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Comparative Studies of TSLP and IL-7 in Normal Early Human Neonatal and Adult B Cell Development

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Comparative Studies of TSLP and IL-7 in Normal Early Human Neonatal and Adult B Cell Development

by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

BCR	B Cell Receptor
BM	Bone Marrow
CB	Umbilical Cord Blood
CD	Cluster of Differentiation
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
cμ	Cytoplasmic IgM
EBF	Early B Cell Factor
ELP	Early Lymphoid Progenitor
FACS	Fluorescence-Activated Cell Sorting
FBM	Fetal Bone Marrow
FL	Fetal Liver
Flt-3	FMS-like Tyrosine Kinase Receptor
Flt-3L	Fetal Liver Kinase-3 Ligand
FMO	Fluorescence Minus One
HC	Heavy Chain
HSC	Hematopoietic Stem Cell
hTSLP	Human Thymic Stromal Lymphopoietin
IL-2RG	IL-2 Receptor Gamma, Common Gamma Chain
IL-7	Interleukin-7
IL-7Rα	IL-7 Receptor Alpha
i.p.	Intraperitonial

JAK	Janus Kinase
KLS	c-Kit^{+} Lin^{-} Sca-1^{+}
KO	Knockout
LC	Light Chain
MPP	Multipotential Progenitor
mTOR	Mammalian Target of Rapamycin
NSG	Non-Obese Diabetic Severe Combined Immune-Deficient Gamma
Pre-BCR	Pre-B Cell Receptor
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RAG-1, 2	Recombinase Activating Gene-1, 2
STAT5	Signal Transducer and Activator of Transcription
TdT	Terminal Deoxynucleotide
TSLP	Thymic Stromal Lymphopoietin

ABSTRACT OF THE DISSERTATION

Comparative Studies of TSLP and IL-7 in Normal Early Human Neonatal and Adult B Cell Development

by

Terry-Ann Milford

Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
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Dr. Kimberly J. Payne, Chairperson

Defining the role of cytokines in promoting human B cell development is important for understanding B cell leukemia and for developing strategies to restore B cell production that is lost during some therapies. The purpose of this study was to investigate the roles of the IL-7R ligands, thymic stromal lymphopoietin (TSLP) and IL-7, in the early stages of normal postnatal and adult human B cell development. Using novel *in vitro* and *in vivo* model systems, we show that TSLP and IL-7 are able to expand B cell precursors individually and in combination from CB hematopoietic stem cells. In this model TSLP increases were seen in the CD34+ pro-B subset and maintained through subsequent stages of development. Adult B lymphopoiesis unlike CB B lymphopoiesis is IL-7– but may not be TSLP–dependent. We also used the expression of IL-7Rα, to identify a lymphoid progenitor that lacks myeloid potential. This information will be important in developing strategies for restoring B cell development after therapy and supports our ongoing leukemia studies by providing a rationale for targeting the TSLP signaling pathway in developing therapies for high-risk B cell acute lymphoblastic leukemia.

CHAPTER ONE

INTRODUCTION

Sites of B Lymphopoiesis

The sites of B lymphopoiesis change during ontogeny. During early to mid-gestation, B cells can be found in the yolk sac, placenta, fetal liver and bone marrow of the fetus. From mid-gestation onward, the bone marrow becomes the primary organ for early B cell development (Magnon & Frenette, 2008). In the BM, hematopoietic stem cells (HSC) interact with stromal cells in specialized microenvironments described as niches (Tokoyoda, Egawa, Sugiyama, Choi, & Nagasawa, 2004). Here, the stromal cells secrete growth factors that direct lineage restriction of developing blood cells. They also express adhesion molecules that maintain cellular contact between the developing cells and the cytokine-secreting stromal cells (Ryan, Nuccie, Abboud, & Winslow, 1991; Tokoyoda et al., 2004). Some of the cytokines that regulate the nascent steps in early B cell differentiation include, Flt-3 ligand, IL-7 and TSLP.

Stages of B Lymphopoiesis

To produce competent, non-self reacting, antibody-producing B cells, HSCs undergo multiple stages of specification, commitment and differentiation, beginning in the bone marrow. Mouse models of B cell development have been essential in understanding the complex network of cytokine-receptor signaling-transcription factor interactions that direct these processes early on. Flow cytometry and PCR are some of the techniques used to characterize the discreet stages of B lymphopoiesis – including pro-B, pre-B, immature B and mature B cells– by the expression of surface antigens and receptors as shown in Figure 1 (Parrish et al., 2009; Pribyl & LeBien, 1996; Rawlings,

Quan, Kato, & Witte, 1995; Scheeren et al., 2010). In mice, surface antigens including B220, AA4.1, CD43, CD19, CD24, Flt3, IL-7Rα and IgM are used to identify six stages (Hardy's Fractions A-F) of developing B cells (Hardy, Carmack, Shinton, Kemp, & Hayakawa, 1991; Hardy & Hayakawa, 2001). Analogous populations are present in humans but some of the surface antigens used to identify them differ (Ichii, Oritani, & Kanakura, 2014). This review will focus primarily on the surface markers used to identify these subsets in human B cell development.

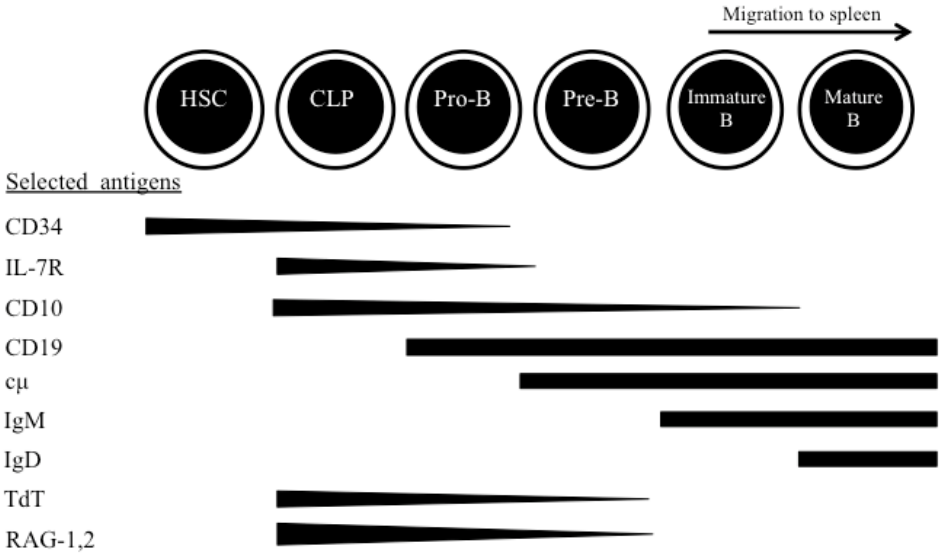

Figure 1. A model of human B cell development based on the mouse. For simplicity, B cell differentiation is viewed as a linear process. As cells differentiate down the B lineage, they express combinations of surface markers and intracellular proteins that aid their identification. A summary of selected antigens and enzymes expressed at different stages of human B lymphopoiesis is shown above. Horizontal triangles indicate decreasing expression of the antigen and lines indicate sustained expression of the antigen.

Mouse HSCs are enriched in the $Kit^{+}Lin^{-}Sca\text{-}1^{+}$ (KLS) fraction of the BM and account for 0.01% – 0.05% of total nucleated cells found there (Challen, Boles, Lin, & Goodell, 2009; Okada et al., 1992; Spangrude, Heimfeld, & Weissman, 1988). They undergo several rounds of restriction to generate progenitors from which erythroid and myeloid populations differentiate, and a common lymphoid progenitor (CLP), from which B, T and natural killer (NK) cells arise (Kondo, Weissman, & Akashi, 1997). The mouse CLP was initially identified as $Lin^{-}IL\text{-}7R^{+}Thy\text{-}1.1^{-}$ $Sca\text{-}1^{lo}c\text{-}Kit^{lo}$ (Kondo, Weissman, et al., 1997). This phenotype was later refined to include $Flk2^{+}$ (Karsunky, Inlay, Serwold, Bhattacharya, & Weissman, 2008). Unlike the mouse, the human HSC was identified in the fetal bone marrow by the expression of a single marker, CD34, and absence of other lineage markers (Baum, Weissman, Tsukamoto, Buckle, & Peault, 1992). While there is some consensus in the field about the phenotype of the mouse CLP, the phenotype of the human counterpart remains debatable.

The combinations of markers used to identify these lymphoid-restricted progenitors in humans, vary according to the tissue being evaluated. In fetal and adult BM, Galy et al. used the phenotype $CD34^{+}Lin^{-}CD38^{+}CD10^{+}$ to identify lymphoid-primed cells (Galy, Travis, Cen, & Chen, 1995). Other groups identified these cells by IL-7Rα (Ryan et al., 1997), CXCR4 and TdT expression together with CD34 (Ishii et al., 1999). Like adult BM, CB cultures of $CD34^{+}CD10^{-}CD19^{-}IL\text{-}7R\alpha^{+}$ cells generated lymphoid cells– NK, B and T cells– in addition to macrophages, suggesting that these cells are permissive depending on the cytokines supplementing the cultures (Reynaud, Lefort, Manie, Coulombel, & Levy, 2003). An alternative phenotype, $CD34^{+}CD38^{-}$ $CD7^{+}$ also describes a putative CLP in CB cultures (Hao et al., 2001). These lymphoid-

restricted cells also express PU.1 and EBF, transcription factors that direct lymphocyte development along with TdT and RAG-1, -2, proteins that regulate immunoglobulin heavy chain rearrangement (Hystad et al., 2007; Reynaud et al., 2003; Rossi et al., 2003). In Chapter 6, we demonstrate that expression of IL-7Rα can be used to identify progenitors from CB that are biased toward lymphoid lineages.

Between the CLP and committed progenitor B cell stages, a few B cell intermediates have been proposed based on expression of surface markers and B cell specific intracellular proteins. Hystad et al. defined the phenotype of the earliest B cell intermediate as $CD34^{+}CD38^{+}CD10^{+}CD19^{-}$ (Hystad et al., 2007). These intermediates share the surface phenotype of the CLP thus making the two populations indistinguishable only by surface marker expression (Galy et al., 1995; LeBien, 2000). Whether these two populations are the same or different at the genetic level remains to be determined. The development of the immunoglobulin heavy chain begins in the early B cell with rearrangement of the D-to-J segments (Davi et al., 1997) and expression of associated genes and proteins including RAG-1,-2, TdT, and the pre-BCR components CD79a (also known as mb-1) and CD179a (also known as VpreB) (Dworzak, Fritsch, Froschl, Printz, & Gadner, 1998; Hystad et al., 2007; LeBien, 2000). The early B cell intermediate also expresses myeloid, T cell and NK cell associated genes (Hystad et al., 2007). Another intermediate, the pre/pro-B cell with the phenotype, $CD34^{+}CD10^{-}CD19^{+}$, is found in the fetal liver, fetal bone marrow and CB (Sanz, Alvarez-Mon, Martinez, & de la Hera, 2003; Sanz et al., 2010). Sanz et al. showed that the pre/pro-B cell is an alternate pathway to generate pro-B cells from CB HSCs (Sanz et al., 2010). Whether this alternate pathway for generating pro-B is active in adult BM remains undetermined.

The next stage of B cell development is the pro-B cell, conventionally described as $CD19^{+}CD34^{+}CD10^{+}$ cells (Loken, Shah, Dattilio, & Civin, 1987; Rossi et al., 2003). TdT, RAG-1, and RAG-2 are expressed as V-to-DJ heavy chain rearrangement is completed (Ghia et al., 1996). Nonproductive VDJ rearrangement in the pro-B cell, results in death by apoptosis (LeBien, 2000). The pro-B to pre-B cell transition is marked by cytoplasmic IgM (μHC) expression, cell surface expression of the pre-BCR and loss of CD34 and TdT (Ghia et al., 1996; LeBien, 2000). The pre-BCR complex is composed of μHC, CD179a, CD179b (surrogate light chains), and CD79a, CD79b (signal transduction heterodimers) (LeBien, 2000). Pre-BCR expression is a critical checkpoint in B cell development. It involves inhibition of further heavy chain rearrangement by suppressing RAG-1, and RAG-2; initiation of light chain rearrangement with re-expression RAG-1, RAG-2 and increased proliferation of pre-BCR expressing cells (LeBien, 2000). The pre-B cell progresses to the immature B cell stage after successful light chain rearrangement, producing IgM on the B cell surface. At this stage, immature B cells migrate from the bone marrow into the periphery where they undergo further maturation.

Transcription Factors Involved in Early B Cell Commitment

To successfully generate B cells, transcription factors act in an inter-dependent regulatory framework to, suppress non-B lineage genes, increase expression of cytokine receptors and surface markers and regulate immunoglobulin gene rearrangement. Based on mouse studies, Ikaros, PU.1, E2A, EBF and PAX-5 are major transcription factors involved in the differentiation of B cells from HSCs (Figure 2). The roles of these

transcription factors in murine B cell differentiation are largely extrapolated to human. A proposed interplay between the transcription factors is illustrated in Figure 2.

Ikaros and PU.1

Ikaros and PU.1 are expressed in HSCs and multipotent progenitors (MPP) (Akashi, Traver, Miyamoto, & Weissman, 2000; Klug et al., 1998). Together, they repress non-B lineage fate and facilitate expression of lymphocyte-associated genes. Ikaros is required for the expression of the transcription factors E2A, EBF and PAX5, necessary for B cell specification and commitment (Bain et al., 1994; Kee & Murre, 1998; Lin & Grosschedl, 1995; Nutt, Urbanek, Rolink, & Busslinger, 1997). In hematopoietic progenitors including the CLP, Ikaros and PU.1 activate lymphoid specific genes (Fuxa & Skok, 2007; Hagman & Lukin, 2006) including Flt-3 (Carotta et al., 2010) and IL-7Rα (DeKoter, Lee, & Singh, 2002). In the absence of Ikaros or PU.1, hematopoietic progenitors show reduced expression of Flt3 (Nichogiannopoulou, Trevisan, Neben, Friedrich, & Georgopoulos, 1999) and IL-7R (DeKoter et al., 2002). B cells are also absent in the fetal liver and bone marrows of these mice (Wang et al., 1996), possibly due to failure to increase expression of these receptors.

E2A and EBF

The E2A gene produces two spliced variants, E12 and E47. Together they regulate the expression of IL-7Rα, RAG-1/-2, EBF and pre-BCR components, CD79a, CD79b, CD179a and CD179b (Beck, Peak, Ota, Nemazee, & Murre, 2009; Kee & Murre, 1998; Ye & Graf, 2007). B cell development is perturbed at the pro-B stage in the

absence of either E12 or E47 (Bain et al., 1994; Bain et al., 1997). EBF is required for B cell specification and is regulated by E2A (Smith, Gisler, & Sigvardsson, 2002), PU.1 and IL-7R (Medina et al., 2004). EBF directly activates PAX-5 (O'Riordan & Grosschedl, 1999); regulates RAG-1 and RAG-2 (Lin & Grosschedl, 1995), the pre-BCR components CD79a (Sigvardsson et al., 2002), CD79b (B29) (Akerblad, Rosberg, Leanderson, & Sigvardsson, 1999), CD179a (Sigvardsson, O'Riordan, & Grosschedl, 1997) and CD179b (Gisler & Sigvardsson, 2002). B cell generation is blocked at an early stage, in the absence of EBF, similar to the E2A absence (Lin & Grosschedl, 1995). Loss of both EBF and E2A produce a more severe deficiency in B cell development than loss of the individual transcription factors (O'Riordan & Grosschedl, 1999).

PAX-5

PAX-5 induction is the first step in B cell commitment (Busslinger, 2004) as it induces the expression B cell specific genes CD19, increases CD79a, CD179a and CD179b (Ichii et al., 2014; Nutt, Heavey, Rolink, & Busslinger, 1999) and blocks the expression of the T cell gene, Notch1 (Monroe & Dorshkind, 2007; Souabni, Cobaleda, Schebesta, & Busslinger, 2002). B cells maintain PAX-5 expression until the mature B cell stage (Adams et al., 1992). In pro-B cells, inactivation of PAX-5 allows macrophage and T cell differentiation *in vitro* and *in vivo*, respectively (Mikkola, Heavey, Horcher, & Busslinger, 2002).

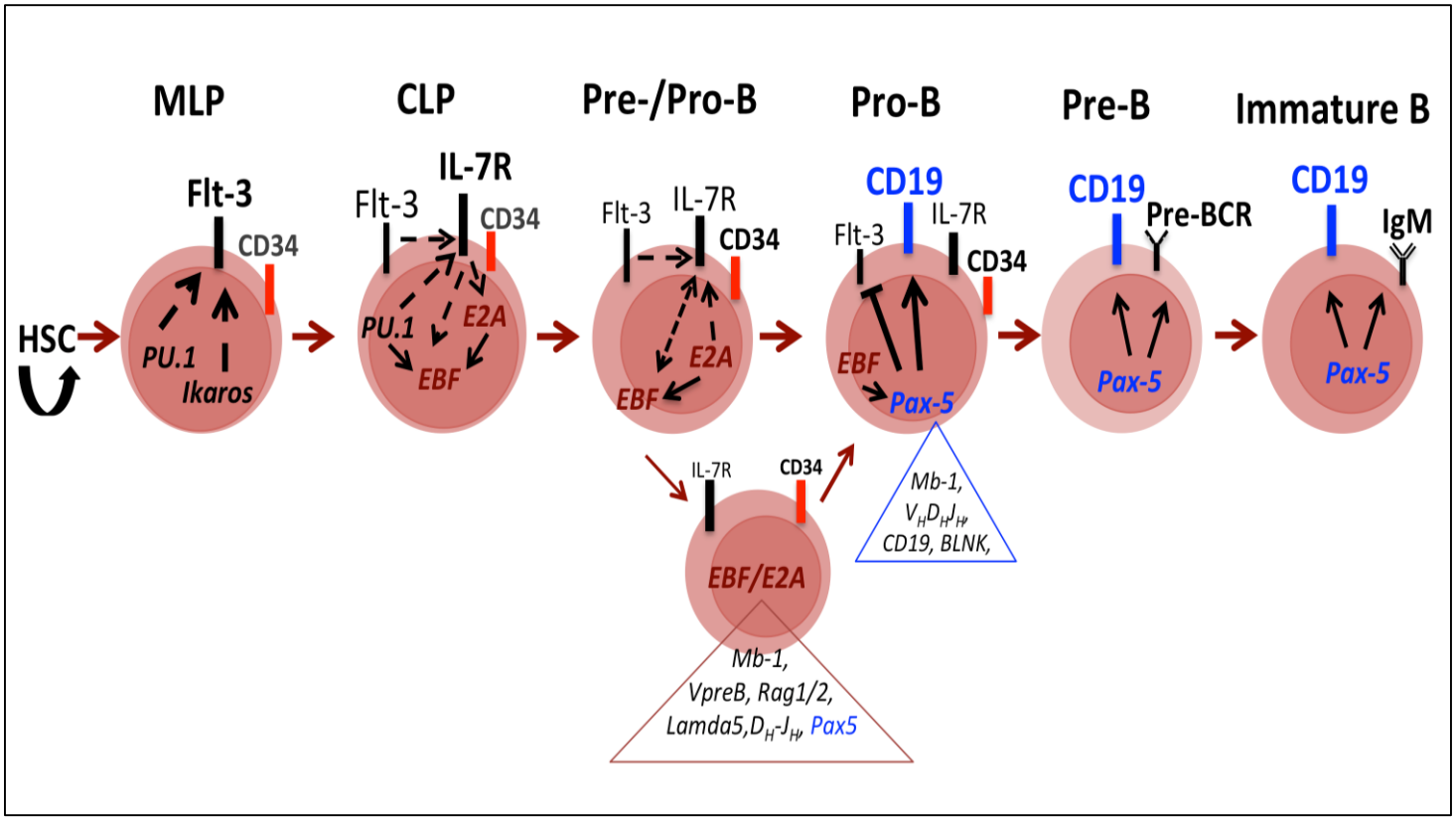

Figure 2. Transcription factors responsible for early B cell differentiation and commitment. Early B cell differentiation generally occurs in the bone marrow, where HSCs advance along the B lineage under the influence of transcription factors. Within the multi-lineage committed progenitor (MLP), PU.1 and Ikaros (black) are involved in lymphoid commitment. In the pre-pro-B and pro-B cells, E2A and EBF (maroon) are expressed. They regulate genes involved in immunoglobulin rearrangement (red triangle) was well as the transcription factor PAX-5 (blue), which is vital for commitment to the B lineage. PAX5 further facilitates expression of CD19 as well as additional genes (blue triangle), required for expression of pre-B cell receptor (pre-BCR) at the pre-B cell stage. Pre-B cells with productive expression of pre-BCR can proliferate, bind antigens a further differentiate into immature B cells with a mature immunoglobulin on the cell's surface. At this stage they migrate from the bone marrow into the periphery where they mature further.

Cytokines in Early B Cell Development

Flt-3 ligand (FL)

Mouse and human FL are cross-reactive, sharing 72% amino acid identity (Lyman & Jacobsen, 1998). Several isoforms of FL exist. Humans express biologically active FL as both transmembrane and soluble forms (Lyman & Jacobsen, 1998; Lyman et al., 1995). Using Northern blot, Lyman et al. found that FL expression was ubiquitous as it was detected in a variety of cells and tissues including PB leukocytes, spleen, thymus, liver, heart and skeletal muscle (Lyman et al., 1994). On the other hand, Flt-3 was primarily detected on hematopoietic stem and progenitor cells (Lyman & Jacobsen, 1998; Zeigler et al., 1994), thus constraining the activities of its ligand. The roles of FL–Flt-3 signaling in B cell development include upregulation of the IL-7R (Borge, Adolfsson, Martensson, Martensson, & Jacobsen, 1999), and synergizing with IL-7 to expand and increase proliferation of primitive hematopoietic progenitors and pro-B cells (Ahsberg et al., 2010; Namikawa, Muench, & Roncarolo, 1996). In accordance with these functions, Flt-3 mutant mice exhibited reduced numbers of progenitors (Mackarehtschian et al., 1995).

Interleukin-7 (IL-7)

IL-7, a 25 kDa protein, originally isolated from the supernatant of the IxN/A6 murine bone marrow stromal cell line was able to stimulate proliferation and on its own support maintenance of B lymphoid progenitors *in vitro* (Namen, Lupton, et al., 1988; Namen, Schmierer, et al., 1988). Human IL-7 was cloned shortly afterward and it stimulated proliferation of both mouse and human B lineage progenitors (Goodwin et al., 1989). Mouse IL-7 and human IL-7 share 81% homology in the coding region of the

nucleotide (Goodwin et al., 1989; Lupton et al., 1990) and 60% at the protein level (Goodwin et al., 1989). Stromal cells of the bone marrow and thymus are the primary producers of IL-7 (Hara et al., 2012). Other cells that produce IL-7 include keratinocytes, intestinal epithelial cells, fetal and follicular DCs (Fry & Mackall, 2002). On the cells' surface, IL-7 binds to a heterodimeric receptor complex of IL-7Rα (also called CD127) and IL-2RG chains (also known as common gamma chain, γC, CD132) (Noguchi et al., 1993). The IL-7Rα chain is shared only by one other cytokine receptor – discussed in the following section–unlike IL-2RG that is shared by other cytokine receptors, including IL-2, IL-4, IL-9 and IL-15. Alternative splicing of the human IL-7Rα gene, produces a secreted form that binds IL-7 (Goodwin et al., 1990) but not TSLP (Lundstrom et al., 2013). In vitro, soluble IL-7Rα (sIL-7Rα) inhibited the effects of IL-7 (Crawley, Faucher, & Angel, 2010), while in vivo, sIL-7Rα enhanced IL-7's activity (Lundstrom et al., 2013). Increased soluble IL-7Rα concentrations have been associated autoimmune conditions such as multiple sclerosis (MS) (Gregory et al., 2007; Lundstrom et al., 2013) and systemic lupus erythematosous (SLE) (Badot et al., 2013).

While mouse and human IL-7 are homologous, their biological functions related to B cells are thought to differ. IL-7 stimulation increased the growth and proliferation of murine pro-B and pre-B cells but not mature B cells *in vitro* (G. Lee, Namen, Gillis, Ellingsworth, & Kincade, 1989; Namen, Lupton, et al., 1988). These *in vitro* findings, correlated with the phenotype of adult IL-$7^{-/-}$ mice that exhibited a block at the pro-B to pre-B cell progression and resulted in reduced numbers of mature B cells in the periphery and spleen (von Freeden-Jeffry et al., 1995). Carvalho et al., later showed that in the absence of IL-7, fetal and perinatal B lymphopoiesis were normal while adult B

lymphopoiesis was perturbed (Carvalho, Mota-Santos, Cumano, Demengeot, & Vieira, 2001), thus demonstrating that IL-7 independent B cell development occurs in the early stages of life (Carvalho et al., 2001). }. Like IL-$7^{-/-}$ mice, *in vivo* B lymphopoiesis is also perturbed at the pro-B cell stage onward by blocking IL-7 with a neutralizing anti-IL-7 monoclonal antibody (Grabstein et al., 1993). Mice deficient for IL-7Rα, exhibited a block at the pre-pro B to pro-B transition, earlier than IL-$7^{-/-}$ mice (Peschon et al., 1994). Kikuchi et al. demonstrated that the arrest at the pre-pro-B cell stage in adult IL-7 and IL-7R deficient mice was because EBF and its target genes were not expressed (Kikuchi, Lai, Hsu, & Kondo, 2005). While IL-7 deficiency results in loss of the earliest B cell precursors, overexpression or administration of recombinant IL-7 *in vivo*, expanded early B cell subsets and produced a lymphoproliferative disorder in cells bearing the immature B cell phenotype (Akdis et al., 2011; Fry & Mackall, 2002).

Aside from IL-7's role in B cell differentiation and proliferation, it also regulates the survival of early progenitors via the Bcl-2 family of proteins. This function is achieved by simultaneously activating the pro-survival members Bcl-2, Bcl-xL and Mcl-1 (Clark, Mandal, Ochiai, & Singh, 2014; Lu, Chaudhury, & Osmond, 1999; Opferman et al., 2003) while repressing the pro-death factors Bax and Bad (Clark et al., 2014; Lu et al., 1999). The precise role of IL-7 in the survival of the earliest B cell progenitors remains debatable, as others show that IL-7 does not regulate Bcl-2, Bcl-xL and Mcl-1 in mouse pre-pro B cells (Kikuchi et al., 2005; Kondo, Akashi, Domen, Sugamura, & Weissman, 1997).

While critical for murine B cell development, IL-7 is thought to be nonessential for human B cell development. Patients with IL-7Rα defects displayed a $T^{-}B^{+}NK^{+}$ SCID

phenotype with normal to increased numbers of functionally deficient B cells (Giliani et al., 2005; Puel, Ziegler, Buckley, & Leonard, 1998; Roifman, Zhang, Chitayat, & Sharfe, 2000). SCID is characterized by deficiencies in T or B cell and can extend to NK cells. Symptoms including pneumonia, meningitis and recurring infections usually occur within the first few months of life. Patients with mutations in IL-2RG and its associated kinase, Janus Kinase 3 (JAK3), also presented with $T^{-}B^{+}NK^{+}$ SCID and had normal to elevated B cell numbers (Fry & Mackall, 2002).

Despite the absence of defects in human B cell numbers due to IL-7Rα mutations, there is a growing body of evidence that supports a role for IL-7 in human B lymphopoiesis. In vitro cultures of human fetal and adult BM derived B cell progenitors demonstrate that they proliferate in response to recombinant IL-7 (Dittel & LeBien, 1995; Goodwin et al., 1989; Namikawa, Muench, de Vries, & Roncarolo, 1996). IL-7R was expressed at similar levels in pro-B and pre-B cells (Dittel & LeBien, 1995). Clinical trials using recombinant hIL-7 (rhIL-7) to treat malignancy, demonstrated increased expansion of pre-B cells in the BM and PB of patients in the study (Sportes et al., 2010). Similar to the clinical trial results, patients with above-normal IL-7 levels, as seen in advancing HIV, showed an increased frequency of immature B cell in the periphery (Malaspina et al., 2006). While fetal B cells differentiated in response to IL-7, Pribyl et al. showed that fetal BM CD34+ HSCs were also capable of IL-7 independent B cell differentiation (Pribyl & LeBien, 1996). In addition to the effects on B cell proliferation and differentiation, another recently identified function of IL-7 in human B cell development is suppression of premature kappa light chain rearrangement in human B cell precursors, (Nodland et al., 2011). The possibility that like the mouse, human B cell

development is IL-7 independent in the perinatal/neonatal period and IL-7 dependency increases with age will be explored in Chapter 2.

Thymic Stromal Lymphopoietin (TSLP)

TSLP, an IL-7 homologue, was originally identified in the supernatant of the murine thymic stromal line, Z210R.1 (Friend et al., 1994). The mature mouse TSLP protein is 121 amino acid (Sims et al., 2000), while the human counterpart is 131 amino acids (Quentmeier et al., 2001). Both share 43% amino acid identity (Quentmeier et al., 2001). Mouse TSLP mRNA was detected in the spleen, thymus, bone marrow and kidney (Sims et al., 2000) while human TSLP has a broader range of expression being found in the heart, liver, testis, prostate, lung, skeletal muscle, kidney, spleen and colon (Quentmeier et al., 2001). Human TSLP is produced by a variety of cells including keratinocytes (Soumelis et al., 2002), epithelial cells, endothelial cells, neutrophils, macrophages, mast cells (Ying et al., 2005) dendritic cells and fibroblasts (Kashyap, Rochman, Spolski, Samsel, & Leonard, 2011). Recent data identified TSLP as a key mediator in allergic and inflammatory conditions, including arthritis, asthma and atopic dermatitis (Bleck, Tse, Curotto de Lafaille, Zhang, & Reibman, 2008; Bleck, Tse, Gordon, Ahsan, & Reibman, 2010; Koyama et al., 2007; E. B. Lee et al., 2010; Ying et al., 2008). TSLP mediates its effects by binding a heterodimeric receptor complex of the IL-7Rα and CRLF2 (cytokine receptor like factor-2, also known as TSLPR) chains (Pandey et al., 2000; Park et al., 2000). Like its cognate cytokine, mouse and human CRLF2 share low homology, ~35 % (Tonozuka et al., 2001). The low homology of

mouse and human TSLP and CRLF2, suggests that the functions between the species may be different

Initial *in vitro* experiments showed that TSLP induced proliferation and differentiation of murine B cell precursors to the IgM+ stage in fetal liver and long-term bone marrow culture (Friend et al., 1994; Ray, Furlonger, Williams, & Paige, 1996), unlike IL-7, which induced differentiation to the earlier IgM– stage (Levin et al., 1999). While the function of TSLP in *in vitro* B cell differentiation appears consistent, the *in vivo* data is less so. Vosshenrich et al., supported the *in vitro* data with *in vivo* studies showing that pro-B cells from fetal liver and pre-B cells from both fetal liver and adult BM responded to TSLP (Vosshenrich, Cumano, Muller, Di Santo, & Vieira, 2003). B cell precursors from adult BM required pre-BCR expression to respond to TSLP (Vosshenrich, Cumano, Muller, Di Santo, & Vieira, 2004). Together these data suggest that TSLP may have both ontogeny and tissue specific activities.

The effects of TSLP in B lymphopoiesis have been evaluated in several transgenic models. In the absence of IL-7, TSLP stimulation increased fetal-derived B cell precursors (Vosshenrich et al., 2003) and restored adult B and T lymphopoiesis in primary and secondary organs (Chappaz, Flueck, Farr, Rolink, & Finke, 2007). In another model, B cells were reduced in the bone marrow but normal numbers were found in the spleen (Osborn et al., 2004). The results of TSLP overexpression were not only localized to the BM but also systemic. Mice displayed increased immature B cell numbers in the periphery (Astrakhan et al., 2007); developed a lethal systemic B lymphoproliferative disorder upon B cell restoration (Demehri et al., 2008); developed a systemic inflammatory condition (Taneda et al., 2001); exhibited cryoglobulinemia and immune

complex disease (Taneda et al., 2001; Tsai et al., 2010) in alternative TSLP transgenic models. Increased myelopoiesis often accompanied TSLP overexpression (Osborn et al., 2004; Tsai et al., 2010).

The absence of the TSLP receptor (TSLPR) chain does not appear to impact mouse B cell development. $TSLPR^{-/-}$ mice, exhibited no apparent loss of B or T cells resulting from the absence of the TSLP receptor-signaling pathway (Carpino et al., 2004). In a different $TSLPR^{-/-}$ mouse model, Jensen et al. showed that FL not TSLP was responsible for IL-7 independent B lymphopoiesis (Jensen et al., 2008).

To date, the effects of TSLP on normal human B lymphopoiesis have been limited to fetal tissues. Based on the *in vitro* findings that TSLP promoted B cell development from murine fetal sources (Levin et al., 1999; Vosshenrich et al., 2003, 2004), Scheeren et al. investigated the role of TSLP in human fetal B cell precursors. They found increased B cell differentiation from fetal-liver derived CD34+ HSCs and increased proliferation of pro-B and pre-B cells from the fetal liver in response to TSLP (Scheeren et al., 2010). Fetal BM pro-B cells also were also TSLP-responsive while pre-B cells were not (Scheeren et al., 2010).

TSLP-induced signals have been implicated in malignant B cell development. Firstly, Brown et al. demonstrated that primary pre-B ALL cells increased proliferation and viability *in vitro* when cultured with TSLP (Brown et al., 2007). Secondly, overexpression of CRLF2 on the B cell surface was recently associated with a high-risk form of pre-B acute lymphoblastic leukemia (pre-B ALL) (Cario et al., 2010; Mullighan et al., 2009; Russell et al., 2009).

