most likely attributed to a proliferative advantage of MLCP cells, pro-B and/or pre-B cells and not of BCR+ B-cells, since we observed that
BCR⁺ cells were TSLP unresponsive. In part, these findings are consistent with a previous publication in which it was observed that TSLP, but not IL-7, promoted the development of mouse B220⁺IgM⁺ B-cells [15]. A notable difference in these studies, however, was the fact that the number of IgM⁺ mouse B-cells that developed in TSLP-supplemented cultures was almost similar to the number of cells that developed in IL-7-supplemented cultures. This is in contrast to our results as we clearly showed that not only in proportion, but also in absolute numbers the human B-cells selectively increased in TSLP-supplemented cultures. The exact underlying differences in IL-7 versus TSLP driven B-cell development remain elusive. One possibility that may explain these findings is that engagement of IL-7 and TSLP to their respective receptors results in differential strength of signal transduction. In line with this, it has been reported that IL-7 activates STAT5 to higher levels than TSLP [13, 15, 43], and that high or intermediate levels of STAT5 activation in human CD34⁺ precursor cells resulted respectively in the induction of erythropoiesis or a proliferative advantage for CD34⁺ cells [44], indicating that strength of signal matters. Alternatively, the timing of receptor activation might be crucial. Treatment of a mouse pre-B cell line with IL-7 only briefly induced STAT5 activation, while treatment with TSLP increased the amount of pSTAT5 in a more sustained manner [45]. Whether this has consequences for the outcome of human B-cell differentiation is unknown.

All the experiments presented here have focused on the role of TSLP and IL-7 using in vitro systems. It will, however, be of significant value to explore the role of TSLP during human B-cell development in vivo. Discrepancies between IL-7 or IL-7Rα deficient adult mice, which have a clear block in B-cell development [46, 47] and humans with a IL-7Rα deficiency, who have normal numbers of B-cells [2, 40, 41] have been reported. The use of humanized mouse models, which have been described as a reliable model to study in vivo human B-cell development [48, 49], will be instrumental to address the pertinent questions on the differences between human and mouse B-cell development.

Materials and methods

Flow cytometry
Monoclonal antibodies to CD34, CD19 (HIB19), IgM conjugated to FITC, PE, PerCP, PeCy7, APC, APCy7 or Alexa-fluor 700, and isotype control IgG1 Alexa-647 were purchased from BD Biosciences (San Jose, CA). Antibody to CD127 (clone eBioRDR5) conjugated to Alexa-647 was obtained from eBioscience. BCR κ light chain (clone A8B5) and
\( \lambda \) light chain (rabbit polyclonal) were obtained from Dako-Cytomation and BD Biosciences, respectively. Rat-anti-human TSLP-R antibody (clone LB1.18B3.1E4) was obtained from Schering Plough Biopharma (Palo Alto, CA) and secondary antibody donkey anti-rat IgG2-Alexa488 was obtained from Molecular Probes (Leiden, the Netherlands).

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 in combination with antibodies specific for CD38 (FITC), \( \lambda \) light chain and \( \lambda \) light chain (PE), CD34 (PE-Cy7), and CD19 (Alexa-fluor 700). Stained cells were analyzed with a LSR II (BD Biosciences) and flow cytometry data were processed with FlowJo software (Tree Star).