It remains unknown if the effects of TSLP in human B cell development are restricted to fetal tissues. We will explore a role for TSLP in conjunction with IL-7 in the development of B cell precursors from postnatal and adult HSC in Chapters 3, 4 and 5.

IL-7 and TSLP Signaling in Mice and Humans

As demonstrated in Figure 3, the binding of IL-7 to the IL-7 receptor induces dimerization of the receptor chains and cross-phosphorylation of the associated JAKs–. JAK1 with IL-7Rα and JAK3 with the common gamma chain. This interaction results in the phosphorylation, dimerization and translocation of signal transducer and activator of transcription 5a/5b (STAT5a/b) to the nucleus (Isaksen et al., 1999; Johnson, Shah, Panoskaltsis-Mortari, & LeBien, 2005). Activation of this pathway is common to B lymphopoiesis in mice and humans (Johnson et al., 2005). IL-7 also activates the phosphoinositol-3-kinase (PI3K)/AKT pathway in human thymocytes but not in B cell precursors (Johnson, Shah, Bajer, & LeBien, 2008)

Like IL-7, TSLP ligation induces phosphorylation of STAT5 (Isaksen et al., 1999), but early studies suggested that activation of STAT5 was independent of the known JAKs (Isaksen et al., 1999; Ray et al., 1996). Rochman et al. later refined these findings, demonstrating that JAK2 associated with CRLF2 and was cross-phosphorylated by IL-7Rα-associated-JAK1 upon TSLP stimulation (Rochman et al., 2010). Unlike IL-7, TSLP signaling also activates the PI3K/AKT pathway in B cell precursors (Brown et al., 2007; Johnson et al., 2008; Tasian et al., 2012).

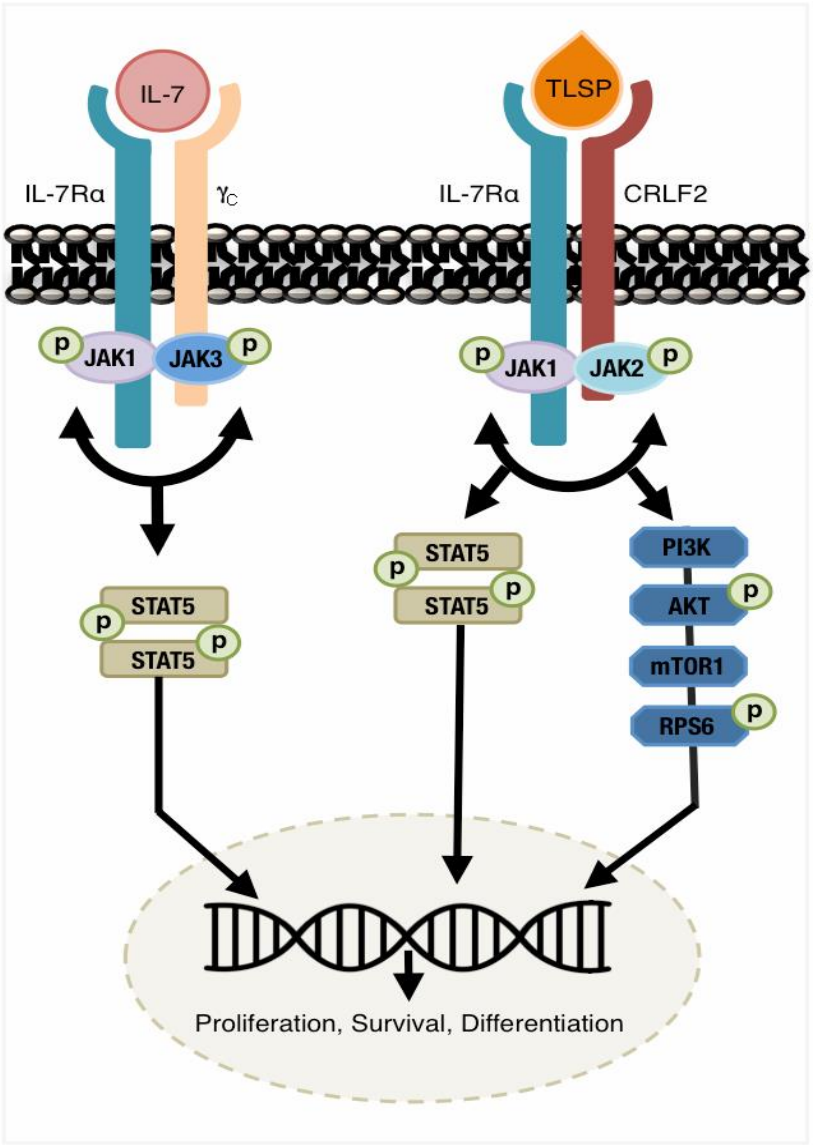

Figure 3. TSLP and IL-7 signaling pathways activated in human B cells**.** The binding of IL-7 to its receptor complex of α and IL2RG, and TSLP to its receptor of IL-7Ra and CRLF2, facilitates the cross-phosphorylation of the JAKs-associated with the respective chains. Phosphorylated-JAK1 in turn phosphorylates STAT5A/B, which dimerizes and translocates to the nucleus. TSLP also activates the PI3K/AKT/mTOR and together with STAT5 they regulate the processes of proliferation, survival and differentiation in B cells.

Models of B Cell Development

Several *in vitro* culture models have been used to explore the stages human of B lymphopoiesis and the cytokines that influence these stages. Both stromal cell-dependent and stromal-cell independent models have been developed to mimic the BM microenvironment by providing intercellular contact and/or proper cytokine stimulation for B cell development. The stromal cell-dependent models consist of culturing hematopoietic progenitors on mouse or human stromal cell lines, for example, MS-5 (Berardi et al., 1997; Johnson et al., 2005), S17 (Cumano, Dorshkind, Gillis, & Paige, 1990; Dorshkind, Johnson, Harrison, & Landreth, 1989; Rawlings et al., 1995) and OP9 (Scheeren et al., 2010; Vodyanik, Bork, Thomson, & Slukvin, 2005). These cell lines secrete cytokines and *in vitro* cultures may also be supplemented with recombinant cytokines to induce early B cell differentiation. Another method of generating a stromal cell-dependent culture model includes the use of primary bone marrow stromal cells isolated from human bone marrow (Parrish et al., 2009; Pribyl & LeBien, 1996). Unlike their cell line counterparts, these primary stromal cells do not produce high concentrations of cytokines (Parrish et al., 2009). This is advantageous because it allows the investigator to titrate varying concentrations of exogenous cytokine and evaluate their effects on developing B cells without the need for elimination of confounding endogenous cytokines.

As B cells develop, their cytokine requirements change. The high concentration of cytokines, like IL-7, that are produced by stromal cell lines and are important for early B cell development may hamper the maturation of the early B cell progenitors and precursors (Ray et al., 1998). The stromal-cell independent model is an attractive

prospect to address this concern because in lieu of stromal cells, the investigator administers combinations of recombinant cytokines at different intervals during B cell culture (Ichii et al., 2010; Kraus et al., 2014; Yoshikawa et al., 2000). This way the effects of the cytokines can be evaluated without intervening stromal cells and the uncharacterized proteins that they may secrete. Together, data from both stromal-cell independent and dependent cultures have assisted in characterizing the discreet stages of B cell development.

In vivo mouse models used to study human B cell development, take advantage of the severe combined immune-deficient (SCID) mutation that does not affect myeloid cells but renders intrinsic B and T cells functionally deficient (Bosma & Carroll, 1991). The deficiency is due to a defect in the activity of the DNA repair enzyme, protein kinase DNA activated catalytic polypeptide, Prkdc, resulting in incomplete VDJ recombination. The antibody- and cellular-mediated immune systems are affected because B and T cell do not mature, thus allowing the engraftment of human cells and tissues and the reconstitution of human hematopoietic cell lineages. Variations of SCID mice have been developed that demonstrate higher engraftment of human cells including the non-obese diabetic (NOD)- SCID mice, in which the IL2RG is deleted (NSG mice) or truncated (NOG mice) (M. Ito et al., 2002; Shultz et al., 2005). Although there are other strains of immune deficient mice that permit the efficient engraftment of human cells (R. Ito, Takahashi, Katano, & Ito, 2012), NSG mice rank highest and were thus selected for *in vivo* experiments presented in Chapters 3 and 4.

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CHAPTER TWO

IL-7 DEPENDENCE IN HUMAN B LYMPHOPOIESIS INCREASES DURING PROGRESSION OF ONTOGENY FROM CORD BLOOD TO BONE MARROW[1]

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Abstract

IL-7 is critical for B cell production in adult mice, however its role in human B lymphopoiesis is controversial. One challenge was the inability to differentiate human cord blood (CB) or adult bone marrow (BM) hematopoietic stem cells (HSCs) without murine stroma. Here, we examine the role of IL-7 in human B cell development using a novel, human-only model based on co-culturing human HSCs on primary human BM stroma. In this model, IL-7 increases human B cell production by >60-fold from both CB and adult BM HSCs. IL-7-induced increases are dose-dependent and specific to CD19+ cells. STAT5 phosphorylation and expression of the Ki-67 proliferation antigen, indicate that IL-7 acts directly on CD19+ cells to increase proliferation at the CD34+ and CD34– pro-B cell stages. Without IL-7, HSCS in CB, but not BM give rise to a small but consistent population of $CD19^{LO}$ B lineage cells that express EBF and PAX-5 and respond to subsequent IL-7 stimulation. Flt3 ligand, but not thymic stromal-derived lymphopoietin (TSLP), was required for the IL-7-independent production of human B lineage cells. As compared to CB, adult BM shows a reduction of *in vitro* generative capacity that is progressively more profound in developmentally sequential populations, resulting in a ~50-fold reduction in IL-7-dependent B lineage generative capacity. These data provide evidence that IL-7 is essential for human B cell production from adult BM and that IL-7-induced expansion of the pro-B compartment is increasingly critical for human B cell production during the progression of ontogeny.

Abbreviations

CB	Umbilical Cord Blood
BM	Bone Marrow
HSC	Hematopoietic Stem Cell
TSLP	Thymic Stromal-Derived Lymphopoietin
IL-7R	IL-7 Receptor
LIN–	Lineage Marker Negative

Introduction

Murine studies have provided evidence that B cell development is controlled by a network of transcription factors and cytokine signaling that coordinates stage-specific expression of B lineage genes (Medina & Singh, 2005; Singh, Pongubala, & Medina, 2007). In mice, signaling through two cytokine receptors, Flt3 (FMS-like tyrosine kinase receptor) and the IL-7R are critical for B cell development. The combined loss of signaling through both of these receptors completely blocks fetal and adult B cell development (Sitnicka et al., 2003, Vosshenrich, 2003 #856) (Vosshenrich, Cumano, Muller, Di Santo, & Vieira, 2003). Flt3 signaling upregulates IL-7Rα expression (Borge, Adolfsson, & Jacobsen, 1999). In adult mice, signaling through the IL-7Rα upregulates expression of Early B cell factor (EBF). EBF in turn regulates expression of a cascade of B cell specific genes (Medina & Singh, 2005; Singh et al., 2007) required for the transition from the common lymphoid progenitor to pro-B cells (Dias, Silva, Cumano, & Vieira, 2005; Kikuchi, Kasai, Watanabe, Lai, & Kondo, 2008; Kikuchi, Lai, Hsu, & Kondo, 2005). During fetal and neonatal life, IL-7-independent expression of EBF allows early B lineage differentiation, but not proliferation, unless IL-7 is present (Kikuchi & Kondo, 2006).

Human B cell development is thought to differ from that in mouse with respect to the requirement for IL-7. Early evidence for the importance of IL-7 in murine B cell production came from *in vitro* studies showing that B cell precursors derived from adult BM increase by approximately 50 fold with the addition of IL-7 (Lee, Namen, Gillis, Ellingsworth, & Kincade, 1989). Further evidence came from reports of blocked B cell development in the initial *in vivo* studies that characterized mutant mice with defects in

IL-7 (von Freeden-Jeffry et al., 1995), or in components of the IL-7 receptor (IL-7R) (DiSanto, Muller, Guy-Grand, Fischer, & Rajewsky, 1995; Peschon et al., 1994). Parallel *in vitro* studies of human B cell development were performed either using fetal hematopoietic sources (Dittel & LeBien, 1995; Kurosaka, LeBien, & Pribyl, 1999; Moreau, Duvert, Banchereau, & Saeland, 1993; Moreau, Duvert, Caux, et al., 1993; Namikawa, Muench, de Vries, & Roncarolo, 1996; Saeland et al., 1991; Wolf, Buckley, Goldfarb, Law, & LeBien, 1991) or using hybrid co-cultures with human progenitors and murine stromal cell lines (Johnson, Shah, Panoskaltsis-Mortari, & LeBien, 2005; Rawlings, Quan, Kato, & Witte, 1995; Taguchi et al., 2007). In those experiments IL-7 either had no effect on B cell production or resulted in IL-7-induced increases that were 10-fold less than those reported in the mouse. *In vivo* human data from clinical reports indicated that patients having severe-combined-immunodeficiency due to defects in IL-7/IL-7R signaling lack T cells, but have peripheral blood B cells (Macchi et al., 1995; Noguchi et al., 1993; Puel, Ziegler, Buckley, & Leonard, 1998). These data have been interpreted to mean that IL-7 is essential for normal murine, but not human, B cell development (Bertrand et al., 2000; Espeli et al., 2006; LeBien, 2000; Munfus, Haga, Burrows, & Cooper, 2007; Roifman, Zhang, Chitayat, & Sharfe, 2000).

More detailed studies in the mouse revealed a dramatic change in B cell precursor requirements during ontogeny. IL-7 knockout mice show a block in adult B lymphopoiesis, but not in the production of B cells during the fetal or neonatal period (Carvalho, Mota-Santos, Cumano, Demengeot, & Vieira, 2001). More recently it has been shown that IL-7-independent expression of EBF in the mouse allows B lineage differentiation early in ontogeny resulting in a “developmental switch” in murine stem

cells with IL-7-independent B cell production occurring during fetal development and through the first two weeks of neonatal life, but not in the adult (Kikuchi & Kondo, 2006).Thus, it is not surprising that *in vitro* IL-7 effects on fetally-derived human B cell precursors (Kurosaka et al., 1999; Moreau, Duvert, Banchereau, et al., 1993; J. R. Pribyl, Dittel, & LeBien, 1995; Saeland, Duvert, Moreau, & Banchereau, 1993; Saeland et al., 1991; Wolf et al., 1991) were minor when compared to those observed for adult B lymphopoiesis in the mouse (Lee et al., 1989). Similarly, clinical data from patients with IL-7/IL-7R signaling defects was obtained early in life and is consistent with observations that fetal/neonatal B cell production is intact in mice with IL-7 defects.

It is also possible that residual, low level IL-7R signaling plays a role in B cell production in patients with defects in the IL-7R or its signaling pathway. Phosphorylation of STAT5 is a signaling event downstream of IL-7R ligation (Lin et al., 1995). Studies that compare B cell development in mice that are STAT5 null with that in STAT deficient mice that express a truncated form of STAT5 reveal that even low levels of residual IL-7R signaling can maintain B cell production in adult mice (Dai et al., 2007; Yao et al., 2006). Experiments to assess for IL-7 function in human patients with defects in components of the IL-7R signaling pathway are limited by the number, type, and quantity of specimens available for study. Thus, *in vitro* studies of human B cell development are essential for determining the role of IL-7 in human B cell development and whether humans exhibit the "developmental switch" that results in the loss of IL-7 independent B cell production observed in the mouse.

The association between ontogeny and IL-7 requirements has not been rigorously examined in humans. *In vitro* studies that address the role of IL-7 in human B cell

production during the neonatal period and through adulthood have been performed using hybrid human-mouse co-culture models (Johnson et al., 2005; Rawlings et al., 1995; Taguchi et al., 2007). The limitations of this model were highlighted by recent reports demonstrating that murine IL-7 can stimulate signaling through the human IL-7Rα (Barata et al., 2006; Johnson et al., 2005; Taguchi et al., 2007). Thus, the production of murine IL-7 by stroma in hybrid human-mouse co-cultures has rendered it difficult to evaluate the precise contribution of human IL-7, and the magnitude of IL-7-dependent effects in human B cell production from HSCs in CB and BM.

Here we describe a novel, entirely human model of *in vitro* B cell development that is based on co-culturing human HSCs on primary human BM stroma. Using this human-only model we show that human IL-7 increases the production of human B cell precursors by more than 60 fold, both from HSCs in adult BM, and from the HSCs in CB that give rise to B cells during the neonatal period. IL-7 effects were mediated through the direct action of human IL-7 on human CD19+ pro-B cells to increase proliferation at both the CD34+ and CD34– stages of B cell development. Adult BM, as compared to CB, shows a reduction of *in vitro* generative capacity that is progressively more profound in developmentally sequential populations, resulting in a ~50-fold reduction in B lineage generative capacity. In the absence of IL-7, HSCS in CB, but not BM give rise to a small but consistent population of $CD19^{LO}$ B lineage cells that express EBF, PAX-5 and respond to IL-7 with increased cell size and upregulation of CD19. These data suggest that in humans the role of IL-7 in B cell production becomes increasingly critical as ontogeny progresses, and that it is an essential factor for B cell production in adults.

Materials and Methods

Cells

CB was collected according to guidelines approved by the Institutional Review Boards at Children's Hospital Los Angeles and Loma Linda University. Adult human BM was purchased from AllCells, LLC, Berkeley, CA; the National Disease Research Interchange (NDRI), Philadelphia, PA; or Poietics Cell Systems of Lonza Walkersville, Inc., Gaithersburg, MD. The leukopheresis product from a patient with pre-B ALL (> 90% CD19+ cells) served as a source of primary human pre-B ALL cells. The use of all human tissues was reviewed and approved by the Institutional Review Boards at Children's Hospital Los Angeles and/or Loma Linda University. Primary murine BM was obtained from normal adult control mice immediately after they were sacrificed as a part of other studies approved by the Institutional Animal Care and Use Committees at Children's Hospital Los Angeles or Loma Linda University.

Lineage marker negative (Lin–) CD34+ cells were isolated by FACS sorting from CD34-enriched CB and BM mononuclear cells as previously described (Payne et al., 2003). Primary stromal cells from adult human or murine BM were obtained by plating whole BM in Dexter's original medium as described previously (Nolta et al., 2002). Non-adherent cells were removed on the second day and adherent cells were used through passage six.

The murine S17 (Collins & Dorshkind, 1987) stromal cell line is a generous gift from Dr. Kenneth Dorshkind, University of California Los Angeles and the murine MS-5 (Ohkawara et al., 1998) stromal cell line is a generous gift from Dr. Kazuhiro Mori.

Cultures

Human CB and adult BM HSCs (typically ~10,000 and ~15,000 respectively) were seeded onto murine stromal cells pre-plated at 3,500 cells/well (hybrid cultures) or onto sublethally irradiated (2000 rads) primary human BM stromal cells plated at 7,000 cells/well (human-only cultures) in 96-well tissue culture plates in B cell medium: RPMI medium (Irvine Scientific, Santa Ana, CA) containing penicillin/streptomycin, L-glutamine, 50 μM 2-mercaptoethanol, and 5% FBS (Omega Scientific, Inc. Tarzana, CA) lot tested for ability to support B cell production. Sublethal irradiation assured that culture progeny did not arise from residual human HSCs that might be associated with the BM-derived stroma. Control cultures were supplemented with IL-3 at 1 ng/ml (first week only) and Flt-3 ligand at 1 ng/ml or 50 ng/ml (R&D Systems, Minneapolis, MN) two cytokines that have previously been shown to support the production of human B lineage cells *in vitro* (Crooks, Hao, Petersen, Barsky, & Bockstoce, 2000; Miller, McCullar, Punzel, Lemischka, & Moore, 1999; Namikawa et al., 1996; Shah, Smogorzewska, Hannum, & Crooks, 1996). Additional supplementation with recombinant human cytokines and/or antibodies is described in fig. legends or as follows: IL-7 at 5 ng/ml, stem cell factor at 25 ng/ml (R&D Systems) neutralizing anti-human IL-7 antibody, or control antibody at 10 ng/ml (BD Pharmingen, San Diego, CA), neutralizing anti-mouse IL-7 antibody (Abcam, Inc. Cambridge, MA) or control antibody (Jackson ImmunoResearch, West Grove, PA), neutralizing anti-human TSLP antibody (R&D) or control antibody (Jackson ImmunoResearch) at 1 ug/ml, neutralizing anti-human Flt-3 ligand or control antibody (R&D) at .6 μg/ml. The ND_{50} for rabbit anti-mouse IL-7 neutralizing antibody was 0.012 to 0.02 μg/ml of antibody for mouse IL-7 at

600 pg/ml. The ND_{50} for rat anti-human IL-7 neutralizing antibody was .0001 to .001μg/ml of antibody for human IL-7 at 167 pg/ml. The ND_{50} for sheep anti-human TSLP was .05 to .25 μg/ml for .5 ng/ml human TSLP. The ND_{50} for mouse anti-human Flt-3 ligand was .02 to .06 μg/ml for 5 ng/ml Flt-3 ligand.

For cultures described in Figure 4A each well contained 3 antibodies: either the neutralizing antibody to Flt-3 ligand, IL-7, and/or TSLP, or the corresponding control antibody at an identical concentration as neutralizing antibody. Wells to which exogenous cytokine were added received the appropriated control antibody for that cytokine. This assured that antibody isotypes and concentrations were the same in all wells. To simplify, only neutralizing antibodies (and not control antibodies) are indicated in the legend to Figure 4A.

CD19+ progeny from human-only cultures were enriched for apoptosis assays using the Human B cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada) and for assays of STAT5 phosphorylation using the Miltenyi Biotech CD19 microbead kit (Miltenyi Biotec, Auburn, CA).

FACS-Sorting and Flow Cytometry

For flow cytometry cells were surface stained as described previously, (Zhang et al., 2005) and resuspended in PBS for FACS sorting using the FACSVantage or FACSAria cell sorter (Becton Dickinson Immunocytometry Systems, [BDIS] San Jose, CA) or in PBS or 1% freshly prepared formaldehyde for analysis using the FACSCalibur flow cytometer. The following antibodies were used: kappa FITC, lambda FITC, IgM

FITC, IgM PE-Cy5 (Cychrome), CD19 APC, CD34 PerCP (BD Pharmingen), and IL-7Rα PE (Beckman-Coulter Immunotech).

To assess and cytoplasmic mu (cμ) and Ki-67 expression, cells were first stained for surface markers then stained with anti-IgM antibodies to detect intracellular cμ, with anti-Ki-67, or with an isotype-matched control antibody (BD Pharmingen) using the Fix and Perm Cell Permeabilization Kit (Caltag Laboratories, Burlingame, CA) as per manufacturer's instructions.

For phospho STAT5 staining, cells were washed, fixed by incubating for 10 min with 1.5% paraformaldehyde at room temperature, pelleted by centrifugation and then paraformaldehyde was decanted off. To permeabilize, cells were vortexed, resuspended in 100% methanol at 4^{o} C, and incubated for 10 min at 4^{o} C or at -20^{o} C overnight. Cells were pelleted by centrifugation, washed in PBS with 10% FBS and .01% sodium azide, then incubated with anti-phospho STAT5 PE or mouse IgG1 PE isotype control antibody (BD Pharmingen) for 30 min at room temperature. Cells were then washed and resuspended in staining buffer for flow cytometry.

To assess apoptosis, harvested cells were incubated with CD19 APC and the apoptosis indicator Annexin V FITC (BD Pharmingen) and prepared for flow cytometry according to manufacturers instructions.

Fluorescence intensity on all flow cytometry dot plots and histograms is shown on a log scale. Forward scatter (FSC) and percent maximum on histograms is shown in a linear scale.

ELISA

Primary murine and human BM stromal cells (non-irradiated, 3.5 X 10^5 cells or sublethally irradiated [2000 rads], 7.0 X 10^5 cells) and murine MS5 and S17 stromal cells (non-irradiated, 3.5 X 10^5 cells) were plated in 200 μl of B cell medium (described above) without cytokines in individual wells of a 96-well plate. At weekly intervals 100 μl of culture medium was harvested, filtered with a 0.2 μm filter, and 100 μl of new media added to each well. Harvested supernatants were frozen, thawed, and IL-7 levels were detected using the Mouse IL-7 or Human IL-7 ELISA kit (R&D Systems).

RT-PCR

cDNA was prepared as described previously. Reverse-transcription (RT)-PCR to detect expression of IL-7 and β2 microglobulin was performed as described previously (Payne et al., 2003). Intron-spanning PCR primers for mouse and human IL-7 and B_2 microglobulin were as follows: human β2 microglobulin, 5'–CTC GCG CTA CTC TCT CTT TC–3' and 5' –CAT GTC TCG ATC CCA CTT AAC–3' (330 base product); human IL-7, 5'–CTC CCC TGA TCC TTG TTC TG–3' and 5'–TCA TTA TTC AGG CAA TTG CTA CC–3' (151 base product); mouse β2 microglobulin, 5'–TGC TAT CCA GAA AAC CCC TC–3' and 5'–GGC ATG CTT AAC TCT GCA GG –3' (259 base product); and mouse IL-7, 5' –TGG AAT TCC TCC ACT GAT CC–3' and 5' –TGG TTC ATT ATT CGG GCA AT–3' (160 base product): (Invitrogen Life Technologies, Paisley, PA). PCR conditions for human IL-7 and B2 were as follows: 10 min 94° C (1 cycle); 94° C for 1 min, 50° C for 45 s, 72° C for 1 min and 15 s (5 cycles); 94° C for 1 min, 55° C for 30 s, 72° C for 1 min and 15 s (29 cycles); 72° C for 5 min (1 cycle). PCR

conditions for mouse IL-7 and B2 were as follows: 15 min 95° C (1 cycle); 94° C for 30 s, 55° C for 30 s, 72° C for 30 s (35 cycles); 72° C for 10 min (1 cycle).

cDNA was amplified for detection of CD19, EBF, Pax-5, and GAPDH (housekeeping gene) using the LightCycler 1.0 system real-time thermal cycler and the LightCycler FastStart DNA MasterPLUS SYBR Green kit (Roche Applied Science, Indianapolis, IN). The small numbers of B lineage cells produced in the absence of IL-7 activity precluded quantitative or semi-quantitative assessment of mRNA levels. The assessment of samples as positive for expression was based on 1) observing reproducible CT values in 2 replicates of the target gene where the GAPDH housekeeping gene showing a CT value of <30 cycles and 2) melting curve analysis showing superimposable melting curves across samples (including pre-B ALL cells as a positive control) and replicates for a given target gene in the absence of a superimposable product in negative control samples. Gene specific primer pairs were designed to span introns using Roche's Assay Design Center at www.roche-applied-science.com. Primers were as follows: GAPDH left 5'- GAG TCC ACT GGC GTC TTC AC; GAPDH right 5'- GTT CAC ACC CAT GAC GAA CA; CD19 left 5'- TCC ACC TGG AGA TCA CTG CT; CD19 right 5'- GAC CTT CCA GCC ACC AGT C; Pax5 left 5'- GTC CCA GCT TCC AGT CAC AG; Pax5 right 5'- GTG CTC ACC GAG GAC ACC; EBF left 5'-AGC TGC CAA CTC CCC CTA T; EBF right Lower 5' GGG AGG CTT GTG GAG GAG. Cycling parameters for all products: initial denaturation of 15 min at 95 degrees followed by 50 cycles of 10 s at 95°C (denaturing); 5 s at 60°C (annealing); 15 s at 72°C (elongation).

Statistics and Data Analysis

In flow cytometry analysis, CD19+ B lineage cells and CD34+ HSC and progenitor populations were identified based on light scatter within a lymphocyte/progenitor light scatter gate and by expression of indicated surface markers. CD19–CD34– non-B lineage cells were identified based on light scatter within a living cell light scatter gate. Gates for determining percentages of cells positive for surface or intracellular markers were set on similarly gated CD19+ B lineage and CD19– cells in samples co-stained for CD19 and isotype-matched control antibodies.

Absolute numbers of culture progeny were determined by multiplying the fraction of cells within a particular subset (gated based on surface immunophenotype: CD19–, CD19+, CD19+CD34+IL-7Rα+, etc.) as a percentage of the cells within living cell light scatter (assessed by flow cytometry) by the total count of living cells generated in the culture (determined by hemocytometer count of cells negative for trypan blue staining or by flow cytometry using Caltag Counting Beads, [Caltag Laboratories]). Relative numbers of cells were determined by dividing the absolute number of cells of a particular type generated in each experimental condition by the absolute numbers of the same type of cells generated under control conditions (Figure 1, 2) or under control conditions plus anti-human IL-7 neutralizing antibodies (Figure 3, 8) in each independent experiment.

Each independent co-culture experiment represents an independent FACS sort of Lin–CD34+ BM or CB cells. In most cases adult BM in independent experiments is from different donors and in all cases CB in each independent experiment is pooled from multiple different donors. The hematopoietic cells for all conditions in each independent

experiment are aliquoted from a pool of cells FACS sorted at the same time from the same donor(s).

Flow cytometry analysis was performed using FlowJo software (Tree Star, Inc. Ashland, OR). Statistical analysis was performed using InStat3 software (GraphPad Software, Inc., San Diego, CA).

Results

Human-Only Co-Cultures Support In Vitro Human B Lymphopoiesis

We developed a novel, human-only *in vitro* model of B cell development based on co-culturing nonfetal human hematopoietic progenitors with primary human stroma from adult BM (Figure 1A). Initial experiments used CB as a source of HSCs. While CB contains fetally-generated B cells, the stem cells present in CB are of interest because they give rise to B cells in the neonatal period and are increasingly used clinically as a source of HSCs for transplant.

B cell production in human-only cultures was compared to that in an established hybrid co-culture model (Crooks et al., 2000; Fluckiger et al., 1998; Hao, Smogorzewska, Barsky, & Crooks, 1998; Rawlings et al., 1997) consisting of human HSCs and murine S17 stroma. (Figure 1A). The emergence of human B lineage cells in human-only cultures paralleled that observed in hybrid cultures with murine S17 stroma. CD19+ B lineage cells were undetectable at one week and rare at two weeks (data not shown). At three weeks, CD19+ B lineage cells were detectable in both human-only and hybrid S17 cultures under control conditions. However the frequency of B lineage cells was low in human-only cultures (Figure 1B, middle panel). With the addition of human IL-7,

human-only cultures produced a substantial population of CD19+ cells (Figure 1C, middle panel). At 3 weeks, the CD19+ B lineage cells present in human-only cultures, like those in hybrid cultures, were B cell precursors that did not express the cytoplasmic mu (cμ) Ig heavy chain (Figure 1B, C, middle and left panels) or surface immunoglobulin (Ig) as evidenced by the absence of kappa and lambda Ig light chains (data not shown). At 5 weeks, surface IgM+ cells were observed in both human-only (Figure 1B, C, right panels) and hybrid co-cultures (data not shown), and while the production of cμ+ and IgM+ B cells in human-only cultures was inefficient (~11 to 16% of CD19+ cells), it was consistently higher than that observed in hybrid co-cultures (~3% of CD19+ cells). These data provide evidence that co-cultures with primary human BM stromal cells support the production of human B cells and establish human-only cultures as an *in vitro* model for studying early stages of human B lymphopoiesis.

Exogenous IL-7 Increases B Cell Production in Human-Only, but not Hybrid Co-Cultures with Murine Stromal Cell Lines

Using human-only and hybrid S17 cultures we characterized the effect of IL-7 on the production of human B cell precursors from CB HSCs. The addition of IL-7 to human-only, but not hybrid S17 cultures, significantly increased the production of CD19+ B lineage cells as compared to cultures without IL-7 (Figure 1B, C, D). Stem cell factor (SCF) had no significant effect on human B cell production, either alone or in combination with IL-7 in human-only or hybrid S17 cultures (Figure 1D). Thus, human-only cultures revealed specific, IL-7 effects on human B cell production.

A titration of IL-7 into human-only cultures showed that IL-7-induced increases in the production of B lineage cells were dose-dependent and specific to CD19+ cells, achieving maximal effects (43-fold) at 10 ng/ml of IL-7 (Figure 1E). In contrast, IL-7-induced increases observed in hybrid S17 cultures at 3 weeks never averaged more than 3-fold at any concentration of IL-7 (Figure 1E). These data provide evidence that IL-7 induces a lineage-specific increase in the *in vitro* production of human B cell precursors that is not detectable in hybrid cultures with the murine S17 stromal cell line.

To determine if the inability to support IL-7-induced increases in human B cell production is specific to murine S17 stroma, we examined IL-7 effects in hybrid cultures with the MS-5 murine stromal cell line, another established model (Berardi et al., 1997; Nishihara et al., 1998; Ohkawara et al., 1998) for *in vitro* human B lymphopoiesis. As with S17 stroma, the addition of IL-7 did not significantly increase the production of human B cell precursors in hybrid MS-5 cultures (data not shown). However, substantial numbers of human B lineage cells were produced in the absence of exogenous IL-7 in hybrid cultures with murine S17 and MS-5 stroma (Figure 1B and data not shown) suggesting that another factor replaces human IL-7 in hybrid human-mouse co-cultures.

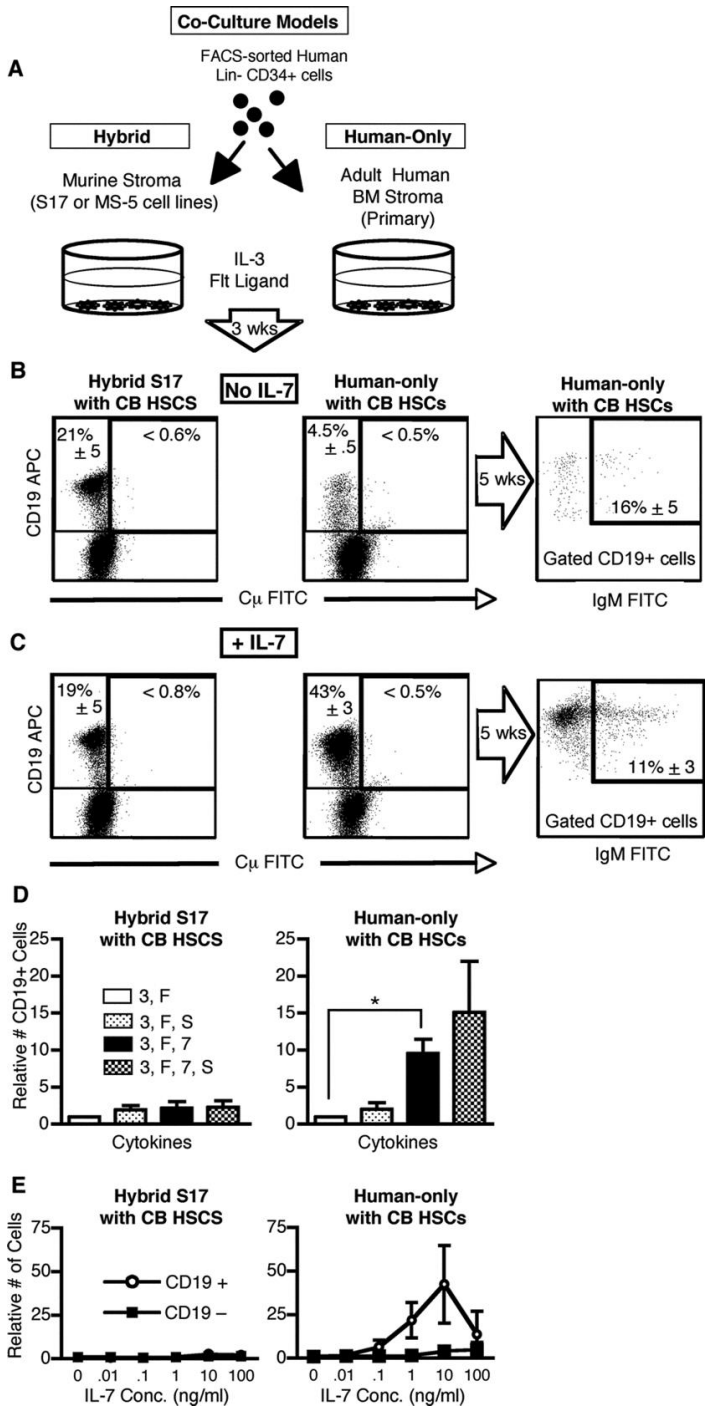

Co-Culture Models
A
FACS-sorted Human
Lin- CD34+ cells
Hybrid
Murine Stroma
(S17 or MS-5 cell lines)
Human-Only
Adult Human
BM Stroma
(Primary)
IL-3
Flt Ligand
3 wks
B
Hybrid S17
with CB HSCS
No IL-7
Human-only
with CB HSCs
Human-only
with CB HSCs
21%
± 5
< 0.6%
4.5%
± .5
< 0.5%
5 wks
16% ± 5
Gated CD19+ cells
CD19 APC
Cμ FITC
IgM FITC
C
+ IL-7
19%
± 5
< 0.8%
43%
± 3
< 0.5%
5 wks
11% ± 3
Gated CD19+ cells
CD19 APC
Cμ FITC
IgM FITC
D
Hybrid S17
with CB HSCS
Human-only
with CB HSCs
Relative # CD19+ Cells
3, F
3, F, S
3, F, 7
3, F, 7, S
*
Cytokines
Cytokines
E
Hybrid S17
with CB HSCS
Human-only
with CB HSCs
Relative # of Cells
CD19 +
CD19 –
0 .01 .1 1 10 100
IL-7 Conc. (ng/ml)
0 .01 .1 1 10 100
IL-7 Conc. (ng/ml)

Figure 1 (*Figure 4).* Exogenous IL-7 increases B cell production in human-only, but not hybrid human-mouse co-cultures with murine stromal cell lines. **(**A) CB CD34+ cells that were negative for lineage markers (Lin–CD34+) were FACS-sorted and placed in co-culture with murine S17 stroma (left panels) or with sub-lethally irradiated, primary stroma from adult human BM (middle and right panels). Co-cultures were supplemented with IL-3 (first week only) and Flt3 ligand (3, F; control conditions), with or without the addition of IL-7 (5 ng/ml). Cells generated in cultures without IL-7 (B) or with IL-7 (C) were harvested at 3 or 5 weeks and stained for flow cytometry to assess for surface expression of the B lineage marker, CD19, and the presence of Ig heavy chain protein (cμ) in the cytoplasm or surface IgM as indicated. Plots are gated on lymphocyte light scatter (gates not shown). CD19+ B lineage cells, as a percentage of the total cells in living cell light scatter (mean $\pm$ SEM) for 4 independent experiments (see "Materials and Methods") are given in left and middle panels. Percentages of IgM+ cells (mean $\pm$ SEM) among total CD19+ cells at 5 weeks are given in right panels (8 independent experiments). (D) Hybrid S17 (left panel) and human-only cultures (right panel) established under control conditions (3, F) as described above were supplemented with stem cell factor (S), with IL-7 (7) or with a combination of IL-7 and SCF and harvested at 3 weeks. Relative numbers of CD19+ and CD19– progeny were determined as described in "Materials and Methods". Data shown are the means $\pm$ SEM of 6 independent experiments. (* $p = .012$, ANOVA) (E) Hybrid S17 (left panel) and human-only cultures (right panel) initiated as described above were supplemented with increasing amounts of IL-7 as indicated. At 3 weeks culture progeny were harvested and relative numbers of CD19– and CD19+ cells were determined. Data shown are the means $\pm$ SEM of 3 independent experiments with the exception of IL-7 at 100 ng/ml for which n=2.

IL-7 Activity is Critical for B Cell Production in Hybrid S17 and MS-5 Cultures

While early studies suggested that mouse IL-7 does not act on human cells (Goodwin et al., 1989; Kroemer, Kroncke, Gerdes, & Richards, 1998), more recent reports indicate that this is not the case (Barata et al., 2006; Johnson et al., 2005). A titration of anti-mouse IL-7 neutralizing antibodies, but not control antibodies, into hybrid cultures with murine stroma gave a dose-dependent reduction in human B cell production. At the highest concentrations, anti-mouse IL-7 neutralizing antibodies decreased B cell production from CB HSCs in hybrid S17 and MS-5 cultures by 10 and 17 fold, respectively, as compared to co-cultures with no neutralizing antibody (Figure 2A). The production of non-B lineage (CD19–) cells was not affected by anti-mouse IL-7 neutralizing antibodies (Figure 2B). These data provide evidence that endogenously produced murine IL-7 is critical for the production of human B cell precursors in hybrid co-cultures with murine S17 and MS-5 stroma.

Production of IL-7 by Primary Human and Murine BM Stroma

To determine if stromal cells can serve as a source of IL-7 for *in vivo* human B cell development we examined the ability of primary human BM stroma to produce IL-7. IL-7 mRNA was detected in primary adult human BM stroma as well as primary murine BM stroma and murine stromal cell lines (Figure 2C). Next we compared the levels of IL-7 protein produced by murine and human stroma grown as in co-cultures. Weekly assessment of IL-7 by ELISA showed that primary adult human BM stroma produce low levels of IL-7 *in vitro* (ranging from .15 to 1.02 pg/ml during a five week culture period)

and that IL-7 production is not affected by sub-lethal irradiation prior to plating for co-culture. In contrast, the murine S17 and MS-5 stromal cell lines produced levels of IL-7 that were at least 40 fold higher than that of primary human BM stroma (Figure 2D). However, *in vitro* IL-7 production by primary adult murine BM stroma, either with or without sub-lethal irradiation, was below the threshold of detection by the ELISA for mouse IL-7. Thus, the high level of *in vitro* IL-7 production by murine S17 and MS-5 murine stromal cell lines does not reflect that of primary murine BM stroma that support *in vivo* IL-7-dependent B cell development in the mouse.

While the production of IL-7 from primary adult human BM stroma was detectable with the much more sensitive ELISA assay for human IL-7, those levels would have been below the sensitivity of the mouse IL-7 ELISA assay. These data provide evidence that primary human adult BM stroma produce IL-7 and that the level of *in vitro* IL-7 production by these cells is consistent with that observed for mouse, an animal model where the physiological relevance of IL-7 in B cell development has been demonstrated *in vivo* (Carvalho et al., 2001; von Freeden-Jeffry et al., 1995).

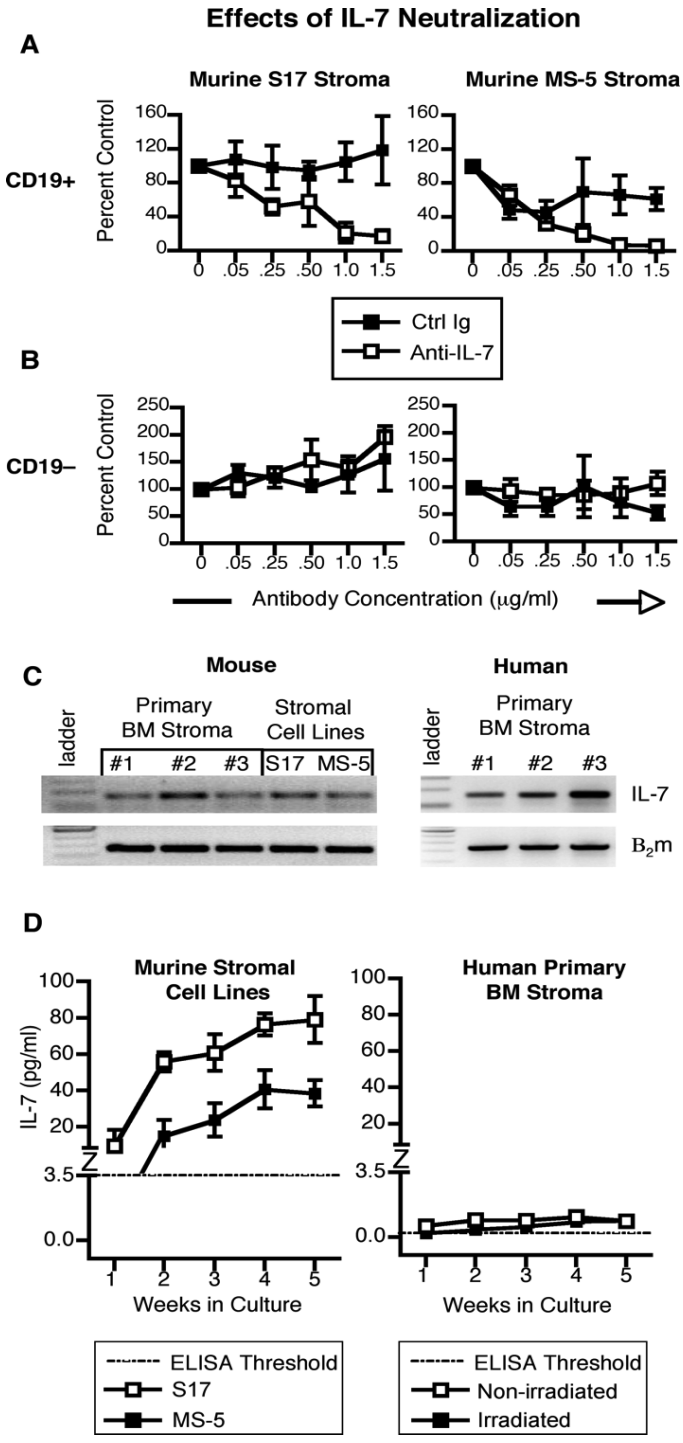

Effects of IL-7 Neutralization
A
Murine S17 Stroma
Murine MS-5 Stroma
CD19+
Percent Control
160
120
80
40
0
0 .05 .25 .50 1.0 1.5
Ctrl Ig
Anti-IL-7
B
CD19–
Percent Control
250
200
150
100
50
0
Antibody Concentration (μg/ml)
C
Mouse
Human
ladder
Primary BM Stroma
Stromal Cell Lines
#1 #2 #3 S17 MS-5
IL-7
B_2m
D
Murine Stromal Cell Lines
Human Primary BM Stroma
IL-7 (pg/ml)
100
80
60
40
20
3.5
0.0
1 2 3 4 5
Weeks in Culture
ELISA Threshold
S17
MS-5
Non-irradiated
Irradiated

Figure 2 (*Figure 5*). B cell production in hybrid human-mouse co-cultures is dependent on murine IL-7 activity. FACS-sorted Lin–CD34+ CB cells were placed in hybrid co-cultures with the murine S17 or MS-5 stromal cell lines and grown under control conditions (3, F) or control conditions supplemented with increasing amounts of anti-mouse IL-7 neutralizing antibody or appropriate control antibody. Culture progeny were harvested at 3 weeks and absolute numbers of CD19+ and CD19– cells were determined as described in "Materials and Methods". Absolute numbers of (A) CD19+ and (B) CD19– progeny generated under increasing control and anti-IL-7 antibody concentrations were compared to absolute numbers of CD19+ and CD19– cells generated under control conditions with no antibody. Relative numbers of CD19+ and CD19– cells (as compared to control conditions with no antibodies) are graphed. Data shown are the means $\pm$ SEM for 3 independent experiments. (C) RT-PCR analysis using primers to detect IL-7 and B_2 microglobulin (B_2m, ubiquitously expressed in nucleated cells). Analyses from three different adult mice (age range 6-12 weeks), from the murine S17 and MS-5 stromal cell lines (n=2) and from three different adult donors (age range 20-30) are shown. (D) ELISA assessment of *in vitro* IL-7 production from indicated stromal cell lines (murine MS-5 and S17) or primary stroma. Stroma were plated under conditions similar to those used for co-cultures (3,5000 non-irradiated MS-5 or S17 cells per well and 7,000 sublethally irradiated primary human stroma per well in 96 well plates). For comparison, non-irradiated primary human stroma were also plated at 3,500 cells per well. Media was harvested at weekly time points and assessed for IL-7 by ELISA. Note the difference in threshold for sensitivity of mouse and human IL-7 ELISA assays (murine: 3.5 - 8.3 pg/mL and human: <0.1 pg/ml). Primary adult murine BM stroma was also assessed for IL-7 production, however data is not shown as the levels of IL-7 production for both irradiated and non-irradiated primary adult murine BM stroma were below the sensitivity of the murine IL-7 ELISA.

IL-7 is Essential for the Production of B Lineage Cells from HSCs in Adult BM, but not from HSCs in CB

To determine the extent to which B cell production is dependent on IL-7 and how this might change during ontogeny, we compared the production of B cell precursors from CB and adult BM HSCs in human-only cultures supplemented with anti-human IL-7 neutralizing antibodies or with IL-7 (Figure 3A). A small population of CD19+ B lineage cells were generated from CB HSCs in cultures where IL-7 activity was neutralized. In contrast, B lineage cells derived from adult human BM were almost totally absent from cultures with anti-IL-7 antibodies, (Figure 3A). These data suggest that HSCs in CB but not adult BM have the potential for IL-7-independent human B lymphopoiesis.

A comparison of human-only cultures initiated with CB and adult BM HSCs showed that IL-7-induced increases in both cases were similar in magnitude and specific to CD19+ cells (Figure 3B). However, the fraction of culture progeny comprised of B lineage cells was significantly less in cultures with HSCs from adult BM (Figure 3A). Therefore, we compared the generative capacities of CB and BM HSCs with respect to B lymphoid (CD19+) and non-B lymphoid (CD19–) progeny and the effects of IL-7 on these capacities (Figure 3C). For each Lin–CD34+ CB cell plated at culture initiation, an average of 5.7 CD19+ cells were generated at 3 weeks in human-only cultures with IL-7. Surprisingly, the average B lymphoid generative capacity of adult BM HSCs in identical conditions was only .11 CD19+ cells/HSC plated. The capacities of CB and adult BM HSCs to generate CD19– progeny were not significantly different and were unaffected by IL-7. Thus, the *in vitro* B lymphoid generative capacity of adult BM HSCs is more than

50 fold less than that of CB HSCs and this decreased generative capacity is specific to CD19+ cells. These data suggest that IL-7 is more critical in maintaining human B cell production during adult life than during the neonatal period and that this may be due in part to a selective decrease in the B lymphoid generative capacity of HSCs in adult BM as compared to CB.

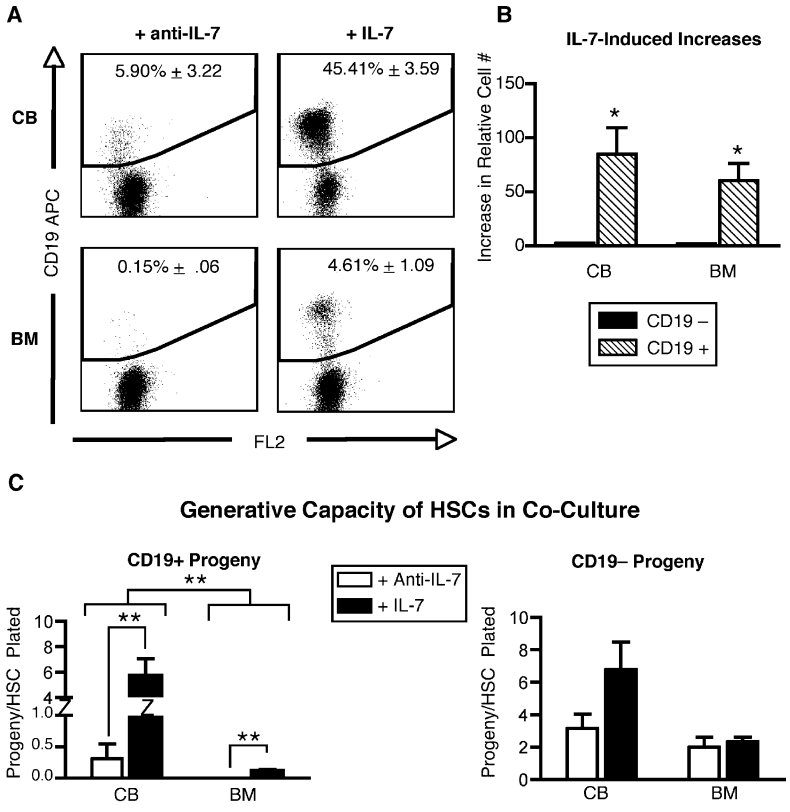


Figure 3 (*Figure 6*). B cell production from adult BM HSCs is profoundly dependent on IL-7. Human-only cultures were initiated with FACS-sorted Lin–CD34+ HSCs from CB or BM under control (3, F) culture conditions (see Figure 1) supplemented with exogenous human IL-7 or with neutralizing anti-human IL-7 antibodies as indicated. At 3 weeks culture progeny were harvested, counted, and stained for flow cytometry to detect expression of the B lineage marker, CD19. (A) Plots shown are gated on lymphocyte light scatter (gates not shown) and display equal numbers of cells. CD19+ B lineage cells, as a percentage of the total cells in living cell light scatter (mean ± SEM) for 6 independent experiments are given. (B) Relative numbers of CD19+ and CD19– culture progeny at three weeks were determined as described in "Materials and Methods". Data graphed are the increases in relative numbers of CD19+ and CD19– cells in cultures supplemented with exogenous IL-7 as compared to cultures supplemented with anti-IL-7 neutralizing antibodies (* $p < .05$, two-tailed paired t test). (C) Shown are CD19+ and CD19– generative capacities of CB and adult BM (donor age 20-30) HSCs under indicated conditions. Generative capacity was determined by dividing the absolute numbers of CD19+ or CD19– progeny generated at 3 weeks by the number of Lin–CD34+ HSCs plated at culture initiation. Data shown in panel C are the means ± SEM of 6 independent experiments (** $p < .01$, two-tailed Mann-Whitney test).

Flt3 Ligand, but not TSLP is Required for the IL-7-Independent Production of B Lineage Cells from CB HSCs

Studies of mouse B cell development suggest that TSLP, a cytokine that signals through the IL-7Rα and/or Flt3 ligand support the production of fetal/neonatal B cells in the absence of IL-7 (Jensen et al., 2008; Vosshenrich et al., 2003). Therefore we examined the extent to which the IL-7-independent production of B lineage cells from CB HSCs in our human-only cultures (Figure 3A, left panel) is dependent on Flt3 ligand and TSLP. The production of CD19+ cells from CB HSCs in human-only cultures supplemented with Flt-ligand and anti-IL-7 was compared to that observed with neutralizing anti-Flt-ligand, neutralizing anti-TSLP, or both. As shown in Figure 4A, the addition of anti-TSLP antibodies had no effect on IL-7 independent production of B lineage cells, while CD19+ B lineage cells were essentially absent in cultures that contained anti-Flt3 ligand. These data show that Flt3 ligand, but not TSLP, is required for the IL-7-independent production of B lineage cells from CB HSCs that we observed in human-only cultures.

B Lineage Cells Produced in the Absence of IL-7 are Capable of Subsequent IL-7 Responses

IL-7 has been reported to increase the expression of CD19 on fetally-derived human B cell precursors (Wolf, Weng, Stieglbauer, Shah, & LeBien, 1993) and in the presence of IL-7, IL-7Rα+ B cell precursors are larger in size than B cell precursors that lack the IL-7Rα (Johnson et al., 2005). Consistent with this report, flow cytometry analysis showed that the B lineage cells generated from CB and BM HSCs in human-only

cultures expressed high levels of CD19 in the presence of IL-7, while cultures supplemented with anti-IL-7 neutralizing antibodies expressed much lower levels of CD19 (Fig 3A). Human-only cultures supplemented with IL-7 also showed higher frequencies of B cells that were larger (as indicated by increased forward scatter (FSC) in flow cytometry analysis) as compared to those produced in cultures with anti-IL-7 neutralizing anti-bodies (see below).

To determine if the B lineage cells generated in the absence of IL-7 were capable of responding to IL-7 by upregulating CD19 and exhibiting increased cells size characteristic of cycling cells, we switched the culture progeny produced from CB HSCs in human-only cultures supplemented with anti-IL-7 neutralizing antibodies to human stromal cell cultures with IL-7 (anti-IL-7 to IL-7 switch cultures). After 3 days, anti-IL-7 to IL-7 switch cultures were compared to cultures that had been switched to human stroma and anti-IL-7 (anti-IL-7 to anti-IL-7 switch cultures) or to human-only cultures that had been both initiated with and switched to IL-7 supplementation (IL-7 to IL-7 switch cultures). The percentage of cells expressing high levels of CD19 ($CD19^{HI}$) was increased in anti-IL-7 to IL-7 switch cultures (Figure 4B, middle panel), as compared to anti-IL-7 to anti-IL-7 switch cultures (Fig 4B, top panel), however, the percentages were not as high as those observed among B lineage cells that were produced under continuous exposure to IL-7 (Fig 4B, bottom panel, and Fig 4D). Cultures with B cell precursors that were never exposed to IL-7 (anti-IL-7 to anti-IL-7 switch cultures) showed a low frequency of cells with the high levels of FSC characteristic of cycling cells. The switch from anti-IL-7 to IL-7 resulted in a significant increase in the frequency of cells that were

FSC^{HI} and an FSC histogram profile that was similar to that seen for IL-7 to IL-7 switch cultures.

IL-7R-signaling, partially via upregulation of EBF, has been reported to be a regulator of the transcription factor network that controls B cell development in the mouse (Singh et al., 2007). Expression of EBF in the absence of IL-7 in fetal and neonatal mice allows IL-7-indepdent B cell differentiation at early points in ontogeny (Kikuchi & Kondo, 2006). Therefore we were interested in examining expression of EBF and its downstream target PAX-5 (O'Riordan & Grosschedl, 1999) in the early B lineage cells generated in our human-only cultures. CD19+ cells generated in switch cultures were FACS-sorted for RT-PCR. Real time RT-PCR showed that B lineage cells generated in all three switch culture conditions expressed mRNA for CD19, PAX-5, and EBF, (Supplemental Data) however the small number of cells generated in cultures initiated with anti-IL-7 precluded a quantitative comparison of mRNA levels. Taken together these data provide evidence that the $CD19^{LO}$ cells generated from CB HSCs in the absence of IL-7 are B lineage cells and have the capacity to respond to subsequent IL-7 stimulation.

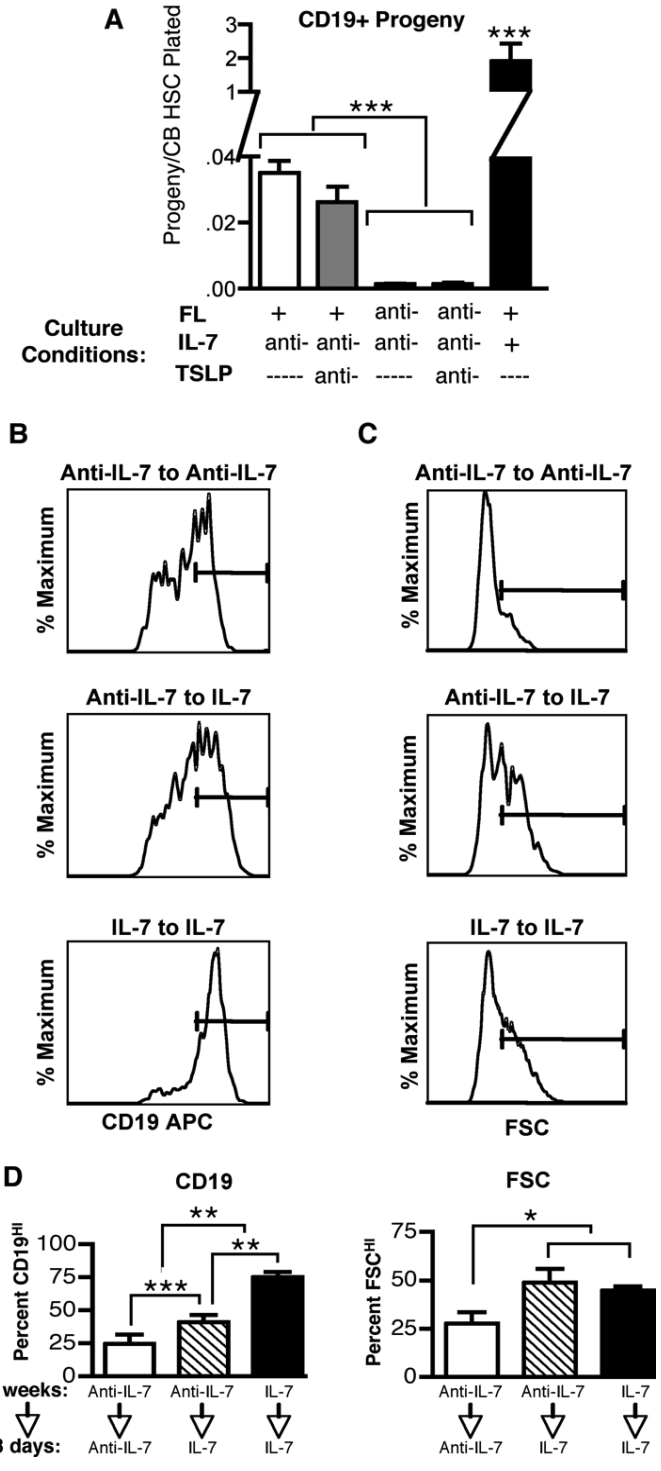

A
CD19+ Progeny
Progeny/CB HSC Plated
3
2
1
.04
.02
.00
***
***
Culture Conditions:
FL + + anti- anti- +
IL-7 anti- anti- anti- anti- +
TSLP ----- anti- ----- anti- ----
B
C
Anti-IL-7 to Anti-IL-7
Anti-IL-7 to IL-7
IL-7 to IL-7
% Maximum
CD19 APC
FSC
D
CD19
FSC
Percent CD19HI
Percent FSCHI
100
75
50
25
0
**
**
***
*
3 weeks: Anti-IL-7 Anti-IL-7 IL-7
3 days: Anti-IL-7 IL-7 IL-7

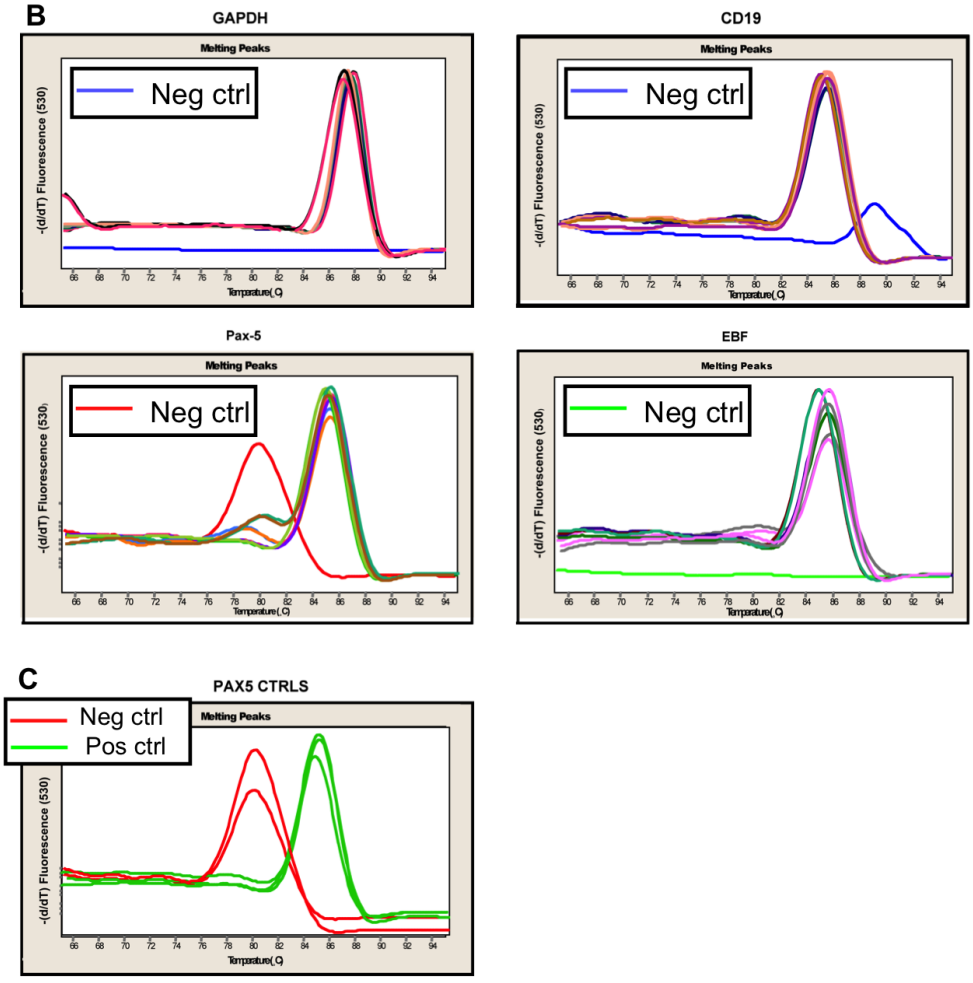
Figure 4 ***(Figure 7).*** IL-7-independent production of human B lineage cells is dependent on Flt3 ligand and produces CD19+ B lineage cells that can respond to IL-7. (A) Human-only cultures were initiated with FACS-sorted Lin–CD34+ HSCs from CB and grown with indicated cytokines (+), with cytokine-specific neutralizing antibodies (anti-) or without the indicated cytokine or its neutralizing antibody (-----). Corresponding isotype control antibodies were added to all wells in which a specific neutralizing antibody was not used but are not indicated in the graph—see "Materials and Methods". At 3 weeks, culture progeny were harvested, counted, stained for CD19 expression and the CD19+ generative capacity was determined as described in Figure 3 legend. Data in panel A are the means $\pm$ SEM of 2 independent experiments with 3 replicates per experiment, (ANOVA, *** $p < .001$ for Bonferroni multiple comparisons post test) (B, C) Human-only cultures were initiated with FACS-sorted Lin–CD34+ HSCs from CB under control (3, F) culture conditions and supplemented with exogenous human IL-7 or with neutralizing anti-human IL-7 antibodies. After 3 weeks, culture progeny were harvested and then switched to a second human-only culture for 3 days under indicated culture conditions (IL-7 to IL-7, anti-IL-7 to anti-IL-7, or anti-IL-7 to IL-7). After 3 days, switch cultures were harvested, stained to detect CD19, and the level of CD19 expression (panel B) and the size (as indicated by FSC, panel C) of gated CD19+ B lineage cells was determined by flow cytometry analysis. Histograms depicting $CD19^{HI}$ and FSC^{HI} gates from a representative experiment are shown. D) Graphed are the percentages of total CD19+ cells that fall within $CD19^{HI}$ and FSC^{HI} gates (mean $\pm$ SEM for 6 independent experiments; * = $p > .05$, ** = $p > .01$, *** = $p > .001$, two-tailed paired t test

A

CT Values For Real Time PCR

Sample	Rep.	CT Value			
		GAPDH (housekeeping)	CD19	EBF	PAX-5
Pre-B ALL (pos ctrl)	#1	19.62	29.67	29.66	29.71
	#2	19.57	29.45	29.80	29.27
B cells generated in IL-7 to IL-7	#1	25.45	26.95	28.86	30.31
	#2	25.70	26.87	28.59	30.00
B cells generated in anti-IL-7 to anti-IL-7	#1	25.22	27.67	26.50	29.15
	#2	25.47	27.63	26.49	29.23
B cells generated in anti-IL-7 to IL-7	#1	23.94	27.58	27.06	29.78
	#2	23.52	27.64	27.54	29.60

B



C

Supplemental Data *(Figure 8)*. Expression of B cell-specific transcripts by CD19+ cells produced in human-only cultures**.** Culture progeny generated in switch cultures as described in Figure 4B (IL-7 to IL-7, anti-IL-7 to anti-IL-7, or anti-IL-7 to IL-7) were harvested, stained for flow cytometry, and CD19+ B lineage cells were FACs-sorted. cDNA was prepared from sorted cells and from primary pre-B ALL cells (pos. ctrl.). Real time PCR to detect GAPDH (housekeeping gene), CD19, Pax-5, and EBF was performed as described in "Materials and Methods." (A) Shown are the CT values for 2 replicates of real time PCR amplification of indicated cDNAs from Pre-B ALL cells and from B lineage cells generated in indicated switch cultures. (B) Melting curves for final round (cycle 50) PCR products generated using primers to detect GAPDH,, CD19, PAX-5, EBF as indicated above each plot. Each plot shows the superimposed melting curves (multiple colors) for PCR products from Pre-B ALL (2 replicates), for FACS-sorted B lineage cells generated in each of the switch culture conditions (2 replicates each), and for a negative control that contained water and no cDNA (1 replicate, color as indicated). Note that all melting curves peak at or near 86 to 88 degrees C as expected for these primers. (C) Shown are additional replicates from independent experiments of positive (Pre-B ALL) and negative control (water) samples using the PAX-5 primer pair which consistently generated a primer artifact peak. The melting curve of the primer artifact showed a consistent peak (~80 degrees C) that was easily distinguishable from the melting curve peak observed in the positive control (~86 degree C) and in CD19+ cells generated in all culture conditions as shown in the lower left plot in panel B.