**Human tissues and purification of fetal cell subsets**

Human fetal tissues were obtained from elective abortions. Gestational age was determined by ultrasonic measurement of the diameter of the skull and ranged from 14 to 20 weeks. Mononuclear cells were isolated from a Ficoll-Hypaque density gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway). For the fetal liver cells, CD34\(^+\) cells were subsequently isolated using a magnetic-activated cell sorter (MACS) direct CD34 progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) resulting in purity of more than 93%. For further purification of hematopoietic stem cells (HSCs) and multi-lineage committed progenitors (MLCPs), the CD34\(^+\)CD38\(^-\) and CD34\(^+\)CD38\(^+\) populations were sorted to a purity of more than 99%. From the CD34 MACS-negative fraction, fetal liver pro-B cells (CD34\(^+\)CD38\(^+\)CD19\(^+\)BCR\(k/\lambda\)^-), pre-B cells (CD34\(^+\)CD38\(^+\)CD19\(^+\)BCR\(\kappa/\lambda\)^-) and BCR\(^+\) B cells (CD34\(^+\)CD38\(^+\)CD19\(^+\)BCR\(\kappa/\lambda\)^+) were isolated by fluorescence-activated cell sorting after enrichment for human B-cells by CD22-positive MACS selection (Miltenyi Biotec) (Suppl. Fig. 1). Similar cell populations were isolated from fetal bone marrow cells, without performing magnetic purification prior to fluorescence-activated cell sorting. Cells positive for several lineage markers (CD56, BDCA2, CD3) were excluded during the cell sorting procedure. The cell sorting was done using a FacsARIA cytometer, interfaced to the Diva software (Becton Dickinson, San Jose, CA). The use of fetal tissue was approved by the Medical Ethical Committee of the Academic Medical Center and was contingent on obtaining informed consent, in accordance with the Declaration of Helsinki.
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Co-cultures of human precursor cells and OP9 cells
The OP9 culture system supporting human B-cell development has already been described (23). In brief, in vitro development of human B-cells was assessed by co-culturing $1 \times 10^5$ CD34$^+$CD38$^-$ HSCs or CD34$^+$CD38$^+$ MLCP cells from fetal liver with $5 \times 10^4$ OP9 cells in MEM$\alpha$ (Invitrogen) with 20% FCS (Hyclone), in the presence or absence of 10 ng/mL purified recombinant human IL-7 (Amgen) or TSLP (obtained from Schering Plough Biopharma). The co-cultures were supplemented every 2 to 3 days with fresh medium, and precursor cells were transferred to fresh stromal OP9 cells every 4 to 5 days of culture.

Proliferation assays
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 $[^{3}H]$-thymidine (1µCi/well). Cytokines were added at the start of the culture and just before addition of the $[^{3}H]$-thymidine. Proliferative responses are expressed as the mean $[^{3}H]$-thymidine incorporation (cpm) of triplicate wells ± SD.

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Supplementary figures

**Supplementary Figure 1.** Gating strategy for the analysis and isolation of HSCs, MLCPs and B cells at various developmental stages derived from fetal BM (A) and fetal liver (B) (see Material and Methods section for more details). As depicted on the cytometry dot plots, five fractions were identified: (i) fetal BM HSCs (CD34⁺CD38⁻CD19⁻); (ii) multilineage committed progenitors (MLCPs) (CD34⁺CD38⁺CD19⁻); (iii) pro-B cells (CD34⁺CD38⁻CD19⁺); (iv) pre-B cells (CD34⁻CD38⁻CD19⁺BCRκ/λ⁺); and (v) BCR⁺ B cells (CD34⁻CD38⁻CD19⁻BCRκ/λ⁻).

**Supplementary Figure 2.** Increasing the concentration of TSLP resulted in enhanced proliferation of sorted fetal liver MLCPs cells (A) and pro-B cells (B), as measured by [³H]thymidine uptake. Fetal liver HSCs (C) and MLCPs (D) were cultured with TSLP or IL-7 and the absolute cell numbers were determined over a period of 12 days. Data are representative of three experiments.
Supplementary Figure 3. Statistical analysis on the relative increase of \[^3H\]thymidine incorporation of the TSLP and IL-7 treated fetal bone marrow MLCP (A) and pro-B cells (B), or fetal liver MLPC (C), pre-B cells (D), or pre-B cells (E), as compared to non-treated cells (X), which is set to 1. Data are pooled from four independent experiments. Statistical analysis was performed using a paired two tailed student's t-test; *P<0.05, **P<0.005.