IL-7 Directly Targets B Cell Precursors in Human-Only Cultures

To identify the cellular targets of IL-7 we used flow cytometry to examine IL-7Rα expression among cells present during *in vitro* B lymphopoiesis (Figure 5A). IL-7Rα was not detectable on primary human BM stromal cells used in co-cultures (Figure 5B). Approximately 35% of CD19+ B cell precursors produced in co-culture expressed the IL-7Rα, regardless of whether they arose from HSCs in CB or adult BM or whether IL-7 was present (Figure 5C and Figure 5D, right panel). We gated CD19– cells into a CD34– subset that included more mature non B-lineage cells and into a CD34+ subset that included HSCs and progenitors present in the 3-week human-only cultures. Of the CD19–CD34– non B lineage cells generated from CB and BM HSCs in human-only cultures, 3-12 % expressed the IL-7Rα (Figure 5C, left panel) while 4-14% of the of the CD19–CD34+ HSC and progenitor subset was IL-7Rα+ (Figure 5C, middle panel). The percentage of cells in each subset that expressed IL-7Rα was not significantly different in cultures initiated with CB and BM HSCs, and the presence or absence of IL-7 activity had no significant impact on the percentage of cells expressing the IL-7Rα in any of the subsets (Figure 5D). These data show that CD19+ B cell precursors represent the major IL-7Rα+ population in human-only cultures and are therefore a potential IL-7 target during *in vitro* B cell development.

To determine if IL-7 acts directly on B cell precursors we used flow cytometry to assess phosphorylation of STAT5, an event downstream of IL-7R signaling (Lin et al., 1995). CD19+ B cell precursors were isolated from human-only cultures and assessed by flow cytometry for purity (based on CD19 expression) and for IL-7Rα expression (Figure 5E, left panel) and then for STAT5 phosphorylation following incubation with or without

IL-7 (Figure 5E, right panel). Phosphorylated STAT5 was detected in B cell precursors incubated with IL-7, but not in B cell precursors cultured without IL-7 (Figure 5E, right panel). The percentage of B cell precursors positive for phosphorylated STAT5 following incubation with IL-7 was virtually identical to the percentage of cells expressing IL-7Rα (Figure 5E, compare left and right panels). These data provide evidence that B cell precursors are directly targeted by IL-7 in human-only cultures.

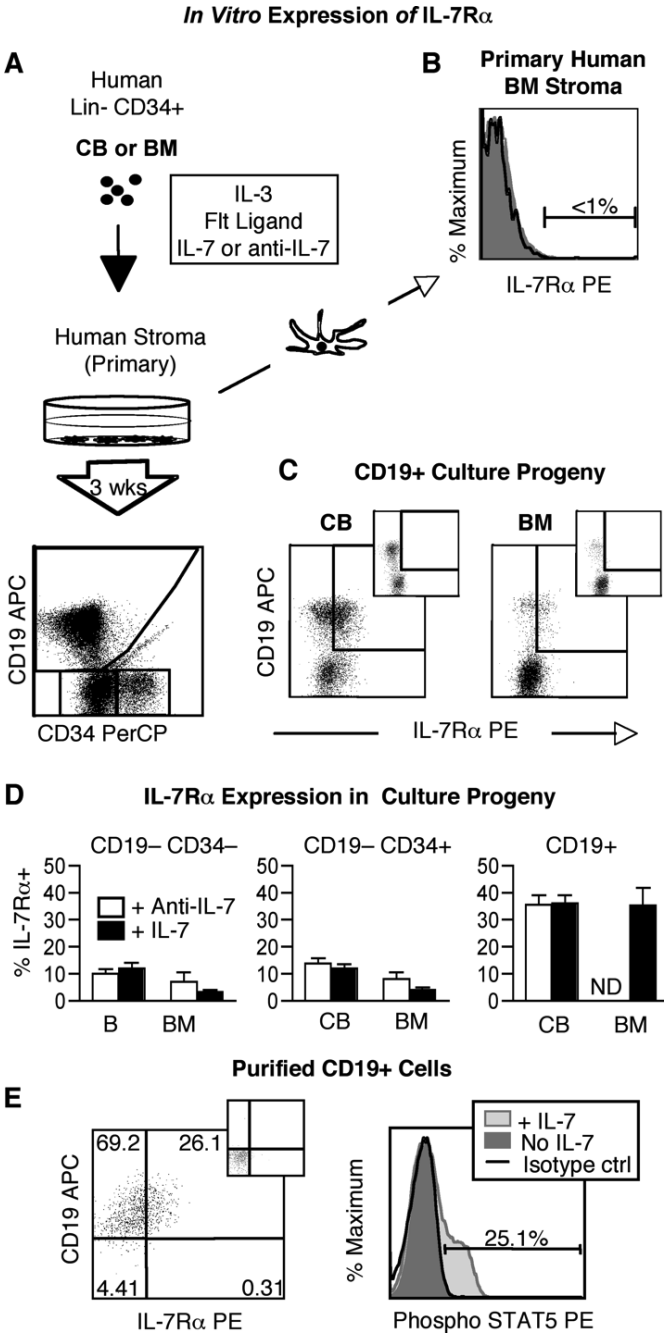

In Vitro Expression *of* IL-7Rα
A
Human
Lin- CD34+
CB or BM
IL-3
Flt Ligand
IL-7 or anti-IL-7
Human Stroma
(Primary)
3 wks
CD19 APC
CD34 PerCP
B
Primary Human
BM Stroma
% Maximum
<1%
IL-7Rα PE
C
CD19+ Culture Progeny
CB
BM
CD19 APC
IL-7Rα PE
D
IL-7Rα Expression in Culture Progeny
CD19– CD34–
CD19– CD34+
CD19+
% IL-7Rα+
+ Anti-IL-7
+ IL-7
ND
B
BM
CB
BM
CB
BM
Purified CD19+ Cells
E
69.2
26.1
4.41
0.31
CD19 APC
IL-7Rα PE
+ IL-7
No IL-7
Isotype ctrl
% Maximum
25.1%
Phospho STAT5 PE

Figure 5 ***(Figure 9).*** IL-7 directly targets B lineage cells**.** (A) Human-only cultures were initiated with FACS-sorted Lin–CD34+ HSCs from CB or BM under control conditions (3, F) and supplemented with anti-IL-7 neutralizing antibodies or exogenous human IL-7. At 3 weeks culture progeny were harvested and stained for flow cytometry to detect co-expression of CD19, IL-7Rα, and CD34. Cells were gated into CD19+, CD19–CD34– and CD19–CD34– subsets as indicated. Plot shown is from a CB culture with IL-7. (B) Flow cytometry analysis of IL-7Rα expression among primary human BM stromal cells used in human-only cultures. Data is representative of flow cytometry analysis of stroma from three different donors. (C) Flow cytometry analysis showing IL-7Rα vs. CD19 expression for human-only cultures of CB or BM HSCs supplemented with IL-7. Insets show isotype antibody control vs. CD19 staining. Plots are gated on lymphocyte light scatter (gates not shown) and display equal numbers of cells. (D) The percentages of cells in indicated subsets (gated as shown in panel A) that express IL-7Rα are graphed. Data are from 3-week, human-only cultures of CB and BM HSCs supplemented with anti-IL-7 or IL-7 as indicated. Percentages are the means $\pm$ SEM of 5 (CB) or 3 (BM) independent experiments with 8 and 6 total replicates, respectively. (E) Human-only cultures were initiated with CD34+ HSCs from CB under control conditions supplemented with exogenous human IL-7. At 3 weeks, culture progeny were harvested and CD19+ B lineage cells were isolated by magnetic separation. Aliquots of purified B cell precursors were co-stained for expression of CD19 and IL-7Rα or with isotype-matched control antibodies (left panel). Plots shown are gated on events falling within lymphocyte light scatter. Quadrants were set based on isotype control staining (inset) and percentages of cells in each quadrant are shown. The remaining purified precursors, after a minimum of 3 hours removal from IL-7, were incubated with or without exogenous human IL-7 for 1 hour and then stained for flow cytometry with an antibody to detect phosphorylated STAT5 or an isotype-matched control antibody as describe in "Materials and Methods". The percentage of cells positive for phospho-STAT5 staining, based on isotype control staining (overlay) are shown (right panel). Data shown is representative of two independent experiments.

IL-7-Induced Increases in B Cell Production are Mediated Through Increased Proliferation of B Cell Precursors

To determine if increased cell survival is involved in the IL-7-induced increases observed in our co-cultures, we examined the effect of IL-7 on apoptosis among culture-generated human B cell precursors. Human-only cultures initiated with CB HSCs were harvested at 3 weeks and CD19+ cells (either enriched or unseparated from CD19– cells) were placed in co-culture with fresh human stroma and supplemented with anti-IL-7 neutralizing antibodies, control antibodies, or IL-7. Cells were harvested at daily time points and flow cytometric analysis of staining with the Annexin V apoptosis indicator was used to determine the percentages of CD19+ B cell precursors undergoing apoptosis. IL-7 had no effect on the percentages of CD19+ that were Annexin V+, whether they were in co-culture alone or together with CD19– cells (data not shown). Similarly IL-7 had no effect on apoptosis among *in vivo*-generated B cell precursors (CD19+K/L–) that were FACS sorted from adult BM and assessed in identical assays (data not shown). These results suggest that IL-7 does not mediate protection from apoptosis and that increased survival of CD19+ B cell precursors is unlikely to be a major mechanism by which IL-7 increases human B cell production.

To determine if increased proliferation is a mechanism for IL-7-induced increases in human B cell production, we used flow cytometry to examine expression of the Ki-67 proliferation antigen in cells harvested from human-only cultures supplemented with IL-7 or with anti-IL-7 neutralizing antibodies (Figure 6A). IL-7 did not change the percentage of CD19– cells that were Ki-67+ in cultures of CB or BM HSCs (Figure 6A, B). In contrast, in

CB cultures the percentage of CD19+ cells expressing the Ki-67 was significantly higher with IL-7 supplementation (Figure 6B). Cultures of BM HSCs generated too few B cell precursors to be assessed for Ki-67 when anti-IL-7 antibodies were added. However, the percentage of B cell precursors expressing Ki-67 was the same in CB and BM cultures with IL-7 (Figure 6 A, B).

To verify that the increases in proliferation observed in cultures supplemented with IL-7 were due to the direct action of IL-7 on B cell precursors, we compared Ki-67 expression (Figure 6C) and size (indicated by FSC, Figure 6D) in IL-7Rα+ and IL-7Rα– B cell precursors generated in cultures with IL-7. For both CB and BM cultures, average cell size and the percentages of cells expressing Ki-67 was significantly higher for IL-7Rα+ than for IL-7Rα– B cell precursors. These data provide further evidence that IL-7 increases the production of human B lineage cells through increased proliferation due to direct targeting of B cell precursors by IL-7.

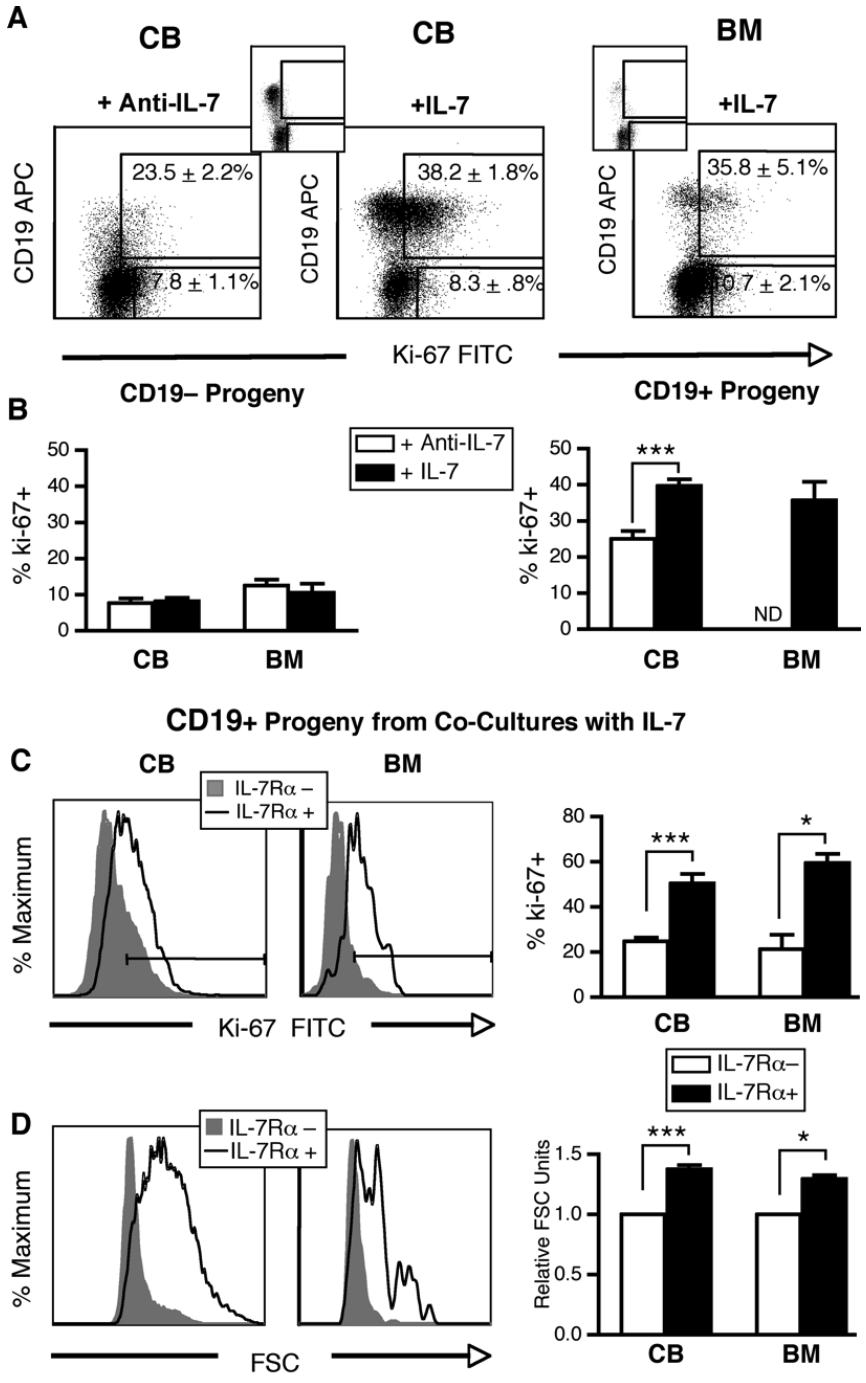

A
CB
+ Anti-IL-7
CB
+IL-7
BM
+IL-7
CD19 APC
23.5 ± 2.2%
7.8 ± 1.1%
38.2 ± 1.8%
8.3 ± .8%
35.8 ± 5.1%
0.7 ± 2.1%
Ki-67 FITC
CD19– Progeny
CD19+ Progeny
B
+ Anti-IL-7
+ IL-7
% ki-67+
***
ND
CB
BM
CD19+ Progeny from Co-Cultures with IL-7
C
IL-7Rα –
IL-7Rα +
% Maximum
Ki-67 FITC
*
D
FSC
IL-7Rα–
IL-7Rα+
Relative FSC Units

Figure 6 ***(Figure 10).*** IL-7-induced increases in the production of B lineage cells are mediated through increased proliferation. Human-only cultures were initiated with FACS-sorted Lin–CD34+ HSCs from CB or BM under control conditions (3, F) and supplemented with anti-human IL-7 neutralizing antibodies or with exogenous human IL-7. At 3 weeks, culture progeny were harvested, counted, and stained for surface expression of IL-7Rα and/or the B lineage marker, CD19. Surface-stained cells were fixed, permeabilized and stained with an antibody specific to the Ki-67 proliferation antigen or an isotype-matched control antibody. (A) The plots shown are gated on living cell light scatter and display equal numbers of cells. Gates shown were set based on isotype control staining among CD19+ and CD19– cells (insets). (B) Graphed are the percentages of CD19– (left panel) and CD19+ cells (right panel) positive for the Ki-67 proliferation antigen (mean ± SEM) for n=5 (BM) and n=8 (CB) experiments. In co-cultures with BM HSCs and with anti-IL-7 neutralizing antibodies, too few B cells were generated to assess Ki-67 expression (ND, not done). (C, D) At 3 weeks co-cultures supplemented with IL-7 were harvested, counted, and stained for surface expression of IL-7Rα and/or the B lineage marker, CD19. Surface stained cells were fixed, permeabilized and stained with an antibody specific to the Ki-67 proliferation antigen or an isotype-matched control antibody. (C) The expression of Ki-67+ among gated CD19+IL-7R– and CD19+IL-7R+ cells (gates not shown) are plotted in histograms (left panels) and graphed (right panel). (D) The forward scatter (FSC, an indicator of cell size) of gated (gates not shown) CD19+IL-7R– (shaded) and CD19+IL-7R+ (overlay) cells are plotted in histograms (left panels). The relative forward scatter of CD19+IL-7R+ cells as compared to CD19+IL-7R– cells is graphed in the panel at right. Data shown in panels C and D are the mean ± SEM for 5 (CB) or 3 (BM) independent experiments. (* $p < .05$; *** $p < .001$, paired t test).

Expression of the IL-7Rα in Context of Human B Cell Development In Vivo

To determine whether the IL-7 targets that we identified in our human-only cultures are reflective of human B cell development *in vivo*, we examined the expression of IL-7Rα in early B lineage cells that were generated *in vivo* in adult human bone marrow. The average frequency of IL-7Rα+ cells among *in vivo*-generated B cell precursors (CD19+K/L–) in adult human BM (Figure 7A, Table 1) was similar (32%) to that observed for B cell precursors generated *in vitro (35%)* from HSCs in CB and adult BM in human-only cultures (Figure 5C, D). The percentages of *in vivo*-generated CD19+K/L– B cell precursors that we assessed in human subjects (Table 1) exhibited a broad range in the percentage of cells that were IL-7Rα+ (17%-56%) and showed no obvious correlation with age, gender, or race, suggesting that there is substantial natural variation among adult individuals. Thus, the IL-7Rα+ population of B cell precursors observed in human-only cultures is reflective of *in vivo* B cell development in adult human BM and suggests a physiological role for IL-7 in human B cell development.

A substantial portion of the B cell precursors generated in human-only cultures initiated with CB and BM HSCs were IL-7Rα–. This was also the case for CD19+K/L– cells in adult BM. To determine if the expression of IL-7Rα correlated with the expression of developmental markers during *in vivo* B cell differentiation we examined IL-7Rα expression among developmental subsets of CD19+ K/L– B cell precursors identified based on expression of CD34+ or cμ (Figure 7B). The percentages of IL-7Rα+ cells were similar for all developmental subsets (cμ+, cμ–, CD34–, and CD34+) of CD19+K/L– cells. Thus, the expression of IL-7Rα among B cell precursors produced *in*

vivo does not appear to correlate with particular stages of development that can be identified based on expression of CD34 or with successful rearrangement of the Ig heavy chain as indicated by the expression of cμ (Figure 7B).

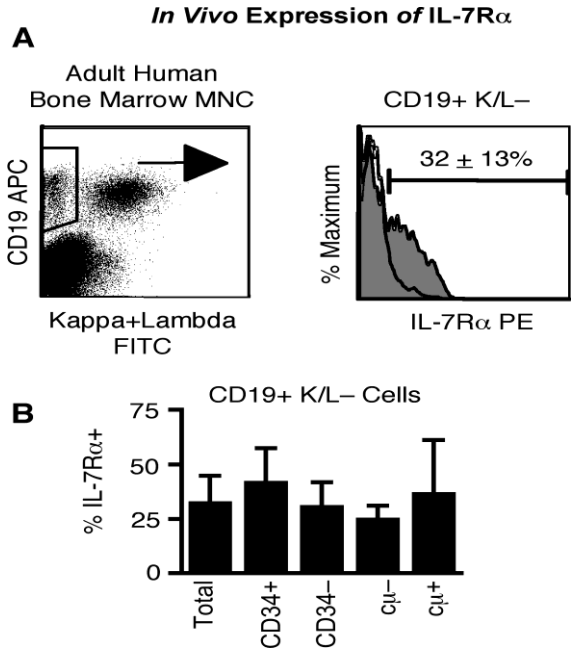


Figure 7 ***(Figure 11).*** The distribution of IL-7Rα+ cells in adult human B cell precursor populations produced *in vivo*. (A) Primary adult human BM mononuclear cells, either fresh or frozen and then thawed, were stained for flow cytometry to assess co-expression of CD19, the kappa and lambda light chains, IL-7Rα, and either CD34 or the cytoplasmic mu (cμ) Ig heavy chain. CD19+ B lineage cells falling within lymphocyte light scatter (gates not shown) that were negative for the kappa and lambda light chains (CD19+K/L–) were gated as shown (left panel) and the percentage of cells expressing of IL-7Rα was determined (right panel, n=10). (B) CD19+K/L– cells were gated into a CD34+ and CD34– subsets (n=7) or into cμ– and cμ+ subsets (n=2). Percentages of total CD19+K/L– or of the cells in each subset that were IL-7Rα+ are graphed. Percentages are the mean ± SD from the indicated (n) numbers of donors

.

Table 1. Expression of IL-7Rα by in vivo-generated CD19+ K/L– B cell precursors

		Donor Information		
Donor	% IL-7Rα+	Age	Gender	Race
1	27.9	------	------	----------
2	24.1	------	------	----------
3	38.6	49	Female	Caucasian
4	56.5	25	Male	Caucasian
5	26.4	25	Male	Caucasian
6	50.6	22	Female	African American
7	23.2	25	Male	Caucasian
8	22.6	22	Male	African American
9	17.4	20	Male	African American
10	32.8	19	Female	Caucasian
Avg	32.0			

Precursor Populations Targeted by IL-7-Induced Expansion in Human-Only Cultures

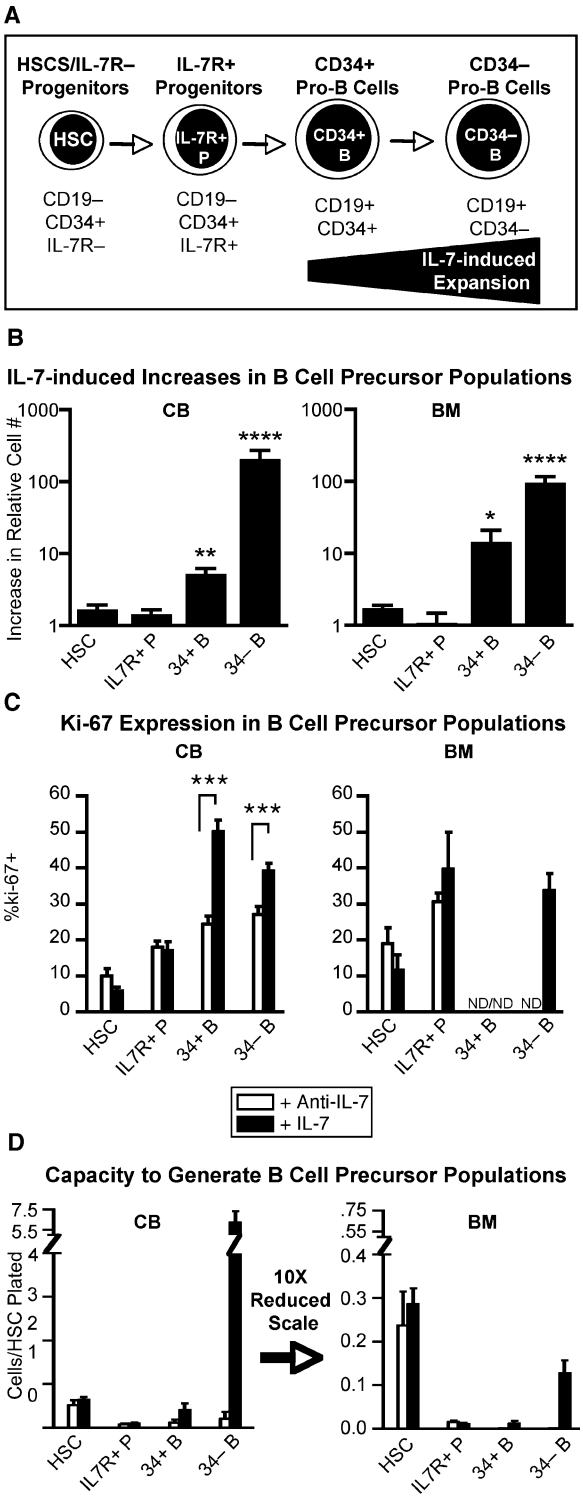
In the mouse, IL-7R signaling has been associated with distinct differentiation and proliferation signals. Thus, while the expression of IL-7Rα may not correlate with markers of development in CD19+ B cell precursors, it is possible that IL-7 may directly target distinct developmental populations of IL-7Rα+ B lineage precursors for proliferation or for differentiation. To identify the developmental targets of IL-7-induced proliferation we examined IL-7-induced increases and expression of the Ki-67 proliferation antigen in four subsets that include developmentally sequential populations of B cell precursors. FACS analysis of the cells present at 3 weeks in human-only cultures was used to identify four populations (Figure 8A): 1) The most primitive IL-7R– HSCs and progenitors (HSC) through which IL-7 effects might be indirectly mediated, 2) IL-7Rα+ progenitors (IL-7R+ P) that would include the earliest CD19– lymphoid-

committed progenitors that express the IL-7Rα (Hao et al., 2001) (Ryan et al., 1997) ; 3) CD34+ pro-B cells (CD34+ B) and 4) CD34– pro-B cells (CD34– B).

Human-only cultures initiated with Lin–CD34+ cells from CB and BM and supplemented with IL-7 showed significantly increased numbers of cells in the CD34+ B and CD34– B subsets at three weeks when compared to cultures with anti-IL-7. The relative numbers of CD34+ B cells increased by only 5 to 14-fold (CB and BM cultures respectively) when IL-7 was present while CD34– B cells went up by 194 to 90-fold (CB and BM cultures respectively). In contrast, the relative numbers of HSCs, and IL-7R+ P were not significantly increased in cultures with IL-7 (Figure 8B). Consistent with the IL-7 effects that we observed on cell numbers, CB cultures supplemented with IL-7 showed significantly higher percentages of Ki-67+ cells among the CD34+ B and CD34– B, but not among the HSC or IL-7R+ P subsets (Figure 8C). The percentage of Ki-67+ B cells among CD34– B cells in BM cultures was similar to that observed in CB cultures, however there were too few CD19+ cells to assess in Ki-67 expression in any BM cultures with anti-IL-7 or in the CD34+ B cells in BM cultures where IL-7 was present (Figure 8C, right panel). Taken together these data provide evidence that IL-7 targets both CD34+ and CD34– pro-B cells for increased proliferation. In contrast, in our culture model we were unable to detect any significant changes in proliferation due to IL-7 effects among developmental subsets that would include CD19– B cell precursors.

In Figure 3 we showed that IL-7 is particularly critical for the production of B lineage cells from adult BM because the capacity of adult BM to generate B lineage cells is greatly reduced as compared to CB. To get a better understanding of the dynamics and potential mechanisms that might be responsible for the changes in B cell development

that occur during ontogeny, we compared the ability of CB and adult BM to generate the developmental subsets observed *in vitro* in human-only cultures. In cultures with adult BM the HSC generative capacity was about half, and the IL-7R+ P generative capacity about 10 fold less than that observed for CB cultures (Figure 8D, Note the 10X scale reduction in the right panel). In cultures with IL-7, the adult BM generative capacities for cells in the CD34+ B and CD34– B subsets were ~40-fold and ~50-fold less, respectively than those observed for CB. While BM cultures generated detectable CD19– subsets, and the capacity to generate these cells was unaffected by IL-7, B lineage precursors that reached the CD19+ stage of development were essentially absent in cultures with anti-IL-7. These data suggest that there is a progressive loss of generative capacity during the course of B lineage differentiation in adult BM, as compared to CB and that as a consequence of this reduced B lineage generative capacity, IL-7 is more critical for B cell development from HSCs in adult B and that as a consequence of this reduced B lineage generative capacity, IL-7 is more critical for B cell development from HSCs in adult BM.


A
HSCS/IL-7R–
Progenitors
IL-7R+
Progenitors
CD34+
Pro-B Cells
CD34–
Pro-B Cells
HSC
IL-7R+ P
CD34+ B
CD34– B
CD19–
CD34+
IL-7R–
CD19–
CD34+
IL-7R+
CD19+
CD34+
CD19+
CD34–
IL-7-induced Expansion
B
IL-7-induced Increases in B Cell Precursor Populations
CB
BM
Increase in Relative Cell #
1000
100
10
1
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HSC
IL7R+ P
34+ B
34– B
C
Ki-67 Expression in B Cell Precursor Populations
CB
BM
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60
50
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30
20
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ND/ND ND
HSC
IL7R+ P
34+ B
34– B
+ Anti-IL-7
+ IL-7
D
Capacity to Generate B Cell Precursor Populations
CB
BM
Cells/HSC Plated
7.5
5.5
4
3
2
1
0
.75
.55
0.4
0.3
0.2
0.1
0.0
10X Reduced Scale
HSC
IL7R+ P
34+ B
34– B

Figure 8 (*Figure 12).* Precursor populations targeted by IL-7-induced expansion. A) Flow cytometry analysis of human-only cultures was used to identify four subsets of cells that include developmentally sequential precursors to mature B cells: HSCs and IL-7R– Progenitors (HSC), IL-7R+ progenitors (IL-7R+ P), CD34+ pro-B cells (CD34+B), and CD34– pro-B cells (CD34–B). Surface immunophenotypes used to identify each population in human-only cultures are indicated at the bottom of the panel. Human-only cultures initiated with Lin–CD34+ cells from CB and BM and supplemented with anti-IL-7 or with exogenous IL-7 were harvested at 3 weeks, and counted and stained for flow cytometry to detect surface CD19, CD34, IL-7Rα and an additional surface or intracellular marker. Cells in each subset were gated as shown in Figure 5A, C and based on isotype control staining. (B) Relative numbers of cells in each subset were determined as described in "Materials and Methods". Data graphed are the increases in relative numbers of cells in each subset in cultures supplemented with exogenous IL-7 as compared to cultures supplemented with anti-IL-7 neutralizing antibodies. Data shown are from 3 independent experiments and 6 total replicates. (C) The percentage of cells in each gated subset that are positive for expression of the Ki-67 proliferation antigen are graphed. Data shown are the means $\pm$ SEM obtained from 3-7 independent experiments (D) The ability of CB and BM Lin–CD34+ cells to generate cells in each subset under indicated conditions was determined by dividing the absolute numbers of progeny in each subset, generated at 3 weeks, by the number of Lin–CD34+ HSCs plated at culture initiation. Data shown were obtained from 3 independent experiments and 6 total replicates (HSC, IL-7R+ P) or from 6 independent experiments (CD34+ B and CD34– B). (* $p < .05$, ** $p <. 01$, *** $p < .001$, **** $p < .0001$, two-tailed paired t test).

Discussion

Defining the role of IL-7 in human B cell development has been difficult for several reasons. First, most early studies of IL-7 and human B cell development used fetal hematopoietic sources. This is particularly relevant as subsequent murine studies revealed that IL-7-dependent B lymphopoiesis is linked with ontogeny–only in the adult mouse does B cell development become dependent on IL-7. Thus, differences in fetal and adult B cell development have been interpreted as fundamental differences in mouse and human B lymphopoiesis. Second, studies of humans that are defective in components of the IL-7R signaling pathway are hampered by limitations in the number, type, and quantity of specimens available for assessment of IL-7 function. Third, studies of nonfetal B lymphopoiesis were hampered by the lack of an *in vitro* model of human B cell development that reconstitutes the human BM stroma microenvironment without the confounding factors introduced by murine stromal cell lines. Here we describe a novel human-only model of *in vitro* B cell development based on co-culturing HSCs on primary human BM stroma. We used this model to examine the role of IL-7 in human B lymphopoiesis from HSCs in adult BM and from the HSCs in CB that give rise to B cells during the neonatal period.

Using the human-only culture model we show that IL-7 increases the production of human B cell precursors from HSCs in CB and adult BM by more than 60-fold as compared to cultures with anti-IL-7 neutralizing antibodies (Figure 3). IL-7-induced increases were dose-dependent (Figure 1E), specific to CD19+ cells (Figure 1E and 5), and mediated through increased proliferation (Figure 6), but not increased cell survival (data not shown). Expression of the IL-7Rα and the induction of STAT5 phosphorylation

following stimulation with IL-7 (Figure 5) indicate that IL-7 acts directly on CD19+cμ– cells present in 3-week human-only cultures (Figure 1) to expand both the CD34+ and CD34– pro-B cell compartments (Figure 8).

The IL-7-induced increases in B cell precursors that we observed in human-only cultures were not detectable in hybrid cultures where human HSCs were co-cultured on murine S17 or MS-5 stromal cell lines (Figure 1, 2). The absence of IL-7 effects that we and others (Rawlings et al., 1995) (Nishihara et al., 1998) have observed in hybrid cultures is likely due to high IL-7 production by MS-5 and S17 stromal cell lines (>40-fold that of primary human or murine BM stroma [Figure 2]) since human B cell production was reduced up to 17 fold (Figure 2A) by high levels of anti-mouse IL-7 neutralizing antibodies. Thus, the human-only model revealed IL-7 effects on human B cell development that were not easily detectable in hybrid human-mouse co-cultures.

A critical role for IL-7 in adult B cell development has been demonstrated *in vivo* in the mouse model (von Freeden-Jeffry et al., 1995)-(DiSanto et al., 1995; Peschon et al., 1994). IL-7 expression by primary murine BM stroma (Figure 2) and the level of IL-7 that elicits *in vitro* B cell precursor responses (1 ng/ml) in the mouse (Medina & Kincade, 1994) are consistent with what we see during *in vitro* human B cell development (Figure 1, 2). The expression of IL-7Rα by B cell precursors (Figure 7) and of IL-7 by stromal cells (Figure 5) in primary human BM suggests that the IL-7 effects we observe *in vitro* are physiologically relevant for *in vivo* human B cell development. Indirect evidence for the *in vivo* role of IL-7 in human B cell development comes from patients with T cell lymphopenia in whom endogenous IL-7 is upregulated and expansion

of the immature B cell pool correlates with increased serum levels of IL-7 (Malaspina, Moir, Ho, et al., 2006), (Malaspina, Moir, Chaitt, et al., 2006).

Data obtained using our human-only culture model provide the first evidence that human B cell production from HSCs in adult BM is dependent on IL-7. Robust IL-7-independent B cell production has been reported in human fetal BM (J. A. Pribyl & LeBien, 1996). In the absence of IL-7, we observed that HSCS in CB, but not BM consistently give rise to a small population of $CD19^{LO}$ B lineage cells (Figure 3) that express EBF and PAX-5 at three weeks and differentiate to generate very small numbers of IgM+ B cells at 5 weeks (Figure 1). When switched to IL-7 at 3 weeks, $CD19^{LO}$ cells generated in the absence of IL-7 respond by upregulating CD19 and exhibiting increased cell size that is characteristic of cycling cells (Figure 4B-D). Studies of B cell development in the mouse model suggest that IL-7-independent expression of EBF allows early B lineage differentiation (but not expansion unless IL-7 is present) during the fetal and neonatal period. This results in what has been described as a "developmental switch" in stem cells with IL-7-independent B cell production occurring through the first two weeks of neonatal life, but not in the adult (Kikuchi & Kondo, 2006). Our data showing that human B cell development from HSCs in adult BM is IL-7-dependent, coupled with our characterization of B lineage cells generated from CB in the absence of IL-7, provide the first evidence that that such a "developmental switch" may also occur in human B lymphopoiesis.

We found that adult BM, as compared to CB, shows a reduction of *in vitro* generative capacity that is progressively more profound in developmentally sequential populations (Figure 3, 8). The ability of adult BM to generate HSCs, IL-7R+ progenitors,

and pro-B cells after 3 weeks in human-only cultures with IL-7 was reduced by half, 10- and 50-fold, respectively, of that observed for CB (Figure 8D). Indeed the CD19+ generative capacity of BM HSCs in the presence of IL-7 was similar to that of CB HSCs in the absence of IL-7 activity (Figure 3). The B lymphoid-specific decrease in generative capacity that we observed for human HSCs in adult BM, as compared to CB, is consistent with a selective loss of B lymphoid generative capacity observed in xenograft transplant models and in the clinical transplant setting (Arakawa-Hoyt et al., 1999; Inoue et al., 2003).

The decline in IL-7-dependent B lymphoid generative capacity that we have observed in adult versus fetal B cell development suggests, that a mechanism other than the "developmental switch" observed in the mouse model may contribute to the decline in IL-7-independent B cell production that occurs during the progression to adulthood. It is possible that IL-7-induced expansion of the pro-B compartment (absent in the IL-7-indepdent B cell production observed by Kikuchi et al.) is essential for B lymphopoiesis from adult HSCs because of their reduced generative capacity. Further studies will be needed to determine the extent to which the decline in B cell generative capacity contributes to the loss of IL-7-independent B lymphopoiesis in adult humans and mice and whether B cells arising from IL-7-dependent and IL-7-independent pathways in humans are functionally distinct.

In summary, data obtained using our human-only culture model provide the first evidence that B cell development from HSCs in adult BM is dependent on IL-7 and characterize the progressive decline in developmentally sequential populations of B cell precursors that correlates with B cell production that is the dependent on IL-7. These

studies have implications for immune reconstitution following stem cell transplant with CB versus BM in the clinical setting.

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CHAPTER THREE

A NOVEL XENOGRAFT MODEL TO STUDY THE ROLE OF TSLP-INDUCED CRLF2 SIGNALS IN NORMAL AND MALIGNANT HUMAN B LYMPHOPOIESIS

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Abstract

The cytokine, TSLP, stimulates *in vitro* proliferation of human fetal B cell precursors, however, its *in vivo* role during normal human B lymphopoiesis is unknown. Genetic alterations that cause overexpression of the TSLP receptor component, CRLF2, lead to B cell acute lymphoblastic leukemia (CRLF2 B-ALL), implicating the TSLP-CRLF2 pathway in leukemogenesis. The utility of classic patient-derived xenografts (PDX) for in vivo studies of the TSLP-CRLF2 pathway in normal and malignant human B lymphopoiesis are limited because available data suggest that mouse TSLP does not activate human CRLF2-mediated signals. First, we show that mouse TSLP does not induce activation of CRLF-2 downstream pathways (JAK/STAT5 and PI3K/AKT/mTOR) that are activated by human TSLP (hTSLP) in CRLF2 B-ALL cells. Next, we engineered xenograft mice to produce hTSLP (+T mice) by injection with stromal cells transduced to express hTSLP. Control (–T) mice were produced using stroma transduced with control vector. Normal serum levels of hTSLP were achieved in +T mice, while hTSLP was undetectable in –T mice. PDX generated from +T as compared to –T mice showed a 3-6 fold increase in normal human B cell precursors that was maintained through later stages of B cell development. Gene expression profiles in CRLF2 B-ALL from +T PDX indicate increased mTOR pathway activation and are more similar to the original patient sample than those from –T PDX. The novel +T/–T xenograft model developed here provides a preclinical model for understanding the TSLP-CRLF2 pathway in B lymphopoiesis and identifying treatments for CRLF2 B-ALL.

Introduction

TSLP is an IL-7-like cytokine that plays key roles at several points in normal hematopoietic cell development and function.(Ito et al., 2005; Siracusa et al., 2011; Ying et al., 2005; Zhou et al., 2005) The role of TSLP in B lymphopoiesis has been evaluated almost exclusively in mice.(Osborn et al., 2004; Ray, Furlonger, Williams, & Paige, 1996; Reche et al., 2001; Sims et al., 2000) Knowledge of TSLP in normal human B-cell development is limited to a single in vitro study showing that TSLP increases production of fetal B cell precursors.(Scheeren et al., 2010) Genetic alterations that cause overexpression of the TSLP receptor component, CRLF2, have been linked to B cell acute lymphoblastic leukemia (ALL), thus implicating the TSLP pathway in leukemogenesis (Cario et al., 2010; Chapiro et al., 2010; Russell et al., 2009; Yoda et al., 2010). The low homologies of murine and human TSLP and CRLF2 (~40%)(Quentmeier et al., 2001; Tonozuka et al., 2001) suggest the need for novel models to study the in vivo role of TSLP in normal and malignant human B lymphopoiesis.

ALL is the most common childhood malignancy and primarily involves the B-lineage (B-ALL). Although cure rates approach 90%, approximately 10-20% of patients still relapse.(Hunger et al., 2012; Raetz & Bhatla, 2012) Genomic profiling has identified several high-risk B-ALL subtypes that are chemo-resistant. (Chen et al., 2012; Mullighan, 2011; Mullighan et al., 2009; Mullighan et al., 2007) Included is CRLF2 B-ALL, a leukemia with genetic alterations causing over-expression of the CRLF2 protein on the surface of B-ALL cells.(Cario et al., 2010; Chapiro et al., 2010; Russell et al., 2009; Yoda et al., 2010) CRLF2 and the IL-7 receptor alpha chain (IL-7Rα) together form the TSLP receptor signaling complex (Figure 1A).(Park et al., 2000; Reche et al., 2001) Binding of TSLP induces CRLF2 and IL-7Rα dimerization leading to activation of the JAK-STAT5(Rochman et al., 2010; Wohlmann, Sebastian, Borowski, Krause, & Friedrich, 2010) and the PIK3/AKT/mTOR pathways, (Zhong et al., 2012; Zhong et al., 2014) as demonstrated in CRLF2 B-ALL. (Tasian et al., 2012) The finding that JAK kinases are mutated in CRLF2 B-

ALL(Harvey et al., 2010) suggested that CRLF2 and mutated JAK cooperate to induce constitutive STAT5 activation in CRLF2 B-ALL.(Malin et al., 2010; Roll & Reuther, 2010) However, approximately half of CRLF2 B-ALL, lack JAK mutations. Thus, the role of TSLP in the leukemogenesis of CRLF2 B-ALL remains unclear and the mechanisms for its contribution to chemoresistance are unknown.

The genetic landscape produced by inherited germline variations contributes to leukemogenesis and disease outcome (Moriyama, Relling, & Yang, 2015) and is a biological component that contributes to racial, ethnic and other health disparities in ALL (Lim, Bhatia, Robison, & Yang, 2014). This is particularly relevant in CRLF2 B-ALL which occurs five times more often in Hispanic children than others (Harvey et al., 2010) and comprises more than half of the ALL cases in children with Down Syndrome (Hertzberg et al., 2010; Mullighan et al., 2009; Russell et al., 2009). Patient-derived xenograft (PDX) models produced by injecting human cells into immune deficient mice provide *in vivo* preclinical models for understanding disease mechanisms and identifying effective therapies in context of the range of genetic landscapes present in the human population. However, engineered cellular models (Reche et al., 2001) suggest that mouse TSLP (mTSLP) is species-specific and unlikely to stimulate CRLF2-mediated signaling in human cells (Park et al., 2000; Reche et al., 2001). Given the role of TSLP in activating the CRLF2 pathway (Tasian et al., 2012) and the identification of CRLF2 as a biological component of health disparities in CRLF2 B-ALL (Harvey et al., 2010; Hertzberg et al., 2010; Mullighan et al., 2009; Russell et al., 2009), it is important that studies to identify disease mechanisms and potential therapies for this leukemia be performed in preclinical models that provide human TSLP (hTSLP).

Our goal was to develop and validate a xenograft model that can be used to study the role of hTSLP in normal and malignant B-lymphopoiesis. Here we describe the development of a novel xenograft model system comprised of mice that provide hTSLP (+T mice) and mice that do not (–T mice). PDX generated from +T mice show functional *in vivo* hTSLP effects, expanding

the production of normal B cell precursors from hematopoietic stem cells (HSCs) and inducing changes in mTOR-regulated gene expression in primary CRLF2 B-ALL cells. These data validate the +T/–T xenograft model system as an important new preclinical model for understanding the role of hTSLP in normal and malignant B lymphopoiesis and for identifying therapies to treat CRLF2 B-ALL.

Materials and Methods

Additional details are available in the Online Supplementary Methods.

Human Samples and Cell Lines

Human CRLF2 B-ALL cell lines MUTZ5 and MHH-CALL4 were from DSMZ (Braunschweig, Germany) and the human stroma line HS-27a (HS27) from ATCC, (Manassas, VA). Primary human CRLF2 B-ALL cells (Online Supplementary Table 1 and) and umbilical cord blood (CB) were obtained in accordance with protocols approved by the Loma Linda University Institutional Review Board (IRB) and with the Helsinki Declaration of 1975, as revised in 2008. Details of HS27 stroma characteristics, CB CD34+ cell isolation, and culture media are described in the Supplementary Methods.

Mice

Studies were performed using NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice (Jackson Laboratory) housed under pathogen-free conditions at Loma Linda University Animal Care Facility. Studies were conducted in accordance with Loma Linda University Institutional Animal Care and Use Committee (IACUC) approved protocols.

Flow Cytometry

Cells were prepared for flow cytometry using specific antibody clones as described in Supplementary Methods. Flow cytometry analysis was performed using a MACSQuant analyzer (Miltenyi Biotec, Inc, Auburn, CA) and FlowJo analysis software (FlowJo, Ashland, OR).

Stromal Cell Transduction

Human HS27 stromal cells were transduced to express hTSLP (+T stroma) or with control vector (–T stroma) at a multiplicity of infection of 0.5-5/cell overnight. After transduction, cells were washed, expanded in culture, and frozen in aliquots. For the generation of +T and –T mice, stromal cells were thawed, cultured, and injected into mice within 15 passages after thawing. Details of lentiviral vector production and ELISAs to determine hTSLP levels are available in Supplementary Methods.

Xenograft Transplantation

HS27 stroma transduced to be +T or –T were injected intraperitoneally into NSG mice at doses of 5 or $10x10^6$ cells at time points described in Results section. Mice were sub-lethally irradiated with 225 cGy, then transplanted by tail vein injection with freshly thawed human umbilical CB CD34+ cells ($1x10^5$) or primary CRLF2 B-ALL cells ($5x10^5$ Patient 1 and $1.5\ x10^6$ Patient 2).

Gene Expression Analysis in CRLF2 B-ALL Cells

Microarray analyses were performed by Miltenyi Biotec's Genomic Services (Miltenyi Biotec GmbH, Bergisch Gladbach) using Agilent Whole Human Genome Oligo Microarrays (Design ID 039494, 8x60K v2). Libraries for RNA sequencing were prepared and sequenced by the UCLA Clinical Microarray Core. Data discussed in this publication are deposited in NCBI's

Gene Expression Omnibus (GEO).(Edgar, Domrachev, & Lash, 2002) Microarray and RNA sequencing data are available through accession numbers GSE65274 and GSE74339 respectively at (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSExxx). Gene Set Enrichment Analysis (GSEA) was performed by Novogenix Laboratories, LLC (Los Angeles, CA). Details of microarray, RNA sequencing, data processing, differential gene expression analysis, GSEA, and quantitative RT-PCR analysis are in the Supplementary Methods.

Online Supplementary Methods

Cells and Cell Culture Media

The human stromal cell line HS-27a (HS27) was selected for use in our model because 1) HS27 is one of a limited number of human bone marrow-derived stromal cell lines; 2) HS27 secretes low levels of growth factors that do not support proliferation of isolated progenitor cells in cocultures[1]; and 3) our laboratory has found that HS27 grow robustly in culture. HS27 stroma were maintained in RPMI 1640 medium (Irvine Scientific) supplemented with penicillin/streptomycin, L-glutamine, 50 μM 2-ME and 10% FBS (Omega Scientific). MUTZ5 and MHH-CALL4 were maintained in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with penicillin/streptomycin, L-glutamine, 50 μM 2-ME and 20% FBS (Omega Scientific, Tarzana, CA). This media was also used for in vitro cultures with and without TSLP (both for phosphoflow assays and for gene expression profiling in response to TSLP).

Flow Cytometry

For detection of surface antigens, cells were stained using standard flow cytometry protocols, followed by fixation and permeabilization as described below to detect intracellular antigens. To assess intracellular IgM, the Fix & Perm® cell fixation

and permeabilization kit (Life Technologies, Grand Island, NY)) was used according to manufacturer's instructions. To discriminate living from dying cells, in some experiments, cells were stained with fixable viability dyes (eBioscience, San Diego, CA), per manufacturer's instructions. All antibodies were monoclonal, anti-human antibodies, unless otherwise stated. Specific antibody clones and conjugates are shown below.

Antibody	**Clone**	**Manufacturer**
Ig κ light chain FITC	G20-193	BD Pharmingen (San Jose, CA)
Ig λ light chain FITC	JDC-12	
CD66b FITC	G10F5	
CD34 APC	8G12	BD Biosciences (San Jose, CA
CD56 PE	MY31	
CD79a PE	HM47	
IgM PE.Cy-5	G20-127	
Anti-Phospho AKT (pS473) Alexa Fluor 488	M89-61	
Anti-phospho STAT5 (pY694) PE	47/Stat5 (pY694)	
Anti-Phospho S6 (pS235/S236) Alexa Fluor 488	N7-548	
CD10 APC.Cy7	HI10A	BioLegend (San Diego, CA)
CD14 APC	M5E2	
CD19 APC	HIB19	
CD19 Pacific Blue	HIB19	
CD34 PerCP	581	
CD38 Brilliant Violet 421	HIT2	
Ig κ light chain Pacific Blue	MHK-49	
Ig λ light chain Pacific Blue	MHL-38	
IgD PE	IA6-2	
TSLPR APC	1B4	
CD19 APC	HIB19	eBioscience (San Diego, CA)
CD19 PE.Cy7	SJ25C1	
CD45 PE.Cy7	HI30	
Ig κ light chain eFluor 450	TB28-2	
Ig λ light chain eFluor 450	1-155-2	
CD19 APC	LT19	Miltenyi Biotec (Auburn, CA)
CD29 PE	TS2/16	
CD90 APC	DG3	
CD127 PE (IL-7Ra)	MB15-18C9	
Anti-Mouse CD45 FITC	30F11	

Statistical Analysis

Statistical analysis was performed using Students t test and data expressed as mean ± SEM. Statistical significance was defined as $p<0.05$.

Phospho-Flow Cytometry Staining

Human CRLF2 B-ALL cell lines were harvested from continuous culture and primary CRLF2 B-ALL cells were obtained as BM from patient-derived xenografts. Cells were rested in culture without cytokines for 2 hrs, and then cultured with mTSLP, hTSLP or no cytokine for 30 mins (S6 and AKT) or 1 hour (STAT5). Cells were then harvested and stained with antibody to phosphorylated STAT5 (pSTAT5), AKT (pAKT) or S6 (pS6) as previously described for STAT5 (Parrish et al., 2009). Primary CRLF2 B-ALL patient-derived samples were also stained with CD19 APC prior to fixation and permeabilization for detection of phospho proteins and CD19+ cells were gated for phospho-flow cytometry plots.

ELISA Assays of TSLP

Concentrations of hTSLP in the supernatant of transduced HS27 stromal culture and in sera collected from NSG mice injected with transduced stroma were measured using the Human TSLP ELISA MAX Kit (BioLegend, San Diego, CA). Stromal cell supernatants were harvested from confluent flasks when stromal cells were passaged (typically two times per week) and sera was obtained from mice at weekly time points at the time of weekly stromal cell injection. Data was collected using the uQuant microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT) and analyzed using the KC Junior, version 1.6 (BioTek Instruments, Inc., Winooski, VT)

Lentivirus Vector and TSLP-Expressing Human Stromal Cells

TSLP vector was generated using standard molecular cloning techniques with complementary DNA encoding human TSLP (purchased from Thermo Scientific Open Biosystems). In this vector, the EF-1α promoter was used to drive TSLP expression. A control vector with GFP was also generated. The lentiviral vector packaging and titering were performed as previously described (Meng et al., 2012). In brief, VSV-G–pseudotyped lentiviral vectors were prepared by calcium phosphate–mediated 3-plasmid transfection of 293T cells. Briefly, 27 μg transfer vector construct, 17.5 μg second-generation gag-pol packaging construct pCMV.R8.74, and 9.5 μg VSV-G expression construct pMD2.G were used for transfection of $12x10^6$ 293T cells overnight in 25 ml DMEM with 10% fetal bovine serum. The cells were treated with 10 mM sodium butyrate during the first of three 12-hour vector supernatant collections. The supernatant was filtered through 0.22-μm-pore-size filters and concentrated 100-fold by ultracentrifugation before freezing and storing at –80° C. All vector stocks were titered by transducing HT1080 cells with analysis for GFP expression by flow cytometry or real-time PCR.

Processing of Samples from Xenograft Mice

Flow cytometry analysis of peripheral blood (PB) was used to establish chimerism with human cells prior to euthanasia. PB was collected using tail-tip excision (tail nick) and placed into a microtainer serum separator tube (BD Gold, 200/cs). Samples were centrifuged at 1500 rpm for 15 mins. Serum was removed for ELISA analysis. Blood cells were subjected to red blood cell lysis (Kang et al., 2002) and remaining white blood cells were stained for human surface markers. NSG mice were euthanized 5-7 weeks after CB CD34+ transplantation or 12 weeks after CRLF2 B-ALL transplantation. On the day of euthanasia fresh bone marrow (BM), spleen and PB were harvested. Single cell suspensions were obtained from the BM by using 26g syringe and PBS to flush the femora and humeri and by filtering cells with a 70 micron cell strainer (BD Biosciences, San Jose, CA). Cells obtained from all tissues were stained immediately for flow cytometry analysis or were frozen for future use.

Microarray Analysis of Gene Expression in CRLF2 B-ALL Cells

Mice engineered to be +T and –T by stromal cell injection were transplanted with primary CRLF2 B-ALL cells from a Hispanic pediatric patient. Following transplant, mice received weekly intraperitoneal injection of $5X10^6$ +T or –T stroma from Transduction 1 (see Results section). Nine weeks post transplant, BM was harvested and frozen for later use. For microarray to evaluate *in vivo* TSLP-induced changes in gene expression, xenograft BM was thawed and human B-ALL cells were isolated by magnetic separation using the Human CD19 microbead kit (Miltenyi Biotec, Inc., San Diego, CA) according to manufacturer's protocol and rapidly frozen on dry ice (Samples

E1-E3 and F1-F3 described below). For microarray to evaluate *in vitro* TSLP-induced changes in gene expression (Fig. 4), xenograft BM was thawed and cultured *in vitro* for 3 days with TSLP at 15ng/ml or without TSLP and with neutralizing anti-TSLP antibody (R&D Systems, Minneapolis, MN) or control antibody (Jackson ImmunoResearch). Following culture, cells were harvested and thawed and human B-ALL cells were isolated by magnetic separation using the Human CD19 Microbead Kit according to manufacturer's protocol and rapidly frozen on dry ice (samples A1-A3, B1-B3, C1-C3 and D1-D3 as described below). Frozen cells were shipped to Miltenyi Biotec (Miltenyi Genomic Services) for RNA isolation, sample preparation, whole genome microarray, and discriminatory gene analysis (DGA) as follows:

Total RNA Isolation

Human total RNA was isolated using the NucleoSpin® RNA kit (Macherey-Nagel, Bethlehem, PA). RNA quality and integrity were determined using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was quantified by measuring A260nm on the ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

RNA Amplification and Labelling

Sample labelling was performed as detailed in the "One-Color Microarray-Based Gene Expression Analysis protocol (version 6.6, part number G4140-90040). Briefly, 50 ng RNA for the samples from groups A, B, E and F, 10 ng RNA for the samples of group C and D, and the complete available amount of RNA for the samples C3 and D1 was

used for the amplification and labelling step using the Agilent Low Input Quick Amp Labelling Kit (Agilent Technologies). Yields of cRNA and the dye-incorporation rate were measured with the ND-1000 Spectrophotometer (NanoDrop Technologies).

Microarray Sample Key

Sample	Source of Cells
E1, E2, E3	CRLF2 B-ALL cells from +T mice
F1, F2, F3	CRLF2 B-ALL cells from –T mice

Hybridization of Agilent Whole Human Genome Oligo Microarrays

The hybridization procedure was performed according to the "One-Color Microarray-Based Gene Expression Analysis protocol (version 6.6, part number G4140-90040) using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). Briefly, 0.6 µg Cy3-labeled fragmented cRNA in hybridization buffer was hybridized overnight (17 hours, 65 °C) to Agilent Whole Human Genome Oligo Microarrays 8x60K v2 (AMADID 039494) using Agilent's recommended hybridization chamber and oven. Following hybridization, the microarrays were washed once with the Agilent Gene Expression Wash Buffer 1 for 1 min at room temperature followed by a second wash with preheated Agilent Gene Expression Wash Buffer 2 (37 °C) for 1 min.

Scanning and Data Analysis

Fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent's Microarray Scanner System G2505C (Agilent Technologies). The Agilent

Feature Extraction Software (FES) 10.7.3.1 was used to read out and process the microarray image files.

Gene Set Enrichment Analysis

Microarray data was evaluated at the level of gene sets to define and quantitate trends in gene expression similar to published data. Ranked gene lists were created and submitted to the online public repository provided by the BROAD Institute for Gene Set Enrichment Analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005) to evaluate Oncogenic Signatures that are enriched in CRLF2 B-ALL cells expanded in +T as compared to –T mice (www.broadinstitute.org/gsea).

Discriminatory Gene Expression Analysis

After probe summarization, quantile normalization and log2 transformation, differentially expressed genes were identified by a combination of effect size and statistical significance. For the analysis of the individual comparisons, only reporters with an at least two-fold median up- or downregulation in +T samples compared to –T samples and an adjusted p-value (Student's t-test, two-tailed, equal variance, Benjamini-Hochberg correction for multiple testing) $p \leq 0.05$ were considered relevant. In an additional filtering step, only Agilent reporters with signal intensity values significantly above local background ($p < 0.01$ as calculated by the Rosetta Resolver® gene expression data analysis system) in at least two samples of the group with higher median expression were selected (Weng et al., 2006). All microarray data reported in this publication were

deposited in the NCBI's Gene Expression Omnibus at National Center for Biotechnology Information and are accessible via the GEO series accession number GSE65274.

Real Time Quantitative RT-PCR

Expression of select genes observed in microarray results was validated using quantitative reverse transcriptase - polymerase chain reaction (qRT-PCR) using primers shown below. Total RNA (2.0μg) was reverse transcribed using poly d(T)20 primers and SuperScript II reverse transcriptase (Life Technologies, Grand Island, NY). The resulting cDNAs were used for qRT-PCR analysis of their mRNA levels on a StepOne Plus 7500 Real-time PCR system (Applied Bioscience Inc., Foster City, CA) using specific primers for each gene (see supplemental table 2 for primer sequences) following the manufacturer's instructions. The fluorescence threshold value was calculated and normalized to the values of 18s rRNA. The fold change in mRNA expression of genes between the TSLP-treated W31 cell group versus untreated group was achieved by the ratio of fluorescence threshold value in the treated groups versus that of the untreated group. The fold change for each gene was paired with that of microarray analysis. Linear regression analysis of the fold change versus that of microarray was performed with Origin 6.0. The graph shows the best-fit line, the formula for the best-fit line ($Y = a + bX$), its correlation coefficient (r), and the probability (P) that the r value is a false positive.

Gene	Forward Primer	Reverse Primer
BIVM	5’-GGCCAGAGGCAATGCAAAG	5’-TGAGGTCTAATACTTTCCGCTGT
PTH2	5’-CTGGTTCTCCACAGGTGATG	5’-CATGTACGAGTTCAGCCACT
FOSL2	5’-AACACCCTGTTTCCTCTCCG	5’-ATCTACCCGGAATTTCTGCGG
RAMP1	5’-GCAGGACCATCAGGAGCTACA	5’-GCCTACACAATGCCCTCAGTG
SLC4A8	5’-TGGGTCCAGTAGGGAAAGGT	5’-ACCGTCACCTGGTCTAGGAA
NR4A2	5’-GTAACTCGGCTGAAGCCATGC	5’-GTCGTAGCCTGTGCTGTAGTT
KCNH8	5’- GCAAGATCAATTTGCATCCACTAT	5’- AAGGCAGGGCAGCGATTA
THSD7A	5’- CCATCTCGAGTCTTTGTTCACATT	5’- CATTTGGGAATTGTGCTTCTCA
RAB26	5’-CCAGGCCCTTCTGACTTTGT	5’-CTGCTTGTTGGGACTGTTGC
RGS1	5’-CTCCCTGGGTGAACAGCTTG	5’-GCTTCTTCAACTCTGCGCCT
NELL1	5’-GATATGAAGCCACCCGTGTT	5’-CTGAAGGTCAGGGTCCATCC
MDF1	5’-GAAGTTGCAGACACACCCATCTC	5’-GTCCAGGACGATGTTGCACAG
KREMEN2	5’-TGAGGACCCAGAGGCCC	5’-CAGACAGCTCCCCCAATCG
CPNE8	5’-CGCCTCCTCCCAATATGGAC	5’-ATTTCTGCAGGACACGGACA

RNA Sequencing and Analysis of Gene Expression in CRLF2 B-ALL Cells

RNA libraries were prepared for sequencing by the UCLA Clinical Microarray Core using TruSeq kits (Illumina). Libraries were sequenced on a HiSeq 2000/2500 at 1 X 50 bp. Transcript levels were estimated using RNA-Seq by Expectation Maximization

(Li & Dewey, 2011); Reads were mapped to the human genome (hg19) using RefSeq annotation. Pairwise differential expression assessments were performed using the method EBSeq(Leng et al., 2013); genes were considered to be differentially expressed based on a False Discovery Rate of less than 5% (Posterior Probability of being Differentially Expressed < 0.05). Gene Ontology (GO) analysis was performed using DAVID. Gene Set Enrichment Analysis (GSEA) was performed using MSigDB. Ranked lists of genes having a fold change of greater than 1.5 were used as input for GSEA. The transcript per million (TPM) values from respective samples were clustered using hierarchical clustering with R. Spearman rank correlation was used as the distance metric followed by average linkage clustering.

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Results

Mouse TSLP does not Activate the Human TSLP Receptor Complex

Activation of the human TSLP receptor complex can be induced by hTSLP, but not by mTSLP, in murine Ba/F3 cells transduced to express human CRLF2 and IL-7Rα (Reche et al., 2001). However, the ability of mTSLP to activate CRLF2-signaling in human cells, particularly those expressing high levels of CRLF2 observed in CRLF2 B-ALL, has not been reported. To determine if mTSLP can induce CRLF2-mediated signals we tested its ability to activate downstream pathways in human CRLF2 B-ALL cells. First, we verified that the CRLF2 B-ALL cell lines and primary CRLF2 B-ALL cells used in our studies express the TSLP receptor components. Expression of IL-7Rα□□ as well as high levels of CRLF2, was observed in both CRLF2 B-ALL cell lines and in the two primary CRLF2 B-ALL samples used in studies described here (Figure 1B).

Next we used phospho-flow cytometry to determine whether mTSLP can activate the JAK-STAT5 and PI3K/AKT/mTOR pathways activated by hTSLP in CRLF2 B-ALL cells (Tasian et al., 2012). hTSLP induced robust increases in phosphorylated STAT5 (pSTAT5) and S6 (pS6, activated downstream of mTOR) and more modest increases in phosphorylated AKT (pAKT) in CRLF2 B-ALL cell lines and primary CRLF2 B-ALL cells (Figure 1C-E). In contrast, stimulation of CRLF2 B-ALL cells with mTSLP showed no effect (Figure 1C-E)(Dann, Selvaraj, & Thomas, 2007) These data show that mTSLP fails to induce the STAT5, AKT, or S6 phosphorylation that are characteristic of CRLF2-mediated signals induced by hTSLP in human CRLF2 B-ALL cells. Thus, TSLP produced in the existing mouse xenograft models does not induce human CRLF2-mediated signals.

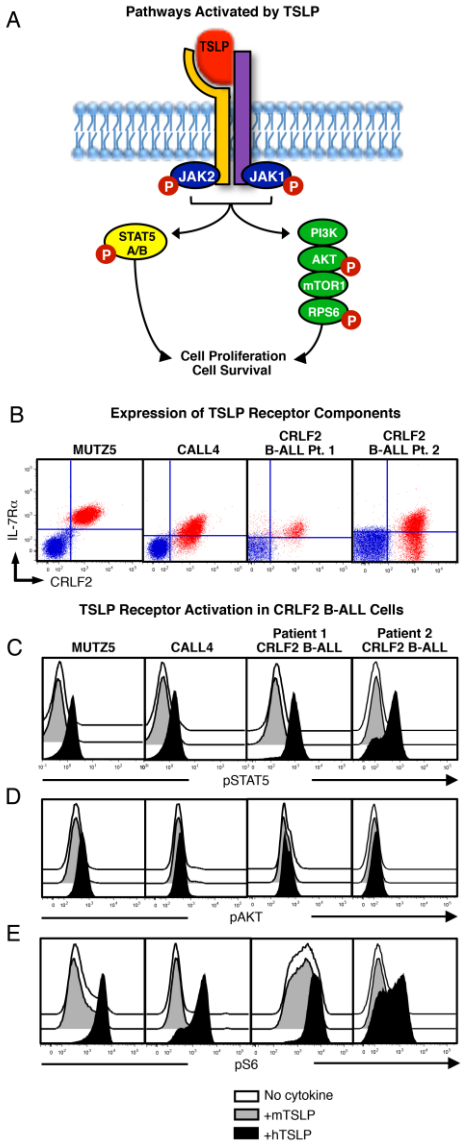


Figure 1 (*Figure 13*). Mouse TSLP does not activate the human TSLP receptor complex. A) Pathways activated downstream of TSLP receptor signaling in human cells. B) CRLF2 B-ALL cell lines (MUTZ5, MHH-CALL4) and primary CRLF2 B-ALL cells used to produce patient-derived xenografts (PDX) used in the studies described here were stained for flow cytometry to detect the TSLP receptor components (IL-7Rα and CRLF2). Plotted in red are CRLF2 B-ALL cells within living cell light scatter. Quadrants shown are set based on unstained controls (blue overlay) C-E) CRLF2 B-ALL cell lines and CRLF2 B-ALL cells from a PDX generated from the primary cells in panel C were stimulated with human TSLP (hTSLP), mouse TSLP (mTSLP), or no cytokine and evaluated for phosphorylated STAT5 (pSTAT5), AKT (pAKT), and S6 (pS6) by phospho-flow cytometry.

Engineering Xenograft Mice to Express Normal Serum Levels of Human TSLP

We developed a strategy for engineering xenograft mice that provide hTSLP to activate CRLF2-mediated signals (Figure 2A) by building on a previous cytokine delivery approach (Dao, Pepper, & Nolta, 1997). Our goal was to produce stable, sustained serum levels of hTSLP, similar to normal levels reported in children (range 13-32 pg/ml(Lee et al., 2010)) by intraperitoneal injection of stromal cells transduced to express high levels of hTSLP (+T mice, Figure 2A). Control mice (–T mice) were produced by injecting stroma transduced with empty vector. Because the only difference between +T and –T mice is the presence of hTSLP in +T mice, this model would allow us to identify the in vivo effects of hTSLP by comparing hematopoiesis in +T and –T PDX generated from these mice.

Human HS27 stromal cells (Graf, Iwata, & Torok-Storb, 2002) were transduced with lentiviral vector containing hTSLP (+T stroma) or with control vector (–T stroma). Culture supernatant collected from stroma after the initial transduction showed hTSLP levels of ~500 pg/ml (Figure 2B, Transduction 1). The ability of +T stroma to generate measureable serum levels of hTSLP *in vivo* was evaluated. NSG mice receiving weekly intraperitoneal injections of $5X10^6$ +T stroma showed serum hTSLP levels that were low but detectable (4-10 pg/ml; data not shown). To achieve higher levels of sustained hTSLP expression NSG mice were injected with 3 doses of $1X10^7$ +T or –T stroma over a 1 week period (loading dose) followed by weekly injections of $1X10^7$ stromal cells. Serum collected at weekly time points showed serum hTSLP levels of ~80 pg/ml following the initial 3 doses of +T stroma but quickly dropped to 15-20 pg/ml with +T stroma

injections (Figure 2C, left panel). hTSLP was not detectable in the sera of mice transplanted with –T stroma (Figure 2C). These data show that +T stroma can generate normal serum levels of hTSLP and that serum levels of hTSLP correspond to the number of stromal cells injected during the previous 1-2 week period. These data suggested that stable, sustained hTSLP production might be more likely with weekly injections of stroma that could deliver slightly higher doses of hTSLP.

A second stromal cell transduction produced hTSLP levels averaging ~900 pg/ml in culture supernatants harvested at early, mid and late passages from +T stroma across multiple thaws of frozen aliquots (Figure 2B, transduction 2). hTSLP was undetectable in supernatant from similarly passaged –T stroma (Figure 2B, Transduction 2). NSG mice transplanted at weekly time points with $5X10^6$ stroma from transduction 2 showed serum hTSLP levels of approximately 20 pg/ml that were stable up to 11 weeks (Figure 2D, and data not shown). Evaluation of BM and spleen of +T and –T mice by flow cytometry showed that HS27 stroma (CD29+CD90+) were not detectable in the either the spleen or BM of PDX mice (Online Supplementary Figure S1). These data provide evidence that the HS27 +T stroma in the peritoneal cavity give rise to normal serum levels of hTSLP in PDX mice.

To verify that the hTSLP produced in our model induces CRLF2-mediated signals, CRLF2 B-ALL cell lines were assessed for activation of the JAK/STAT5 and PI3K/AKT/mTOR pathways. Supernatant from +T stroma induced robust pSTAT5 and pS6 and minimal pAKT (Figure 2E-G). In contrast, CRLF2 B-ALL cells stimulated with supernatant from –T stroma showed background levels of pSTAT5, pAKT, and pS6, similar to cells incubated with medium alone (Figure 2E-G). These data show that +T

stroma, but not –T stroma, used in our model produce hTSLP that can activate signaling in human CRLF2 B-ALL cells.

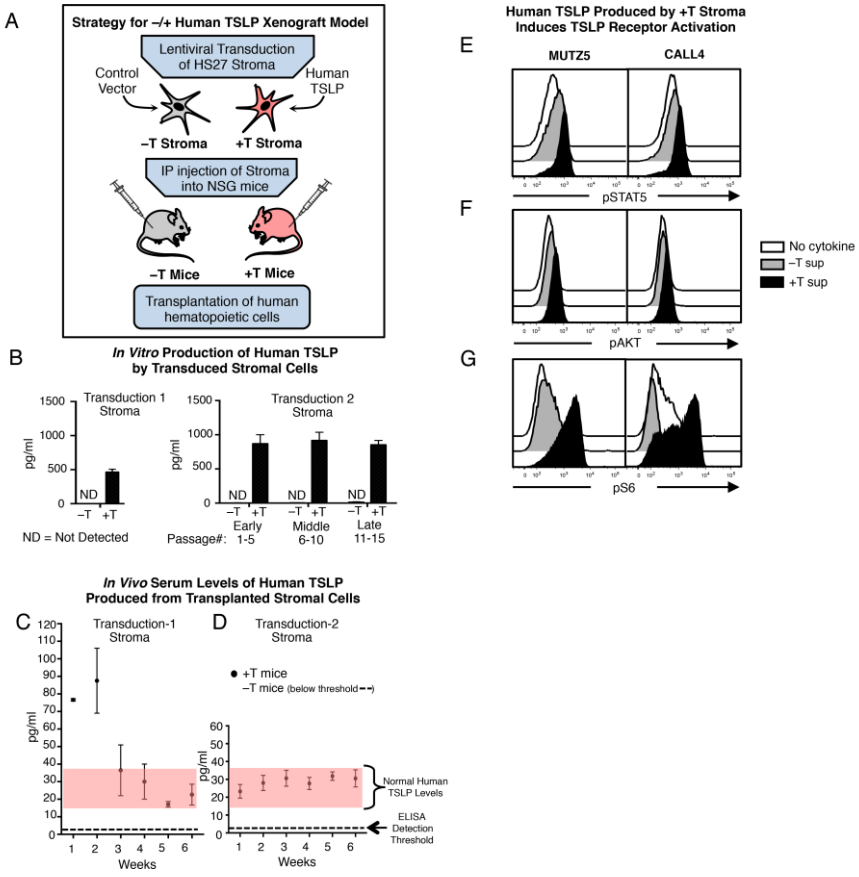
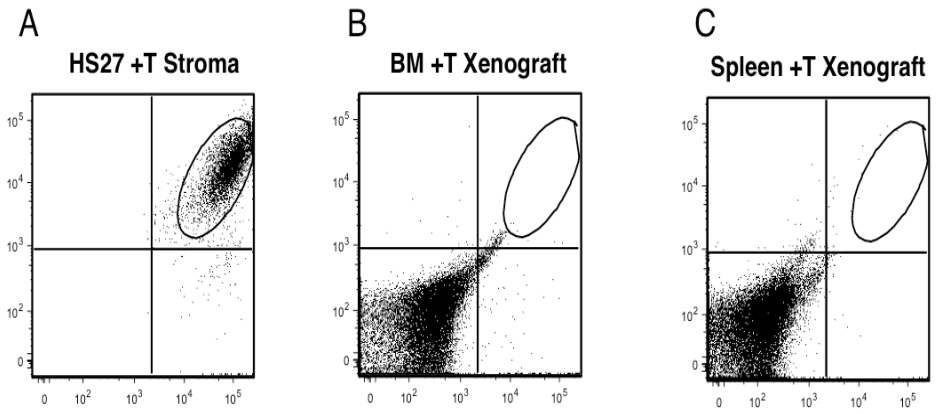


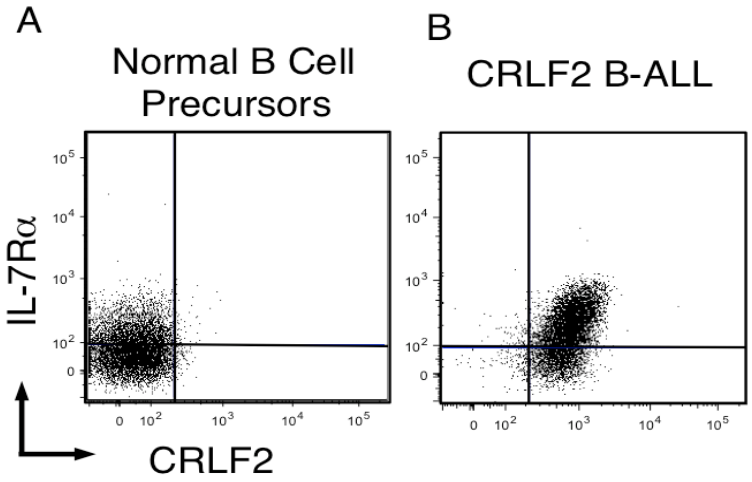
Figure 2 (*Figure 14*). Engineering xenograft mice to express normal serum levels of human TSLP. (A) Strategy for developing the –/+hTSLP xenograft model. (B) Culture supernatant from HS27 +T and HS27 –T stromal cells was collected after multiple passages following the initial transduction (Transduction 1) and assessed by ELISA for hTSLP protein production. Supernatant from Transduction 2 was collected at early (passage 1-5), mid (passages 6-10) and late (passages 11-15) post thaw passages and hTSLP levels were evaluated by ELISA. C-D) Serum levels of hTSLP in blood collected at indicated time points from +T or –T mice injected with stroma as determined by ELISA were as follows: (C) loading dose (3 doses of $1X10^7$ in first week) followed by weekly doses of $1X10^7$ stromal cells (Transduction 1), graphed are the means ± SEM of n = 2-7 mice at each time point. (D) injection of $5X10^6$ stromal cells (Transduction 2) at weekly intervals; graphed are the means ± SEM n ≥ 24 mice at each time point. (E-G) CRLF2 B-ALL cell lines were stimulated with stromal cell supernatant (Transduction 2) and levels of pSTAT5, pAKT and pS6 were measured by phospho-flow cytometry.



Supplementary Figure 1 (*Figure 15).* HS27 stroma are not detectable in bone marrow or spleen of xenograft mice. (A) Flow cytometry plots showing human CD29 and CD90 expression on HS27 stromal cells transduced to express human TSLP (+T HS27 stroma). Similar staining was observed for HS27 stroma transduced with empty vector (–T HS27 stroma). +T or –T HS27 stroma were injected at weekly time points into sublethally irradiated NSG mice for 13 weeks. Mice were euthanized and bone marrow (BM) and spleen were harvested and stained for flow cytometry to detect human CD29 and CD90 co-expression as a means of identifying HS27 stroma in these tissues. Plotted are 25,000 events from (B) BM and (C) spleen of +T mice showing that CD29+CD90+ HS27 stroma are not detectable in BM or spleen of +T NSG mice. Similar data were obtained in –T NSG mice. Data shown are representative of n=5 +T mice and n=4 –T mice.

hTSLP Produced in Xenograft Mice Exerts In Vivo Functional Effects on Normal Human B Lineage Cells

We evaluated the ability of hTSLP in +T PDX to induce *in vivo* functional effects on human B lineage cells. hTSLP has been shown to increase the *in vitro* production of normal human B cell precursors(Scheeren et al., 2010) despite their low levels of CRLF2 expression (Online Supplementary Figure S2). Thus, expansion of normal human B cell precursors provides a sensitive *in vivo* functional test of the hTSLP produced in our xenograft model. We compared normal B cell production in +T and –T NSG mice transplanted with umbilical CB CD34+ cells from 3 different donors (Figure 3A, S3A and S4A). Five weeks post-transplant mice were euthanized and bone marrow (BM) harvested for flow cytometry to evaluate the production of B lineage and other hematopoietic cells. Mouse and human cells were identified based on expression of mouse CD45 (mCD45) or human CD45 (hCD45), respectively (Figure 3B, S3B and S4B). Flow cytometry analysis of hCD19 expression on gated human cells showed an increase in the percentage of B lineage cells in +T as compared to –T PDX mice generated from all 3 CB donors (Figure 3C, S3C and S4C). Pooled data comparing BM cellularity in all three experiments showed that the total number of human cells (hCD45+) was increased by 3-4 fold in +T as compared to –T PDX and this was due to significant increases in CD19+ (B lineage) cells but not CD19– cells (Figure 3D). These data suggest that hTSLP in +T PDX selectively expands human B lineage cells rather than exerting global effects on hematopoiesis.



Supplementary Figure 2 (*Figure 16*). Expression of CRLF2 and IL-7Rα on normal human B cell precursors and CRLF2 B-ALL harvested from xenograft mice. (A) Bone marrow was harvested from xenograft mice transplanted with human CD34+ cord blood cells and stained for flow cytometry. Plotted is CRLF2 and IL-7Ra expression on B cell precursors (hCD19+ and Ig kappa & lambda–). (B) Bone marrow was harvested from a patient-derived xenograft generated using Patient 1 CRLF2 B-ALL cells. Plotted is the CRLF2 and IL-7Rα staining on human CRLF2 B-ALL (hCD19+) cells. Gates were set based on isotype control staining.

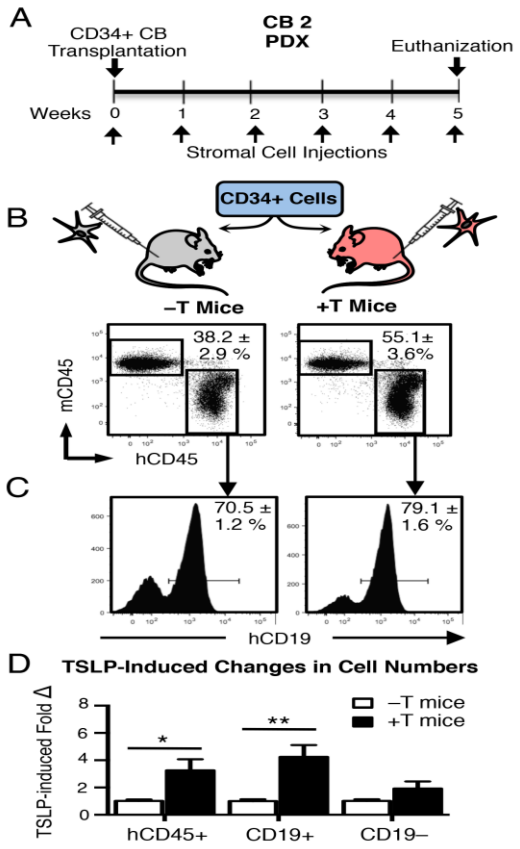
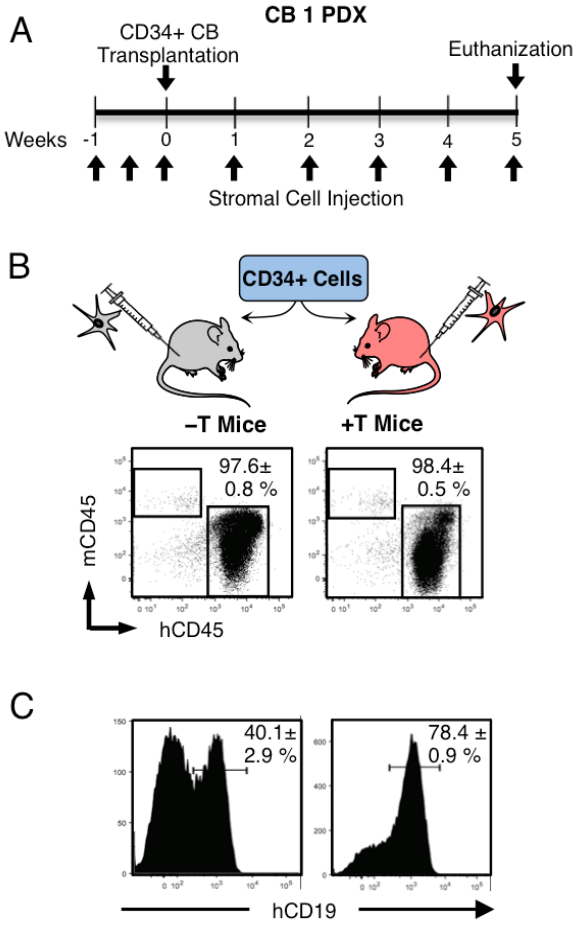
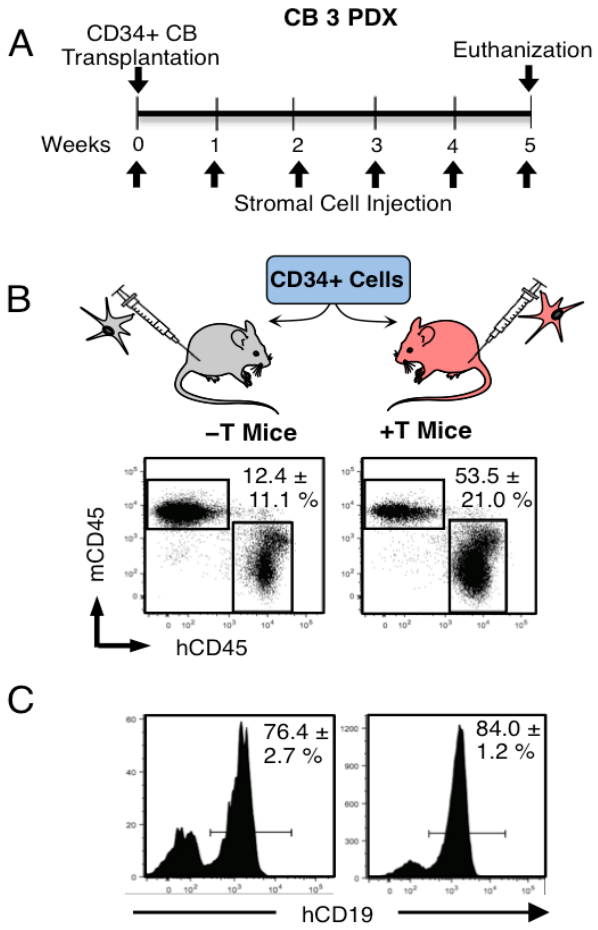


Figure 3 (*Figure 17*). hTSLP produced in xenograft mice exerts in vivo functional effects on normal human B lineage cells. (A) –T and +T NSG mice were transplanted with $1X10^5$ CD34+ human cord blood (CB) cells and injected with weekly doses of $5X10^5$ stromal cells (Transduction 2 stroma). (B) Five weeks post-transplant, mice were euthanized, BM harvested and cells were stained for flow cytometry to detect mouse CD45 (mCD45), human CD45 (hCD45), and hCD19. Total living cells were gated and mCD45 vs. hCD45 is plotted with gates to identify mouse and human leukocytes respectively, in the BM. (C) hCD45+ cells were gated and hCD19 expression is shown by histogram. Percentages are mean ± SEM. Data shown in A-C were obtained from CB2, n=2 –T PDX and n= 4 +T PDX; data from two additional CB samples (CB1 and CB3) are shown in Online Supplementary Figures S3-S4. (D) Graphed are the fold change in total human cells (hCD45+), human B lineage cells (CD19+), and human non-B lineage cells (CD19–). Fold change was obtained by normalizing cell numbers in +T animals to the average cell numbers in –T animals for each experiment. Data shown in Panel D are pooled from 3 different experiments performed with CB samples 1-3. Total n=9 –T PDX mice and n=14 +T PDX mice. Statistical significance was calculated using a 2-tailed, unpaired t-test (* $P \leq 0.05$; ** $P \leq 0.05$).



Supplementary Figure 3 (Figure 18). Functional effects of hTSLP produced in +T xenograft mice on normal human B lineage cells generated from CB 1. (A) –T and +T NSG mice were transplanted with $1X10^5$ CD34+ human cord blood cells (CB 1) and injected with weekly doses of $1X10^7$ stromal cells (transduction 1 stroma). (B) Five weeks post-transplant, mice were euthanized, BM harvested and cells were stained for flow cytometry to detect mouse CD45 (mCD45), human CD45 (hCD45), and hCD19. Total living cells were gated and mCD45 vs. hCD45 is plotted with gates to identify mouse and human leukocytes respectively, in the BM. (C) hCD45+ cells were gated and hCD19 expression is shown by histogram. Percentages are mean $\pm$ SEM. Data shown in A-C were obtained from CB 1, n=3 –T PDX and n= 5 +T PDX.



Supplementary Figure 4 (*Figure 19*). Functional effects of hTSLP produced in +T xenograft mice on normal human B lineage cells generated from CB 3. A) –T and +T NSG mice were transplanted with $1X10^5$ CD34+ human cord blood cells (CB 3) and injected with weekly doses of $5X10^6$ stromal cells (transduction 2 stroma). B) Five weeks post-transplant, mice were euthanized, BM harvested and cells were stained for flow cytometry to detect mouse CD45 (mCD45), human CD45 (hCD45), and hCD19. Total living cells were gated and mCD45 vs. hCD45 is plotted with gates to identify mouse and human leukocytes respectively, in the BM. C) hCD45+ cells were gated and hCD19 expression is shown by histogram. Percentages are mean ± SEM. Data shown in A-C were obtained from CB 3, n=4 –T PDX and n= 5 +T PDX.

B Cell Precursor Production is Increased in +T PDX Mice

To confirm that normal human B cell precursors are expanded in +T PDX, we compared the production of human B cell subsets (Figure 4A) and non B-cell lineages in –T and +T PDX. Staining of PDX BM for markers of human non-B cells showed that human CD19– cells are comprised primarily of monocytes (CD14+), but also include granulocytes (CD66B+), NK cells (CD56+), and CD34+ cells. The number of cells in each non-B lineage was similar (data not shown) in the BM from –T and +T PDX, indicating that the production of these cells is not skewed. However, the production of B cell precursors was increased 3-6 fold at each stage of B cell development in +T PDX as compared to –T PDX, beginning with the earliest CD34+ pro-B cells (Figure 4B). This increase was maintained through the mature B cell stage in BM and spleen (Figure 4B) with no skewing of B cell subsets in the BM (Figure 4C) or spleen (data not shown). These data provide evidence that hTSLP produced *in vivo* in +T PDX has a functional effect on normal human B cell precursors that express low levels of CRLF2.

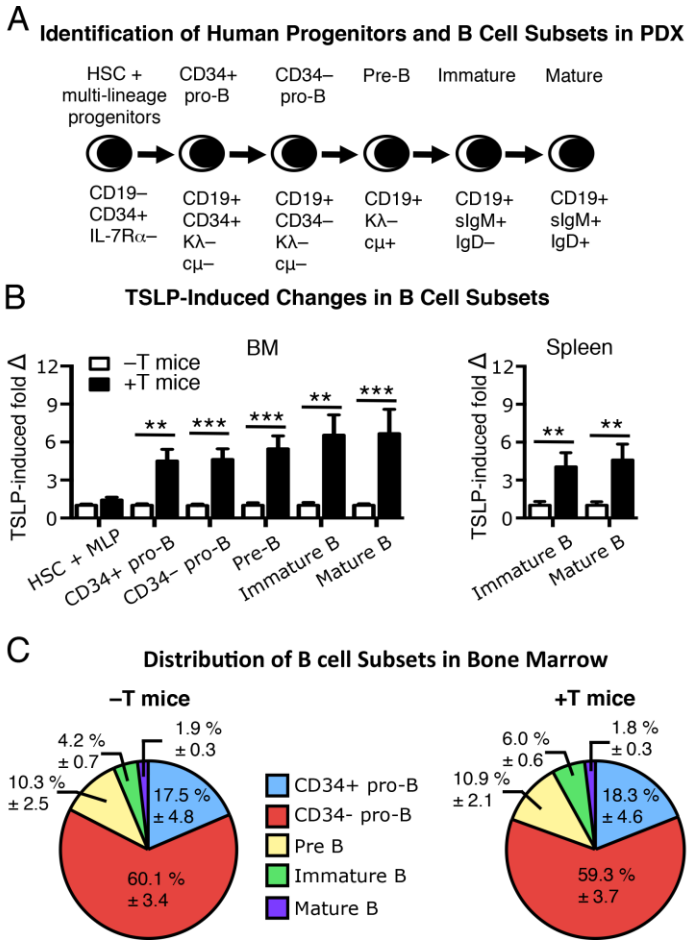


Figure 4 (*Figure 20*). B cell precursor production is increased in +T PDX. BM and spleen were harvested from –T and +T PDX mice generated by from three different CB samples as shown in Figure 3, and in Online Supplementary Figures S3-S4. (A) Harvested cells were counted by hemocytometer and were stained for flow cytometry and to identify progenitors and B cell subsets in the BM and spleen based on the indicated immunophenotypes. (B) Total living cell counts and percentages of living cells in each subset were used to calculate the number of cells in each subset. Graphed is the fold changed in each subset obtained by normalizing cell numbers in +T animals to the average cell numbers in –T animals for each experiment. (C) Graphed is the frequency of each subset within the total CD19+ B lineage pool. Data shown are pooled from experiments performed using PDX generated from CB 1-3. n=9 –T PDX mice and n =15 +T PDX mice. Statistical significance was calculated using a 2-tailed, unpaired t-test (** $P \leq 0.01$; *** $P \leq 0.001$).

hTSLP in Xenograft Mice Induces mTOR-Regulated Genes in Primary CRLF2 B-ALL

To determine the ability of hTSLP in +T xenografts to activate CRLF2 downstream pathways in CRLF2-B-ALL cells we used primary samples obtained two different Hispanic pediatric patients. (Patient characteristics shown in Online Supplementary Table S1.) First, we evaluated the ability of NSG mice transplanted with HS27 stroma to support engraftment of primary CRLF2 B-ALL cells. PDX from Patient 1 were generated using NSG mice injected with +T stroma from transduction 1 (stroma injection schedule, Figure 5A). Serum levels of hTSLP produced in these mice were detectable but below normal levels (4-10 pg/ml) and therefore provided a sensitive test of in vivo hTSLP activity in +T PDX. +T and –T NSG mice were transplanted with primary CRLF2 B-ALL cells (Figure 1B, right panel) from Patient 1. Mice were euthanized at 9 weeks when peripheral blood chimerism reached 80%. BM was harvested and stained for mCD45 and human-specific markers (CRLF2 and CD19) to identify CRLF2 B-ALL cells (Figure 5B). Flow cytometry analysis showed BM chimerism of human CRLF2 B-ALL cells was >90% in +T and –T PDX (Figure 5B). These data show that NSG mice support engraftment of primary B-ALL cells when injected with –T or with +T stroma.

The leukemia cell burden in the BM of –T and +T PDX generated from Patient 1 and from Patient 2 showed no consistent differences (Online Supplementary Figure S6). Thus, our next question was whether hTSLP in +T animals induces gene expression profiles associated with pathway activation downstream of CRLF2-mediated signaling in primary CRLF2 B-ALL cells. Human CRLF2 B-ALL cells were isolated from the BM of

+T and –T PDX generated from Patient 1 by magnetic separation. Gene expression was compared by whole genome microarray performed in triplicate. Differential gene expression analysis identified 280 gene reporters that were upregulated and 281 gene reporters that were downregulated at least 2 fold (Figure 5C). Microarray was validated by quantitative RT-PCR (qRT-PCR) analysis of selected differentially expressed genes (Table S2). Changes in gene expression by microarray and qRT-PCR were strongly correlated as indicated by linear regression (Figure 5D). GSEA(Mootha et al., 2003; Subramanian et al., 2005) of whole genome microarray data showed a strong enrichment for genes regulated downstream of mTOR activation in CRLF2 B-ALL cells from +T PDX as compared to –T PDX (FDR q-value = 0.022, Figure 5E). These data provide evidence that the hTSLP in +T PDX, even when present at low levels, acts on CRLF2 B-ALL cells to increase mTOR pathway activation downstream of CRLF2.

Supplementary Table 1 (Table 2). CRLF2 B-ALL Patient Characteristics.

Patient Designation	Gender	Age	Immunophenotypic Markers	*CRLF2 and IKZF1* Status	Ethnicity
Patient 1	M	10 years	CD10+ CD19+ CD20+ CD22 surface, dim CD22 cytoplasmic, dim CD34 partial CD38 partial CD45 dim CD58 dim CD79a+ HLA-DR+	*CRLF2 High* *No IKZF1* deletion	Hispanic
Patient 2	M	18 years	CD9+ CD10 partial CD19+ CD20 partial CD22 surface, dim CD22 cytoplasmic, dim CD34 partial CD38+ CD45 dim CD79a+ HLA-DR+ TdT+ CD3 cytyoplasmic– Kappa– Lambda– MPO–	*CRLF2 High* *IKZF1* deletion	Hispanic

IKZF1 deletion = deletion of one allele of *IKZF1 (Ikaros)* was determine as described[10]. Both patients expressed high levels of CRLF2 as assessed by flow cytometry in Figure 1.

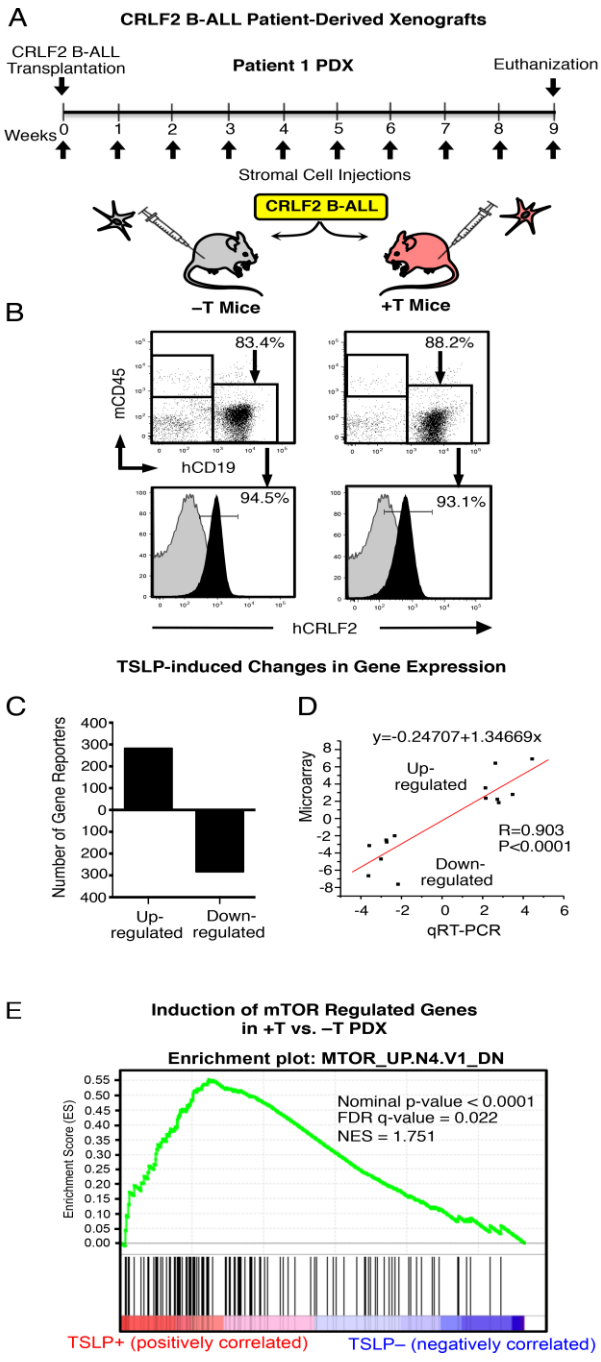

A
CRLF2 B-ALL Patient-Derived Xenografts
CRLF2 B-ALL Transplantation
Patient 1 PDX
Euthanization
Weeks 0 1 2 3 4 5 6 7 8 9
Stromal Cell Injections
CRLF2 B-ALL
–T Mice
+T Mice
B
83.4%
88.2%
mCD45
hCD19
94.5%
93.1%
hCRLF2
TSLP-induced Changes in Gene Expression
C
Number of Gene Reporters
400 300 200 100 0 100 200 300 400
Up-regulated
Down-regulated
D
y=-0.24707+1.34669x
Microarray
Up-regulated
R=0.903
P<0.0001
Down-regulated
-4 -2 0 2 4 6
qRT-PCR
E
Induction of mTOR Regulated Genes in +T vs. –T PDX
Enrichment plot: MTOR_UP.N4.V1_DN
Enrichment Score (ES)
0.55 0.50 0.45 0.40 0.35 0.30 0.25 0.20 0.15 0.10 0.05 0.00
Nominal p-value < 0.0001
FDR q-value = 0.022
NES = 1.751
TSLP+ (positively correlated)
TSLP– (negatively correlated)

Figure 5 (*Figure 21*). hTSLP in +T xenograft mice induces mTOR-regulated genes in primary CRLF2 B-ALL. (A) –T and +T NSG mice were transplanted with primary CRLF2 B-ALL cells from Patient 1 and injected weekly with $5X10^6$ stromal cells (transduction 1). (B) 9 weeks post-transplant, mice were euthanized, BM was harvested and cells were stained with mCD45 and hCD19 to identify human B-ALL cells. CRLF2 staining (black overlay) on gated hCD19+ cells is shown in bottom panels. Isotype control is shown in gray. (C) Human hCD19+ B-ALL cells were isolated by magnetic separation and analyzed using whole genome microarray to identify genes that are differentially regulated in +T as compared to –T PDX. Graphed are the numbers of Agilent gene reporters up or downregulated (≥2 fold, $p \leq .05$) in CRLF2 B-ALL cells from +T as compared to –T PDX. (D) qRT-PCR validation of whole genome microarray. Regression analysis of fold changes measured by microarray versus fold changes measured by qRT-PCR. (E) Gene Set Enrichment Analysis (GSEA) of whole genome microarray data shows that CRLF2 B-ALL cells harvested from +T PDX exhibit an increase in expression of genes regulated downstream of mTOR signaling as compared to cells from –T PDX. The top half of the GSEA enrichment plot shows the enrichment score for each gene and the bottom half shows the values of the ranking metric moving down the list of the ranked genes

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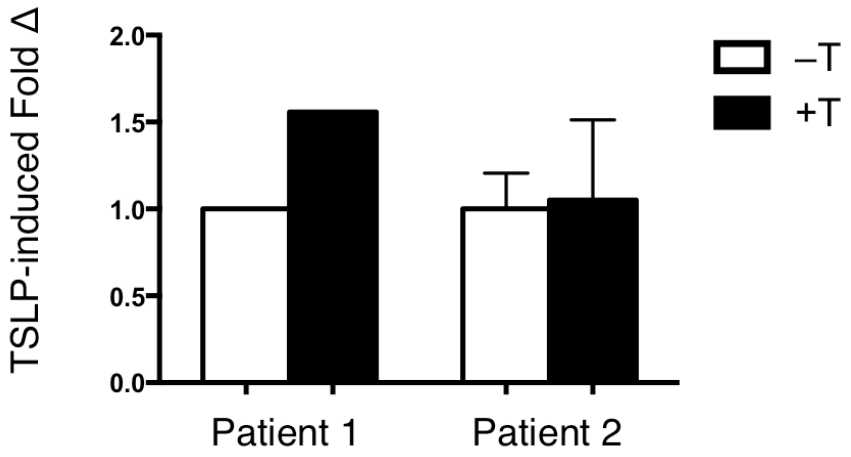


Supplementary Figure S6 (*Figure 22*). CRLF2 B-ALL leukemia cell burden in BM of –T and +T PDX. Bone marrow cells from +T and –T PDX generated from Patient 1 and Patient 2 and shown in Figure 5 and 6 were harvested, counted by hemocytometer, and stained for flow cytometry to identify living CRLF2 B-ALL cells. Total living cell counts and percentages of living CRLF2 B-ALL cells within the total living cells were used to calculate the number of CRLF2 B-ALL cells per PDX. Graphed is the fold change in living leukemia cells in +T PDX as compare to –T PDX obtained by normalizing CRLF2 B-ALL cell numbers in +T animals to the cell numbers in –T animals for each experiment.

Supplementary Table 2 (Table 3). qRT-PCR validation of genes that are differentially regulated in CRLF2 B-ALL cells expanded in +T as compared to –T xenograft mice.

Gene	Microarray (fold change)	qRT-PCR (fold change)
BIVM	4.440	**6.907**
PTH2	3.477	**2.784**
FOSL2	2.793	**1.843**
RAMP1	2.725	**2.227**
SLC4A8	2.628	**6.417**
NR4A2	2.138	**3.548**
KCNH8	2.154	**2.355**
THSD7A	–3.628	**–6.648**
RAB26	–2.727	**–2.726**
RGS1	–2.749	**–2.498**
NELL1	–3.586	**–3.143**
MDF1	–2.168	**–7.621**
KREMEN2	–2.336	**–2.003**
CPNE8	–3.008	**–4.695**

The table shows the list of genes that were selected from the microarray dataset for validation by qRT-PCR analysis and their respective fold changes as determined by microarray and by qRT-PCR.

+T PDX Show Gene Expression Profiles that are More Similar to the Original Patient Sample

Next we performed experiments to determine whether +T PDX provide an in vivo environment more like the patient than –T PDX and if this could be achieved with short term in vivo hTSLP exposure. PDX were established using CRLF2 B-ALL cells from Patient 2. Injection of stromal cells from transduction 2 were not initiated until two weeks prior to euthanasia (Figure 6A). At euthanasia BM was harvested and stained for flow cytometry to verify CRLF2 B-ALL engraftment (Figure 6B). Human leukemia cells were isolated from the BM of +T and –T PDX by magnetic separation for RNA sequencing. Differential gene expression analysis identified 503 genes that were upregulated and 117 that were downregulated at least 2-fold in CRLF2 B-ALL cells from +T as compared to –T PDX (Figure 6C). Similar to what we observed with Patient 1, GSEA analysis of RNA sequencing data showed an enrichment for genes regulated downstream of mTOR in CRLF2 B-ALL cells from +T as compared to –T PDX (FDR q-value = 0.00048, Figure 6D). A comparison of +T PDX to –T PDX showed similar immunophenotypes to each other and to that reported for the respective original Patient 1 and Patient 2 samples (Online Supplementary Table 1 and Figure S7), indicating that the differences in mTOR-regulated gene expression are unlikely due to selective survival of different clones. These data demonstrate that in vivo hTSLP-induced changes in mTOR regulated gene expression can be observed after short term stimulation and validate our model in a second primary CRLF2 B-ALL patient sample.

To determine whether +T PDX provide an in vivo environment that is more similar to the patient than –T PDX, we compared gene expression profiles of +T and –T

PDX to the original ex vivo CRLF2 B-ALL patient sample. Human leukemia cells were isolated from the BM of +T and –T PDX generated from Patient 2, and from the original primary Patient 2 sample by magnetic separation. RNA sequencing was performed to determine whole genome expression. Analysis of gene expression by unsupervised clustering and Spearman rank correlation show that gene expression in +T PDX, as compared to –T PDX, is significantly more closely correlated to the original patient sample (Figure 6E). These data validate the use of +T/–T xenograft mice as an in vivo preclinical model system for studies to understand the role of hTSLP in normal human B cell production and in the development and progression of CRLF2 B-ALL.

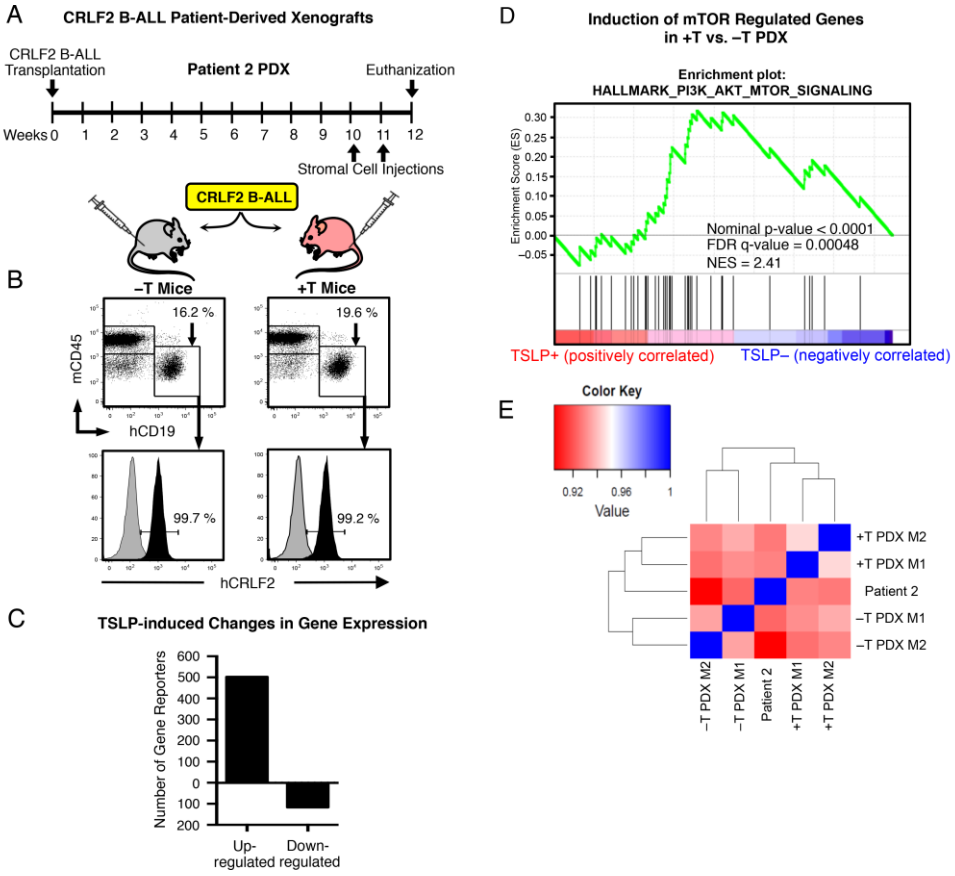
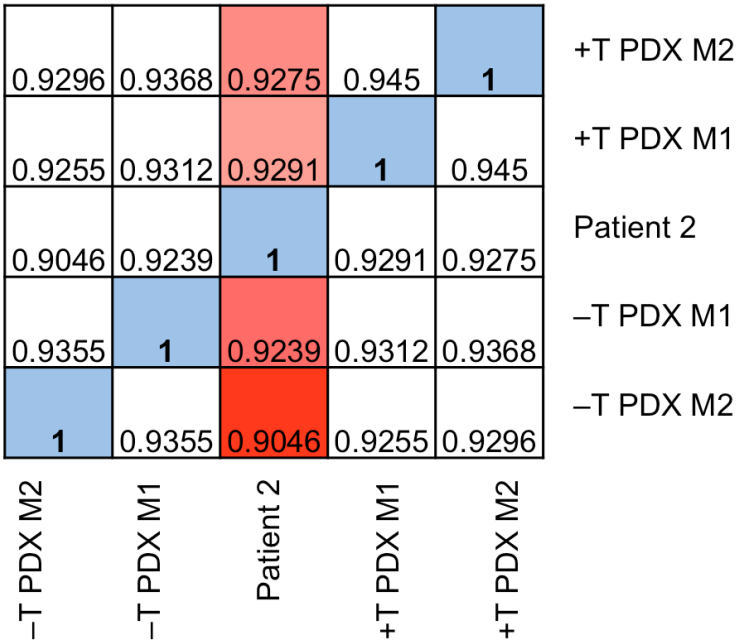
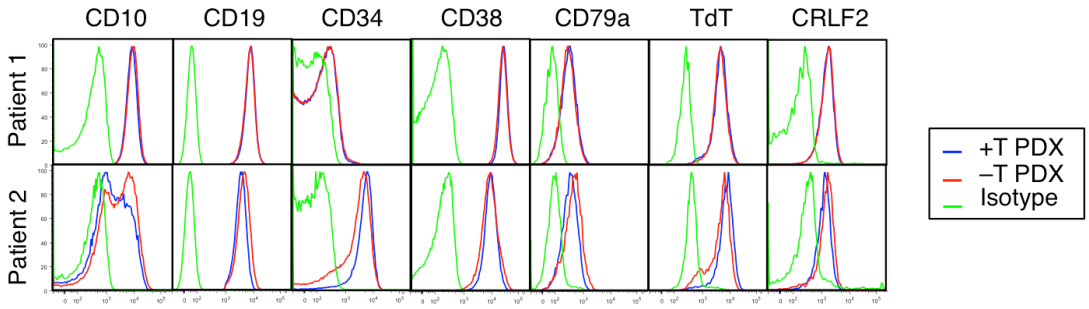

A
CRLF2 B-ALL Patient-Derived Xenografts
CRLF2 B-ALL Transplantation
Patient 2 PDX
Euthanization
Weeks 0 1 2 3 4 5 6 7 8 9 10 11 12
Stromal Cell Injections
CRLF2 B-ALL
B
–T Mice
+T Mice
16.2 %
19.6 %
mCD45
hCD19
99.7 %
99.2 %
hCRLF2
C
TSLP-induced Changes in Gene Expression
Number of Gene Reporters
600
500
400
300
200
100
0
100
200
Up-regulated
Down-regulated
D
Induction of mTOR Regulated Genes in +T vs. –T PDX
Enrichment plot:
HALLMARK_PI3K_AKT_MTOR_SIGNALING
Enrichment Score (ES)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
–0.05
Nominal p-value < 0.0001
FDR q-value = 0.00048
NES = 2.41
TSLP+ (positively correlated)
TSLP– (negatively correlated)
E
Color Key
0.92
0.96
1
Value
+T PDX M2
+T PDX M1
Patient 2
–T PDX M1
–T PDX M2
–T PDX M2
–T PDX M1
Patient 2
+T PDX M1
+T PDX M2

Figure 6 (*Figure 23*). +T PDX show gene expression profiles that are more similar to the original patient sample. (A) –T and +T NSG mice were transplanted with primary CRLF2 B-ALL cells from Patient 2. Beginning at ten weeks after leukemia transplant mice received two injections with $5X10^6$ stromal cells (transduction 2 stroma) one week apart. (B) Twelve weeks post-transplant, mice were euthanized, BM was harvested and cells were stained with mCD45 and hCD19 to identify human B-ALL cells. CRLF2 staining (black overlay) on gated hCD19+ cells is shown in bottom panels. Isotype control is shown in gray. (C) Human hCD19+ B-ALL cells were isolated by magnetic separation and RNA sequencing with differential gene expression analysis was performed to identify genes that are differentially regulated in +T as compared to –T PDX. Graphed are the numbers of significantly up or down-regulated gene (≥2 fold, p≤ .05) in CRLF2 B-ALL cells from +T as compared to –T PDX. (D) GSEA of RNA sequencing data shows that CRLF2 B-ALL cells harvested from +T PDX exhibit an increase in expression of genes regulated downstream of mTOR signaling as compared to cells from –T PDX. The top half of the GSEA enrichment plot shows the enrichment score for each gene and the bottom half shows the values of the ranking metric moving down the list of the ranked genes. (E) RNA sequencing was performed on CRLF2 B-ALL cells isolated by magnetic separation from 2 +T PDX mice (+T PDX M1 and M2) and 2 –T PDX mice (–T PDX M1 and M2) and from the original Patient 2 primary sample used to generate them**.** Graphed is a dendrogram of unsupervised hierarchical clustering and heatmap of Spearman rank correlation values showing that gene expression in +T PDX is significantly more closely correlated to the original sample than is –T PDX. p<.0001. Spearman rank coefficients are shown in Online Supplementary Figure S5. Significance of differences in correlation coefficients was determined using Fisher's r-to-z transformation.



Supplementary Figure S5 (*Figure 24*). Spearman rank coefficients for comparisons of gene expression in +T and –T PDX to original patient sample. A) CRLF2 B-ALL cells from Patient 2 were used to generated –T and +T PDX mice as described in Figure 6. RNA sequencing was performed on CRLF2 B-ALL cells isolated from 2 +T mice (+T PDX M1 and M2) and 2 –T PDX mice (–T PDX M1 and M2) and from the original Patient 2 primary sample used to generate them. Spearman rank coefficients are shown for comparison of gene expression in +T and –T PDX and in the primary patient sample. The comparisons between +T and –T PDX and the primary patient sample are highlighted to match the heatmap in Figure 6E.



Supplemental Figure S7 (*Figure 25*). Surface immunophenotype CRLF2 B-ALL from –T and +T PDX. +T and –T PDX were stained for flow cytometry to determine immunophenotype with respect to marker expression on the original primary CRLF2 B-ALL patient sample (see Online Supplementary Table 1). Plotted is expression of indicated markers on gated living human CD19+ leukemia cells in –T and +T PDX samples from Patient 1 and Patient 2

.

Discussion

PDX provide a critical complement to other models in understanding normal and malignant human B lymphopoiesis in context of the broad background of human genetic variation. A limitation of PDX is the reduced cross-species activity of some cytokines. Classic immune deficient mice, including NSG mice, selectively support B lineage differentiation. This is because IL-7, a cytokine important for human B cell production (Parrish et al., 2009) shows high species cross reactivity (Johnson, Shah, Panoskaltsis-Mortari, & LeBien, 2005) while myeloid supportive cytokines do not. Here we show that mTSLP does not induce the increases in STAT5 and PI3K/AKT/mTOR phosphorylation induced by hTSLP in CRLF2 B-ALL cells (Figure 1). CRLF2 B-ALL cells express abundant IL-7Rα and CRLF2 as compared to normal B cell precursors (Figure 1 and S1) and thus provide a sensitive test for mTSLP activity. These data establish that the classic mouse xenograft models do not provide cross-species activation of the human TSLP receptor.

The goals of the studies described here were to develop a xenograft model that expresses hTSLP and to validate the effects of hTSLP produced in the model on normal and malignant B cell precursors. We engineered NSG mice that express normal serum levels of hTSLP (+T mice) or that lack hTSLP (–T mice) (Figure 2) by intraperitoneal injection with stroma transduced to express hTSLP (+T stroma) or with a control vector (–T stroma) (Figure 2). hTSLP produced by +T stroma induced in vitro STAT5 and PI3K/AKT/mTOR pathway activation (Figure 2) and showed *in vivo* functional effects as indicated by selective expansion of normal B cell precursors (Figure 3-4, S3, S4) and upregulation of the mTOR pathway in primary CRLF2 B-ALL cells (Figure 5-6). The

studies described here establish and validate a novel hTSLP+/– xenograft model system while providing the first data on the *in vivo* impact of hTSLP on normal B cell development and primary CRLF2+ B-ALL cells.

Normal serum levels of hTSLP were achieved in our model, however, a novel aspect of our model is the ability to modulate serum levels of hTSLP based on the timing and number of stromal cells injected intraperitoneally. The ability to vary hTSLP levels or initiate hTSLP at time points after transplant of hematopoietic cells offers advantages for experimental design. The ability to modulate serum levels of hTSLP is also biologically relevant because TSLP production is increased by environmental factors such as pollutants and allergens and is upregulated in some autoimmune diseases and solid tumors (Ziegler et al., 2013). TSLP is primarily produced by cells residing outside the BM (immune cells, smooth muscle cells, gut and skin epithelial cells),(Takai, 2012) although human BM stroma do produce hTSLP (Allakhverdi et al., 2013). Thus, the major source of hTSLP that acts on normal and malignant B cells *in vivo* in patients is likely to be produced outside the marrow as it is in our model.

Little is known about cellular mechanisms that contribute to the initiation, progression and maintenance of CRLF2 B-ALL or the biological factors that contribute to the health disparities associated with this disease. Studies of the relationship between inherited genetic variation and susceptibility to ALL provide some clues to the relationship of ALL to ethnicity (Xu et al., 2013). Patient-derived xenograft (PDX) models are particularly critical for health disparities diseases where inherited genetic variation or alterations such as Down Syndrome are likely to play a role in disease progression (Liem et al., 2004; Lock, Liem, & Papa, 2005). The ability to generate PDX

mice that provide hTSLP make it possible to study the initiation, progression and maintenance of CRLF2 B-ALL from normal human progenitors transduced to overexpress CRLF2 on a range of genetic backgrounds.

The +T and –T PDX generated in the studies described here did not show consistent differences in the leukemia cell burden (Online Supplementary Figure S6). However, changes in gene expression that contribute to chemoresistance would not necessarily be expected to impact leukemia burden in untreated PDX, but would instead be revealed following chemotherapy. CRLF2 B-ALL is a leukemia at high risk for relapse and the mTOR pathway is known to play a role in chemoresistance in multiple malignancies (Jiang & Liu, 2008). The ability of +T PDX to model TSLP-induced mTOR activation makes this an important model for understanding and identifying therapeutic strategies for overcoming chemoresistance in CRLF2 B-ALL. Our finding that gene expression +T PDX is significantly more like the original patient sample than –T PDX (Figure 6E, S5) support the use of +T PDX mice as a preclinical model of CRLF2 B-ALL. PDX generated from +T and –T mice and CRLF2 B-ALL samples obtained from Hispanic or Down Syndrome pediatric patients will allow us to study disease mechanisms 1) in context of a genetic background associated with CRLF2 B-ALL health disparities and 2) in context of TSLP-CRLF2 mediated signals present *in vivo* in the patient.

The model developed here provides an important new tool for understanding the mechanisms of TSLP activity in normal and malignant B lymphopoiesis. It also provides a preclinical model for identifying therapies to effectively treat CRLF2 B-ALL in an environment that can induce the TSLP-mediated signals present *in vivo* in patients and in context of the genetic background that leads to health disparities in this disease.

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CHAPTER FOUR

TSLP OR IL-7 PROVIDE AN IL-7Rα SIGNAL THAT IS CRITICAL FOR HUMAN B LYMPHOPOIESIS

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Abstract

Thymic stromal lymphopoietin (TSLP) and IL-7 are cytokines that signal via the IL-7 receptor alpha (IL-7Rα) to exert both overlapping and unique functions during early stages of mouse B cell development. In human B lymphopoiesis the requirement for IL-7RA signaling is controversial and the roles of IL-7 and TSLP are less clear. Here we evaluated human B cell production using novel in vitro and xenograft models of human B cell development that provide selective IL-7 and human TSLP (hTSLP) stimulation. We show that in vitro human B cell production is almost completely blocked in the absence of IL-7RA stimulation and that either TSLP or IL-7 can provide a signal critical for the production and proliferation of human CD19+ PAX5+ pro-B cells. Analysis of primary human bone marrow (BM) stromal cells show that they express both IL-7 and TSLP providing an in vivo source of these cytokines. Using novel xenograft models we show that the in vivo production of human pro-B cells under the influence of mouse IL-7 is reduced by anti-IL-7 neutralizing antibodies, and this loss can be restored by hTSLP at physiological levels. These data establish the importance of IL-7Rα-mediated signals for normal human B cell production.

Abbreviations

BM	Bone Marrow
CB	Umbilical Cord Blood
FMO	Fluorescence Minus One
hTSLP	Human Thymic Stromal Lymphopoietin
IL-7Rα	IL-7 Receptor Alpha
TSLP	Thymic Stromal Lymphopoietin

Introduction

IL-7Rα signaling is required for B lymphopoiesis in adult mice, although fetal B cell production is IL-7RAα-independent (von Freeden-Jeffry et al., 1995). IL-7Rα is a component of two distinct cytokine receptor complexes with different secondary chains, one activated by IL-7 and the other by TSLP (Zhang et al., 2001). In adult mice where IL-7 is knocked out, there is a block in B cell precursor production that can be restored by TSLP, but only at the supra-physiological levels achieved in TSLP transgenic mice (Chappaz, Flueck, Farr, Rolink, & Finke, 2007).

The role of IL-7Rα signals in human B cell development is controversial (Corfe & Paige, 2012; Sportes, Gress, & Mackall, 2009). Patients with defective IL-7R signaling exhibit severe combined immunodeficiency (SCID) characterized by a loss of T cells (Macchi et al., 1995; Noguchi et al., 1993; Puel, Ziegler, Buckley, & Leonard, 1998; Russell et al., 1995). Although B cell function may be compromised in these patients, B cell numbers are normal (Giliani et al., 2005), suggesting that human B lymphopoiesis differs from that in mice with respect to the requirement for IL-7Rα-mediated signals. However, these data are obtained during the first year of life consistent with fetal B cell production through an IL-7Rα-independent process similar to mouse. Early in vitro studies also suggested that human B cell production is IL-7-independent, however, these were often performed using fetal hematopoietic sources or culture models that produced mouse IL-7 which was later shown to activate human IL-7Rα (Johnson, Shah, Panoskaltsis-Mortari, & LeBien, 2005). Although fetal B cell production is not dependent on IL-7 or TSLP, both of these cytokines can stimulate in vitro proliferation of human fetal B cell precursors (Scheeren et al., 2010).

Our previous in vitro studies, performed using a human-only co-culture model, showed that human B cell production beyond the fetal period is increasingly dependent on a signal that can be provided by IL-7 (Parrish et al., 2009). The ability of TSLP to replace IL-7 in providing this signal is unknown and the importance of IL-7R–IL-7Rα stimulation for in vivo human B lymphopoiesis has not been demonstrated. Recently we developed a novel xenograft model and it showed that physiological levels of human TSLP (hTSLP) increase human B cell production, in vivo, in context of IL-7 (Francis et al., 2015). Here we use novel in vitro and xenograft models, engineered to provide selective IL-7 and hTSLP stimulation, to evaluate the requirement for IL-7R IL-7Rα signals in post-fetal human B cell production and the ability of hTSLP to induce these signals at physiological levels.

Results and Discussion

TSLP or IL-7 can Provide a Signal Critical for the In Vitro Production of Human B Cell Precursors

To investigate the role of IL-7Rα signals induced by TSLP and IL-7 in early stages of human B cell production, we developed a human-only co-culture model that supports the production of human pro-B cells and provides selective IL-7 or TSLP stimulation. Human umbilical cord blood (CB) CD34+ cells were cultured with primary human bone marrow (BM) stromal cells in media supplemented with human serum. Selective cytokine stimulation was achieved with combinations of exogenous human IL-7 or TSLP and with neutralizing antibodies to counter activity of endogenously produced IL-7 and TSLP (Fig 1A). CB CD34+ cells were grown under selective cytokine

conditions for 3 weeks. Culture progeny were harvested and stained for flow cytometry to identify CD19+ PAX5+ B lineage cells. Control cultures that lacked TSLP or IL-7 produced small numbers of B lineage cells (Fig 1B). In contrast, TSLP and IL-7 cultures showed robust production of CD19+ PAX5+ B lineage cells (Fig 1B). Cultures with TSLP or IL-7 produced similar numbers of B cell precursors (Fig 1C).

To determine if TSLP-induced proliferation contributes to the increased production of human B cell precursors from CB CD34+ cells, we compared BrdU incorporation in cells generated under selective cytokine conditions (Fig 1D). Proliferation in TSLP cultures was significantly increased as compared to control cultures lacking IL-7Rα stimulation and similar to cultures containing IL-7 (Fig 1E). These data demonstrate that TSLP can replace IL-7 in providing signals critical for *in vitro* production and proliferation of human CD19+ PAX5+ pro-B cells and that human B cell production is almost completely blocked in the absence of IL-7Rα stimulation.

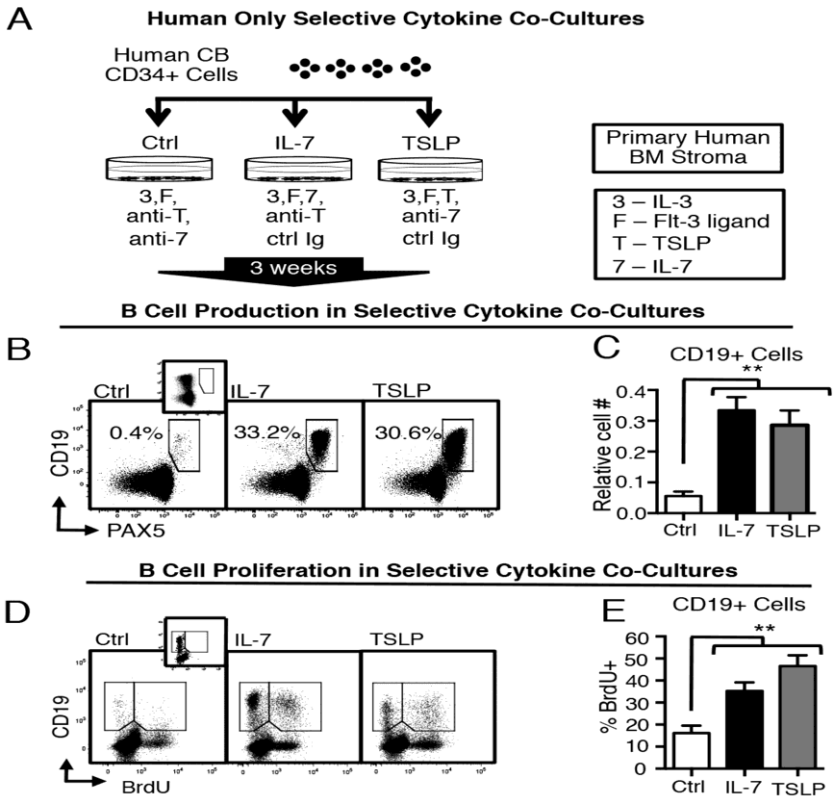


Figure 1 (*Figure 26*). TSLP can replace IL-7 in supporting the in vitro production and proliferation of human CD19+ PAX5+ B cell precursors. (A) Human-only cultures were produced by plating primary human CB CD34+ cells on primary human BM stroma. Selective cytokine stimulation was achieved by supplementing cultures with exogenous IL-7 or TSLP or neutralizing antibodies to TSLP (anti-T) or IL-7 (anti-7) as indicated. Cultures without a particular neutralizing antibody were supplemented, as a control, with non-specific isotype-matched antibodies (ctrl Ig). All culture conditions included IL-3 and Flt-3 ligand. Cultures were maintained for 3 weeks, then harvested and stained for flow cytometry. (B) Dot plots of CD19 vs. PAX5 staining in each culture condition are shown (representative of n=3 independent experiments). PAX5 fluorescence minus one (FMO) control is displayed in inset. (C) Graphed are the relative numbers of CD19+ cells (mean± SEM) generated in vitro under indicated conditions at three weeks (n=14). D) Representative dot plots of CD19 vs BrdU staining in indicated conditions. BrdU FMO control is displayed in the inset of the Ctrl dot plot. (E) Graphed is the percent BrdU+ cells (mean± SEM) in the total CD19+ population generated under indicated conditions at three weeks (n=8). Statistical analysis was performed using one-tailed, t-test, *$p<0.05$, **$p<0.001$. Error bars represent mean ± SEM.

Human Bone Marrow Stromal Cells Express TSLP, as well as IL-7

To determine if the BM provides an in vivo source of TSLP we evaluated the ability of primary human BM stroma to produce TSLP. For comparison we evaluated IL-7, which we have previously shown to be expressed by human BM stroma (Parrish et al., 2009). RT-PCR analysis of stromal cells cultured from healthy donors showed TSLP and IL-7 transcripts (Fig 2A) that resulted in protein production as evidenced by TSLP and IL-7 in supernatants harvested from cultured cells (Fig 2B). These data demonstrate that human BM stroma provide an in vivo source of both cytokines in the normal human BM microenvironment where B cell precursors are generated.

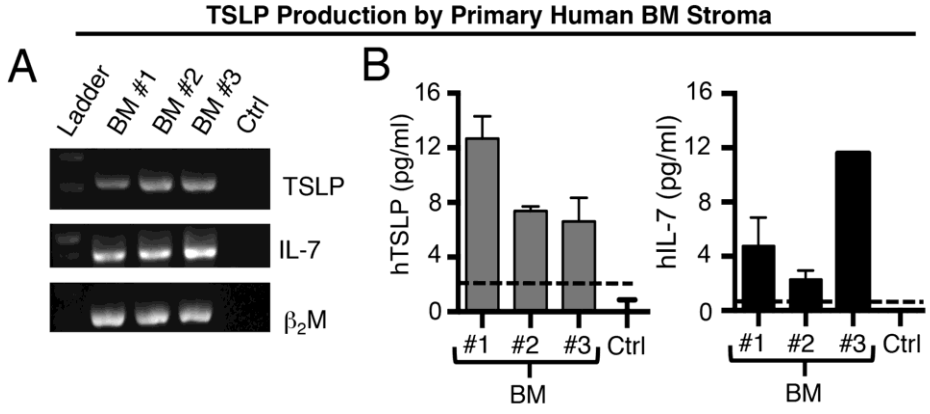


Figure 2 (*Figure 27*). IL-7 and TSLP are produced by human BM stroma. (A) RT-PCR, was used to detect TSLP, IL-7 or beta-2 microglobulin (β_2M, control) transcripts in primary human BM stromal cells from different human donors – pediatric (BM #1 and BM #2) and adult (BM #3) or culture medium as a negative control. Each patient sample was assessed in two or more different PCR reactions. (B) Supernatant from confluent BM stromal cell cultures were assessed by ELISA for TSLP and IL-7 protein production. Data are expressed as mean ± SEM of triplicate values for TSLP, and duplicate values for IL-7. Dashed lines (---) represent ELISA threshold of detection.

IL-7Rα Signals Induced by TSLP or IL-7 Increase the In Vivo Production of Human B Cell Precursors

Next we evaluated the *in vivo* role of IL-7Rα-mediated signals in human B cell development and the ability of physiological levels of TSLP to replace IL-7 in the induction of these signals. Mouse TSLP does not show cross-species activity on human cells (Francis et al., 2015) although IL-7 does (Barata et al., 2006; Johnson et al., 2005). Thus, classic xenografts provide IL-7, but not TSLP that can stimulate IL-7Rα signals. We used a novel human-mouse xenograft that provides normal serum levels of hTSLP (+T mice) and control (–T mice) that lack hTSLP (Francis et al., 2015) as an in vivo system for human B cell precursor production. To study in vivo human B cell precursor production under selective IL-7 and hTSLP stimulation, we established +T and –T xenografts with CB CD34+ cells and treated for two weeks with antibodies that neutralize mouse and human IL-7 (Grabstein et al., 1993) (Fig 3A). This allowed us to compare B lymphopoiesis in mice with 1) no hTSLP and reduced IL-7 (–T–7 mice); 2) no hTSLP and normal IL-7 (–T+7 mice); and 3) physiological hTSLP and reduced IL-7 (+T–7 mice).

Human progenitors and B cell subsets in the bone marrow of xenograft mice were identified by flow cytometry (Fig S1 in online Supporting Information) and cell numbers were compared (Fig 3). No differences in numbers of human hematopoietic stem cells or multi-potential progenitors was observed, including the earliest IL-7R+ progenitors (CD19–CD34+IL-7R+) (Fig 3B). In contrast, mice with normal IL-7 or with physiological levels of hTSLP showed a human pro-B compartment that was increased by 2-3 fold as compared to that observed in –T–7 mice (Fig 3C). Increases observed with

physiological hTSLP extended to the pre-B cell stage, although significant increases in pre-B cells were not achieved in –T+7 mice (Fig 3C). Later stages of B cell development were not impacted, which was not surprising, given the short anti-IL-7 treatment period (Fig 3C). No significant differences were detected in the numbers of non-B cells generated in mice with hTSLP or IL-7 as compared to –T–7 xenografts (Fig 3D). T lineage cells are not generated in xenograft mice at this time point and so were not a part of the non-B lineage compartment. These data show that the in vivo production of human pro-B cells under the influence of IL-7 is reduced by anti-IL-7 neutralizing antibodies, and this loss can be restored by hTSLP at physiological levels.

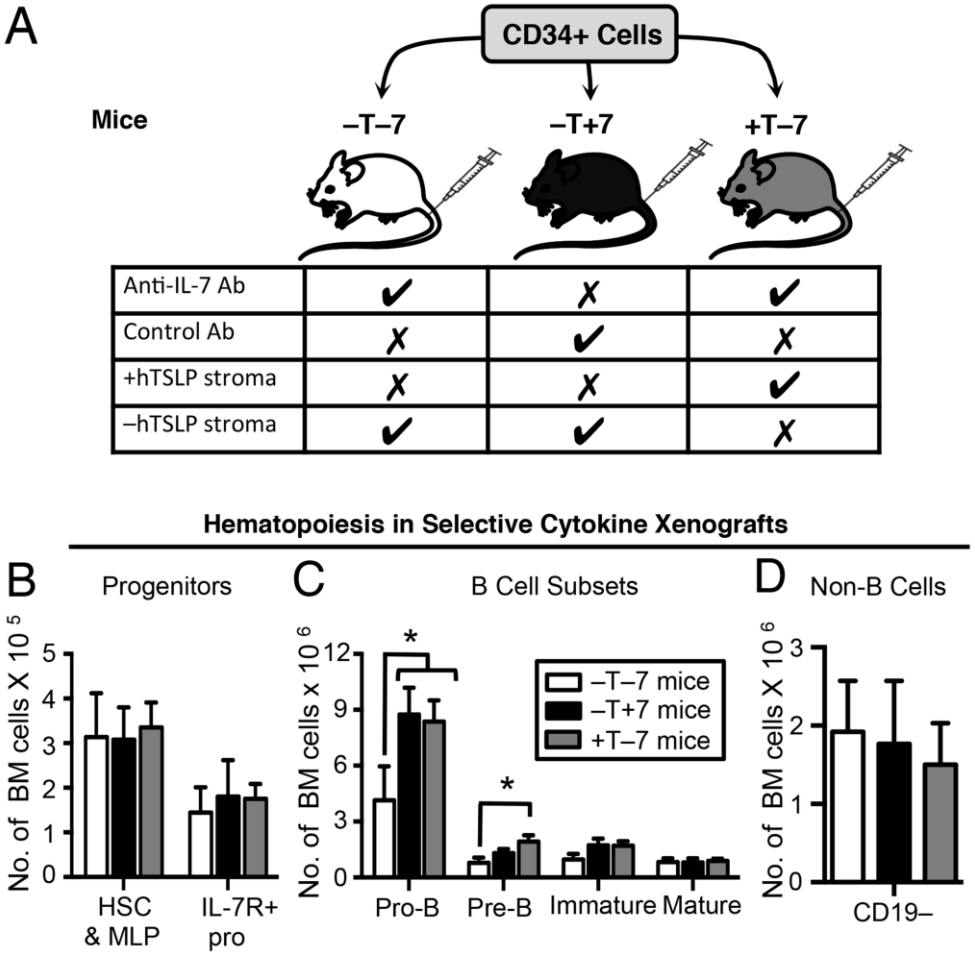
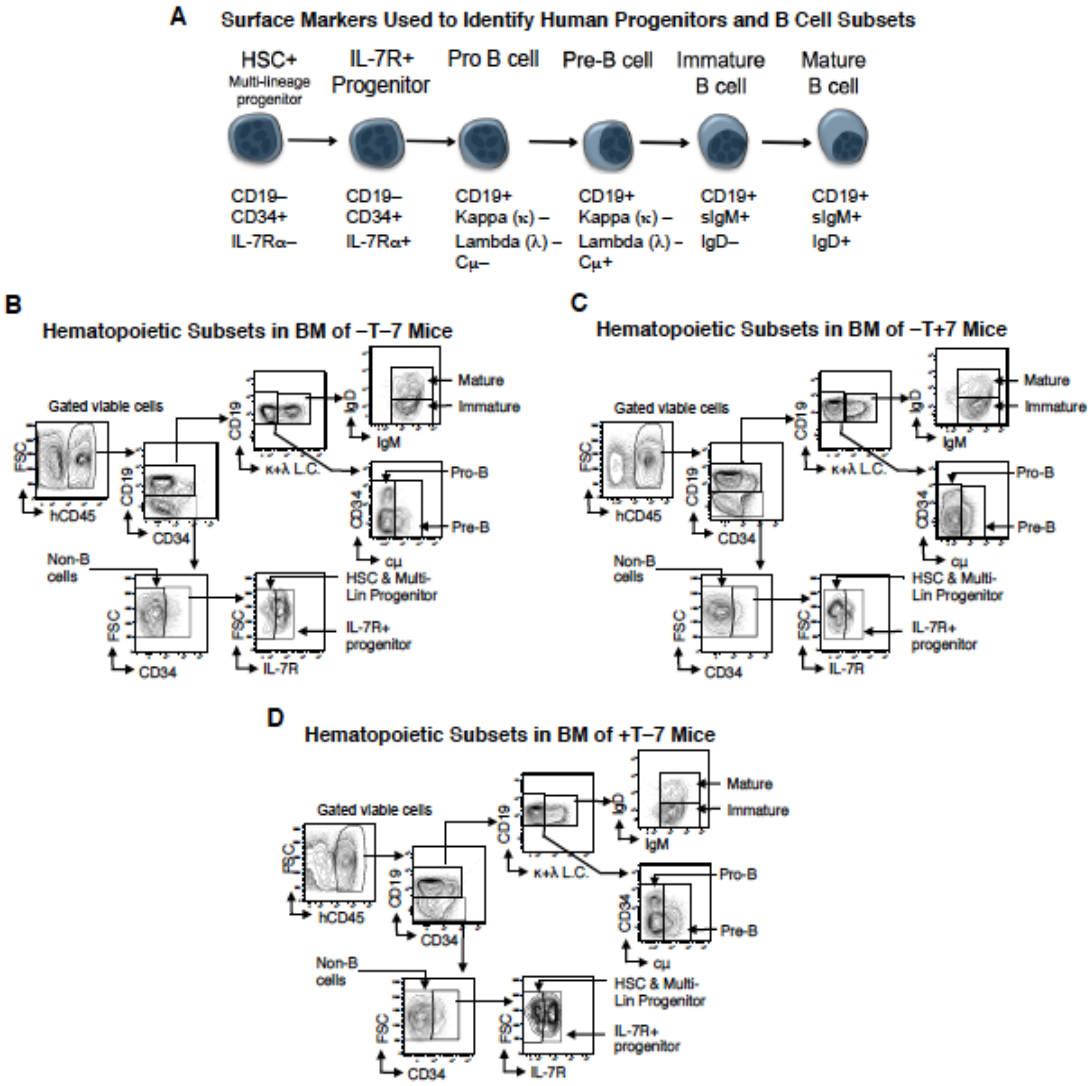


Figure 3 (*Figure 28*). IL-7 and TSLP increase the in vivo production of human B cell precursors in human-mouse xenografts. (A) Immune deficient NSG mice were engineered to express physiological levels of hTSLP (+T mice) or without hTSLP (–T mice) as described (Francis et al., 2015). CB CD34+ cells were injected by tail vein into –T and +T mice. Five weeks later, –T mice and +T mice were treated for two weeks with anti-human/mouse IL-7 antibody or isotype-matched control antibody to generate –T–7 mice (no hTSLP and reduced IL-7), –T+7 mice (no hTSLP and normal IL-7), and +T–7 mice (physiological hTSLP and reduced IL-7). At 7 weeks post-transplant, mice were euthanized and BM harvested and stained for human specific markers to identify hematopoietic subsets (for gating see Fig S1 in online Supporting Information.) Graphed are the absolute numbers of cells in (B) progenitor populations, (C) B cell subsets, and (D) non-B cells in the BM of xenograft mice. Data shown were obtained from two different CB donors with a total of four mice per group. Statistical analysis was performed using one-tailed, unpaired t-test, *$p<0.05$ compared to –T–7 mice. Error bars represent mean ± SEM.



Supplemental Figure S1 (*Figure 29*). Flow cytometry to identify hematopoietic subsets in xenografts with selective IL-7 and hTSLP stimulation. Human CB CD34+ cells were injected by tail vein into mice engineered to express hTSLP (+T mice) or that lacked hTSLP (–T mice) as described in methods and previously reported (Francis, et al 2015). Beginning 5 weeks after injection of CD34+ cells, mice were treated for two weeks with neutralizing anti-human/mouse IL-7 antibody or isotype-matched control antibody to generate mice with no hTSLP and reduced IL-7 stimulation (–T–7 mice); mice with no hTSLP and normal IL-7 (–T+7 mice); and mice with physiological hTSLP and reduced IL-7 (+T–7 mice). Mice were euthanized seven weeks post-transplant and BM harvested and stained for human specific markers to identify progenitors, developmentally sequential B cell subsets and non-B cells in the BM of indicated xenograft groups. (A) Surface immunophenotypes used to identify indicated hematopoietic subsets in xenograft mice. (B-D) Flow cytometry plots showing gating and representative data from n=4 xenografts in each group (B) –T–7 xenografts (C) –T+7 xenografts and (D) +T–7 xenografts. Gates were set based on isotype controls.

Concluding Remarks

In summary, we used in vitro selective cytokine cultures to show that the production of human B lineage cells beyond the fetal period is dependent on an IL-7Rα-mediated signal that can be provided by either TSLP or IL-7. We demonstrate that both of these cytokines are produced by normal human BM stroma. The validity of these findings for in vivo B lymphopoiesis is supported by the reduction in human B cell precursors observed following treatment with IL-7 neutralizing antibodies in xenografts and the ability of hTSLP to restore this loss.

These studies provide in vivo evidence that IL-7Rα signals are required for normal human B cell production and proliferation beyond the fetal period and suggest that TSLP can replace IL-7 in providing these signals. Our data provide a potential explanation for the recent report that B lineage commitment is not blocked in SCID patients who have defects in IL-7 signaling components (common gamma chain and JAK3), but have an intact TSLP receptor signaling pathway (Kohn et al., 2014). Our studies suggest that therapies to stimulate or block IL-7- and TSLP-mediated IL-7Rα signals are likely to impact B cell production. Consistent with this, data from clinical trials showing that the administration of recombinant human IL-7 can increase newly formed B cells in the periphery and expand the bone marrow lymphoid compartment (Mackall, Fry, & Gress, 2011).

Materials and Methods

Additional details are available in the online Supporting Information.

Human Samples

Umbilical CB and BM were obtained in accord with Loma Linda University Institutional Review Board (IRB) protocols and the Helsinki Declaration of 1975, as revised in 2008. Isolation of CB CD34+ cells and detection of TSLP production in BM stroma are described in online supplementary methods.

Selective-Cytokine Co-Cultures

CB CD34+ cells were seeded on primary human BM stroma and maintained as described (Parrish et al., 2009) with media containing 5% human AB serum (Omega Scientific, Tarzana, CA). All cultures, including control, were supplemented with IL-3 and Flt-3 Ligand. For selective cytokine stimulation, hTSLP and/or IL-7 (R&D Systems Inc., Minneapolis, MN) were added. If neither TSLP nor IL-7 was added, cultures were further supplemented with neutralizing antibodies to counter activity of endogenously produced TSLP and/or IL-7. Cultures without cytokine-specific neutralizing antibody were supplemented with an isotype-matched control. Co-cultures were harvested at 3 weeks. BrdU (Sigma-Aldrich, St. Louis, MO) was added for the final 24 hours in some cultures.

Animal Studies

Studies were performed using NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (Jackson Laboratory) under protocols approved by the Loma Linda University Institutional Animal Care and Use Committee (IACUC). Mice were engineered by

intraperitoneal injection with stroma transduced to express hTSLP (+T mice) or with control vector (–T mice) as described (Francis et al., 2015). +T and –T mice were transplanted with CD34+ cells by tail vein injection, after sub-lethal irradiation. Five weeks after CB transplantation, anti-mouse/human IL-7 neutralizing antibody or isotype-matched control (BioXCell, West Lebanon, NH) was injected as described in previous studies that used this anti-IL-7 antibody to inhibit in vivo mouse B lymphopoiesis (Grabstein et al., 1993).

Supplementary Methods

Human Samples

Umbilical cord blood (CB) and bone marrow (BM) were obtained in accordance with protocols approved by the Loma Linda University Institutional Review Board (IRB) and with the Helsinki Declaration of 1975, as revised in 2008. Umbilical cord blood (CB) was obtained from placentas after caesarean section delivery of full-term neonates at Loma Linda University Medical Center. Mononuclear cells were isolated using Ficoll-Paque Plus (GE Healthcare Bio-Sciences, AB) or by red blood cell (RBC) lysis (Kang et al., 2002). CD34+ cells were isolated by magnetic separation with CD34+ microbeads (Miltenyi Biotec, Auburn, CA) and stored in liquid nitrogen for subsequent use. Pediatric BM samples were obtained as waste samples remaining after clinical procedures under an LLU Institutional Review Board (IRB)-approved protocol. Adult BM and stromal cells were purchased from Lonza Inc. (Walkersville, MD). Stromal cells were isolated from pediatric and adult BM as previously described (Nolta et al., 2002) and maintained in IMDM medium supplemented with 15% horse serum, 15% FBS, hydrocortisone, 2-mercaptoethanol, penicillin-streptomycin and L-glutamine.

In Vitro Co-Cultures

Human-only in vitro co-cultures were initiated as previously described (Parrish et al., 2009) which primarily yield pro-B cells. Briefly, CB CD34+ cells were seeded onto pre-plated primary human BM stromal cells, at 10,000 to 20,000 cells per well in 96-well plates. For some experiments, numbers were scaled up to give equivalent cell densities in 6-well and 12-well plates. Cultures were maintained in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with penicillin/streptomycin, L-glutamine, 50 uM 2-mercaptoethanol and 5% human AB serum lot tested to support human B cell production (Omega Scientific, Tarzana, CA). All cultures, including control, were supplemented with IL-3 (1 ng/ml; first week only) and Flt-3 Ligand (1 ng/ml, continuously), two cytokines that support early lymphoid progenitors (Crooks, Hao, Petersen, Barsky, & Bockstoce, 2000; Shah, Smogorzewska, Hannum, & Crooks, 1996). For selective cytokine stimulation human TSLP (10 ng/ml) or human IL-7 (5 ng/ml) or no cytokines (control cultures) were added. All cytokines were from R&D Systems Inc. (Minneapolis, MN). If TSLP or IL-7 was not added, cultures were further supplemented with an anti-human neutralizing antibody to counter activity of any endogenously produced TSLP or IL-7. Cultures without a specific neutralizing antibody (anti-IL-7 or anti-TSLP) were supplemented with an isotype-matched control for the neutralizing antibody. Anti-human neutralizing or control antibody for IL-7 was used at 10 ng/ml (BD Pharmingen, Franklin Lakes, NJ). Anti-human neutralizing or control antibody for TSLP was used at 1 ug/ml (R&D Systems and Jackson ImmunoResearch, West Grove, PA). Media, cytokines, and antibodies were replenished weekly. Cultures were maintained for

three weeks, and then harvested. For BrdU incorporation, selective cytokine cultures were labeled with 10 □M BrdU (Sigma-Aldrich, St. Louis, MO) for the final 24 hours of culture.

Flow Cytometry

For detection of surface antigens, cells were stained using standard flow cytometry protocols, followed by fixation and permeabilization as described below to detect intracellular antigens. To discriminate living from dying cells, in some experiments, cells were stained with fixable viability dyes (eBioscience, San Diego, CA), per manufacturer's instructions.

To assess intracellular IgM, the Fix & Perm® cell fixation and permeabilization kit (Life Technologies, Grand Island, NY)) was used according to manufacturer's instructions. All antibodies were monoclonal, anti-human antibodies, unless otherwise stated. Intranuclear detection of BrdU was performed using BrdU Flow Kit option no. 2 (BD Pharmingen) according to manufacturer's instructions. Briefly, cells were harvested, surface stained, washed then fixed with BD cytofix/cytoperm buffer (BD Biosciences) for 15-30 minutes at room temperature. Cell suspensions were washed, then stored overnight in freezing medium (90% FBS and 10% DMSO) at –80ºC. The following day, thawed cells were washed, re-fixed in BD cytofix/cytoperm buffer and washed with BD perm/wash buffer. Cells were treated with DNase (300 ug/ml, Sigma-Aldrich**)** for 1 hour at 37ºC, washed then stained with anti-BrdU FITC or isotype-matched control for 20 minutes at room temperature. Cells were washed then resuspended in 1% PFA for acquisition. To assess intracellular PAX-5, cells were first surface stained for viability

and surface markers, then fixed and permeabilized with FoxP3 fix/perm buffer (eBioscience) for 30-60 minutes at 4ºC in the dark. Cells were washed, resuspended in permeabilization buffer and stained with PAX-5 PE or isotype-matched controls for 30 minutes at room temperature. Cells were washed then resuspended in 1% PFA for acquisition. In some experiments, cells were simultaneously stained for PAX-5 and BrdU. In these experiments cells were first stained for PAX-5 according to manufacturer's directions, followed by BrdU staining according to manufacturer's instructions.

Antibody	**Clone**	**Manufacturer**
BrdU FITC	B44	BD Biosciences (San Jose, CA)
Ig κ light chain FITC	G20-193	
Ig λ light chain FITC	JDC-12	
IgM PE Cy-5	G20-127	
CD19 APC	HIB19	
CD34 PerCP	8G12	
CD34 APC	8G12	
CD34 APC.Cy7	581	Biolegend (San Diego, CA)
IgD PE	IA6-2	
CD19 APC	HIB19	eBioScience (San Diego, CA)
CD19 PE.Cy7	SJ25C1	
CD45 PE.Cy7	HI30	
CD127 PE	eBioRDR5	
Ig κ light chain eFluor 450	TB28-2	
Ig λ light chain eFluor 450	1-155-2	
PAX-5 PE	1H9	
Anti-Mouse CD45 FITC	30F11	Miltenyi Biotec (Auburn, CA)
CD19 APC	LT19	
CD34 APC	AC136	
CD127 PE	MB15-18C9	

Polymerase Chain Reaction

Total RNA was extracted from 8 to 9 x 10^5 human stromal cells with RNA-STAT 60 reagent (Tel-Test Inc., Friendswood, TX). cDNA was synthesized using Omniscript Reverse Transcriptase (Qiagen, Valencia, CA). The expression of TSLP, IL-7 and □-2 microglobulin as a control, were assessed by touch down PCR. The primers used were: TSLP forward 5'-TCG TAA ACT TTG CCG CCT AT-3' and reverse 5'-TGG TGC TGT GAA ATA TGA CCA-3' (324 bp); IL-7 forward 5'- CTC CCC TGA TCC TTG TTC TG and reverse 5'- TCA TTA TTC AGG CAA TTG CTA CC-3' (151 bp); □-2 microglobulin forward 5'-CTC GCG CTA CTC TCT CTT TC-3' and reverse 5'-CAT GTC TCG ATC CCA CTT AAC-3' primers.

Touch-down PCR conditions for TSLP were as follows: 95°C for 5 min (1 cycle); 95°C for 1 min, 61°C for 30 sec. 72°C for 1 min (2 cycles); 95°C for 1 min, 59°C for 30 sec. 72°C for 1 min (2 cycles); 95°C for 1 min, 57°C for 30 sec. 72°C for 1 min (2 cycles); 95°C for 1 min, 55°C for 30 sec. 72°C for 1 min (2 cycles); 95°C for 1 min, 53°C for 30 sec. 72°C for 1 min (2 cycles); 95°C for 1 min, 51°C for 30 sec. 72°C for 1 min (30 cycles); 72°C for 10 min (1 cycle); and final hold step of 4°C.

Touchdown PCR conditions for IL-7 were as follows: 95°C for 5 min (1 cycle); 95°C for 1 min, 63°C for 45 sec. 72°C for 1 min (2 cycles); 95°C for 1 min, 61°C for 45 sec. 72°C for 1 min (2 cycles); 95°C for 1 min, 59°C for 45 sec, 72°C for 1 min (2 cycles); 95°C for 1 min, 57°C for 30 sec. 72°C for 1 min (2 cycles); 95°C for 1 min, 55°C for 45 sec. 72°C for 1 min (2 cycles); 95°C for 1 min, 53°C for 45 sec. 72°C for 1 min (30 cycles); 72°C for 10 min (1 cycle); and final hold step of 4°C.

Amplified PCR products were visualized with a 2.2% FlashGel (Lonza, Walkerswood, MD). PCR products were purified with QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced at the Genomics Core (University of California, Riverside, CA).

Animal Studies

Studies were performed using NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (Jackson Laboratory). Mice were housed under specific pathogen-free conditions in the Loma Linda University animal facility and studied in accordance with an Institutional Animal Care and Use Committee (IACUC) approved protocols. Mice were engineered by intraperitoneal injection with HS-27 stromal cells (ATCC, Manassas, VA) that had been transduced to express human TSLP (+T mice) or transduced with empty vector (–T mice) as previously described (Francis et al., 2015). Transduction of stroma and injection of stromal cells were performed as described (Francis et al., 2015). Mice were transplanted by tail vein injection with freshly thawed, CB CD34+ cells after sub-lethal irradiation at 225 cGy. Five weeks after CB transplant, 3 mg MAb anti-mouse/human IL-7 (clone: M25) or IgG2b isotype-matched control (clone: MPC-11, BioXCell, West Lebanon, NH) was injected, i.p. every 3–4 days for 2 weeks as described in previous studies that used this anti-IL-7 antibody to inhibit in vivo mouse B lymphopoiesis (Grabstein et al., 1993). NSG mice were euthanized by CO_2 asphyxiation, seven weeks after CB CD34+ transplantation and BM was harvested and frozen for flow cytometry analysis.

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TMM performed the majority of biological experiments, analyzed and interpreted data, and participated in experimental design and writing the manuscript. RJS, OLF, SRM, and XBZ produced xenografts, IB, AJW, MNC, MCN, and ARB performed laboratory experiments. CLM provided patient samples. XB and SD provided vital reagents. CLM, BTS, JCS, DJW and SD provided conceptual advice. KJP designed experiments, analyzed and interpreted data, wrote the manuscript and was the principal investigator and takes primary responsibility for the paper. The authors report no potential conflicts of interest.

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CHAPTER FIVE

UNPUBLISHED DATA: EFFECTS OF TSLP ON B CELL DIFFERENTIATION FROM HUMAN ADULT BONE MARROW HAEMATOPOIETIC STEM CELLS

Abstract

Studies from mice suggest that during ontogeny, B cell progenitors respond differently to TSLP. The ability of TSLP to expand B cell production from adult bone marrow HSCs has not been evaluated in humans. Using an innovative human selective cytokine co-culture, we demonstrate that in the absence of IL-7, TSLP does not increase the production of B cell progenitors. This data suggests that TSLP may not be as important for adult lymphopoiesis.

Introduction

In mice and men, the bone marrow (BM) is the primary organ of post-natal B lymphopoiesis and identifying the cytokines that drive the differentiation and growth of B cell progenitors in this organ has been a major topic of study. TSLP and IL-7, members of the same cytokine family (Sims et al., 2000), were initially found to have overlapping yet distinct functions in promoting B cell development in mice. In culture, both mouse TSLP and IL-7 stimulated the proliferation of B cell precursors. TSLP facilitated differentiation to the IgM+ stage, while IL-7 supported differentiation to an earlier IgM– stage (Levin et al., 1999). In addition to that difference, data from other studies suggest that TSLP may play a role at different points in mouse ontogeny. B cell progenitors from fetal liver were TSLP-responsive, while those from the BM were responsive only after the pre-B cell receptor was expressed (Vosshenrich, Cumano, Muller, Di Santo, & Vieira,

2003, 2004). Furthermore, in the absence of IL-7, intrinsic TSLP levels were unable to sustain B cell production in the bone marrow of these (Vosshenrich et al., 2003). However when overexpressed, TSLP restored the B cell compartment in adult IL-7 deficient mice (Chappaz, Flueck, Farr, Rolink, & Finke, 2007).

While the ability of TSLP to replace IL-7 has been evaluated in the mouse, little is known about its role in modulating human B cell differentiation from HSCs. To date, a single study exists, which examined human TSLP in fetal B lymphopoiesis. Like the mouse, human fetal B cell precursors, proliferated in response to human TSLP (Scheeren et al., 2010). Our group recently found that human TSLP increased the numbers of B cell precursors generated in in vivo (Chapter 3) (Francis et al., 2015) and in vitro (Chapter 4) models of CB lymphopoiesis. Here we set out to determine if human TSLP also promoted the growth and differentiation of B cell progenitors from adult bone marrow HSCs, similarly to fetal HSCs. These preliminary findings suggest that TSLP may not be a major player in human adult B cell development.

Materials and Methods

Cell Sources

Human adult BM and stromal cells were purchased from Lonza Inc. (Walkersville, MD) and mononuclear cells were isolated by density-gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Bio-Sciences, AB) or by red blood cell (RBC) lysis buffer (Kang et al., 2002). The adult BM mononuclear cells were enriched for CD34+ hematopoietic stem cells (typically >80% purity as determined by post isolation flow cytometry analysis) using a direct CD34+ cell isolation kit (Miltenyi Biotec, Auburn, CA). CD34+ cells were frozen and stored in liquid nitrogen until further

use. Adult BM stromal cells were isolated from total BM as previously described (Nolta et al., 2002). The stromal cells were maintained in IMDM medium supplemented with 15% horse serum, 15% FBS, hydrocortisone, 2-mercaptoethanol, penicillin-streptomycin and L-glutamine. All cells were used in accordance with the Institutional Review Board (IRB) guidelines of Loma Linda University.

In Vitro Co-Cultures

Human only co-cultures were initiated as previously described (Parrish et al., 2009). Briefly, adult BM CD34+ cells were seeded unto pre-plated primary human BM stromal cells, at 20,000 to 32,000 cells per well in 96-well plates. For some experiments numbers were scaled up to give equivalent cell densities in 6-well and 12-well plate. Cultures were maintained in B cell medium [RPMI 1640 medium (Irvine Scientific), penicillin/streptomycin, L-glutamine, 50 uM 2-ME and 5 % human AB serum lot tested to support B cell production (Omega Scientific)]. Cultures were supplemented with 1 ng/ml IL-3 during the first week only and 1 ng/ml Flt-3 Ligand, 10 ng/ml TSLP and/ or 5 ng/ml IL-7. All cytokines were from R&D Systems Inc. (Minneapolis, MN). Cultures were further supplemented with anti-human neutralizing or control antibody for Flt3-Ligand at 0.05 ug/ml (R&D Systems); anti-human neutralizing or control antibody for IL-7 at 10 ng/ml (BD Pharmingen); or anti-human neutralizing or control antibody for TSLP at 1 ug/ml (R&D Systems and Jackson ImmunoResearch, respectively). Each week, half of the old medium was removed and replaced with fresh medium supplemented in the same way. Neutralizing antibodies were added at saturating concentration. Cultures were maintained for three weeks then harvested.

Flow Cytometry

In vitro cultures were harvested, surface stained then resuspended in 1% PFA as previously described (Zhang et al., 2005). Data were acquired on either a FACSCalibur (BD Immunocytometry Systems) or MACSQuant® (Miltenyi Biotec), and then analyzed with FlowJo software v8 or v9 (FlowJo, Ashland, OR). All antibodies are monoclonal, anti-human antibodies unless otherwise stated. Clones are included in parentheses. From BD Biosciences (San Jose, CA): CD19 APC (HIB19), CD24 FITC (ML5), CD34 PerCP (8G12), and IgM PE.Cy5 (G20-127). From Biolegend (San Diego, CA): CD19 Pacific Blue (HIB19), CD24 PE.Cy7 (ML5) and CD34 APC.Cy7 (581). From eBioscience (San Diego, CA): CD19 APC (HIB19), CD127 Alexa-Fluor 647 (eBioRDR5) and CD127 PE (eBioRDR5). From Miltenyi Biotec (Auburn, CA): CD19 APC (LT19) and CD127 PE (MB15-18C9).

Statistics

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

Results and Discussion

It is not known if TSLP-induced effects on human B cell development extend to the adult bone marrow or if they are restricted to HSCs from fetal and post-fetal sources. To evaluate the effects of TSLP in adult B cell development, BM CD34+ cells were enriched by magnetic separation and cultured on primary human BM stroma as previously described (Parrish et al., 2009). As shown in Figure 30A, four culture conditions were created from combinations of IL-3, Flt-3 ligand, TSLP and/or IL-7. In

control conditions with IL-3 and Flt-3 ligand, a few B cell progenitors were produced (Figure 30B and C). While the size of this B cell progenitor population significantly increased with the addition of IL-7, TSLP supplementation did not increase the number of B cell precursors above control conditions (Figure 30B and C). Conditions with both TSLP and IL-7 stimulation generated similar numbers of B cell precursors to the IL-7 only condition (Figure 30B and C). Allakhverdi et al. demonstrated that human CD34+ progenitors express both IL-7Rα and TSLPR chains and are responsive to TSLP in an allergy model (Allakhverdi et al., 2009). Therefore we do not attribute the failure to impact B cell production on the absence of the receptor components.

As TSLP has been shown to promote the differentiation of adult mouse B cells to the IgM+IgD– immature B cell stage (Levin et al., 1999), we also evaluated IgM expression on B cell progenitors generated in our selective cytokine cultures. No IgM+ immature B cells were produced in control, TSLP, IL-7 nor TSLP+IL-7 conditions (Figure 30B). Signaling through IL-7R is known to upregulate CD19 expression in human B cell progenitors (Billips et al., 1995; Parrish et al., 2009; Wolf, Weng, Stieglbauer, Shah, & LeBien, 1993). As expected, pro-B cells generated with IL-7 had higher levels of CD19 than control (Figure 30B, D and data not shown). However, pro-B cells from TSLP conditions did not exhibit increased CD19 expression.

Collectively, these initial data suggest that B cell progenitors, produced from adult bone marrow CD34+ cells, are not responsive to TSLP stimulation, like their neonatal CD34+ cells are in our selective cytokine cultures (Chapter 4). It will be of interest to evaluate adult B cell differentiation in the +TSLP mice, as they permit the differentiation of B cell progenitors through the mature B cell stage.

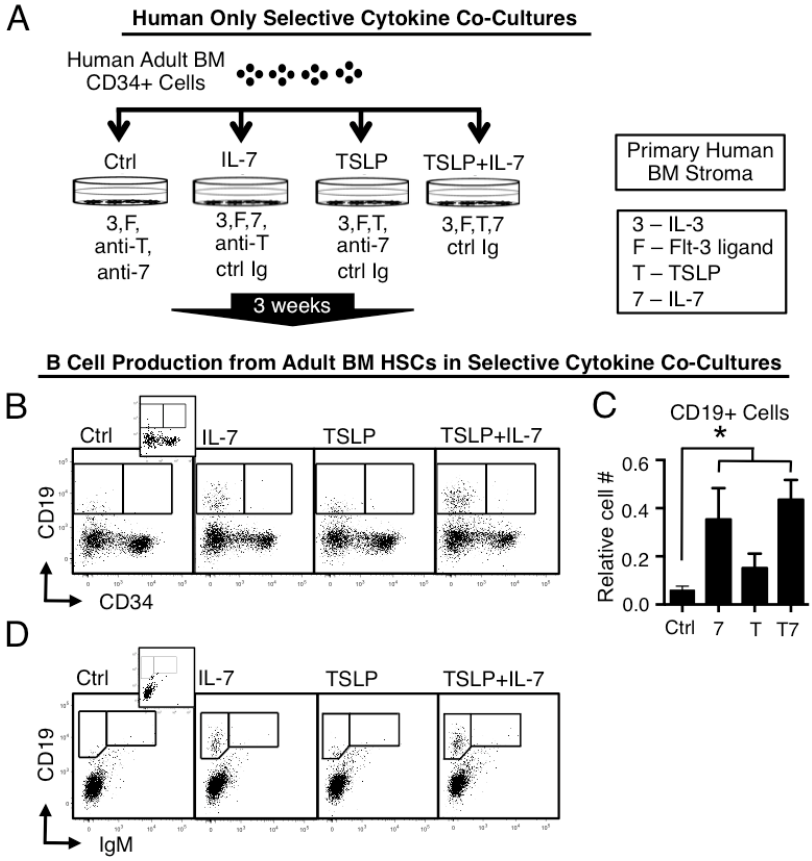

Figure 30. TSLP does not increase B cell production from human adult bone marrow (BM) HSCs in selective cytokine co-cultures. (A) Experimental design of selective cytokine co-cultures. Adult BM CD34+ HSCs were cultured on primary human BM stromal cells with combinations of IL-3 (1 ng/ml, for the first week only), Flt-3 ligand (1 ng/ml) and TSLP (10 ng/ml) and/or IL-7 (5 ng/ml) for 3 weeks as illustrated. Neutralizing antibodies to TSLP and/or IL-7 were added to the cultures to counter the effects of any endogenously produced cytokines if TSLP or IL-7 was not added. Cultures were harvested and stained with antibodies to identify (B) B cell precursors (CD19+ CD34+ or CD34–) generated in vitro. (C) Graphed is the relative number of total B cell precursors (CD19+) produced in each condition. Relative cell number was determined by normalizing the number of CD19+ cells generated in each condition to the total CD19+ cells generated in all 4 conditions. (D) Cultures were also stained with antibodies to distinguish immature B cells (CD19+IgM+) from B cell precursors (CD19+ IgM-). Data shown are representative of, or pooled from 6 independent co-cultures initiated with adult BM HSCs. Statistical significance was determined by unpaired t test, 2-tailed (*, $p < 0.05$).

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CHAPTER SIX

IL-7 RECEPTOR ALPHA EXPRESSION IDENTIFIES A MULTI-LYMPHOID PROGENITOR IN HUMAN CORD BLOOD LYMPHOPOIESIS

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Abstract

IL-7 receptor (IL-7R) signaling is essential for mouse B cell development, however its role in human B lymphopoiesis is controversial. Here we provide the first evidence that expression of a single marker, the IL-7Rα chain, can identify a lymphoid-restricted CD19–CD34+ human progenitor population (IL-7R+ progenitors) that is completely devoid of myeloid potential. Single cell assays show that IL-7R+ progenitors include bi-potential B-NK cells, as well as lineage-committed B and NK progenitors. *In vitro*-generated IL-7R+ progenitors produced B and NK cells in xenograft mice. IL-7R+ progenitors were able to differentiate into T lineage committed cells with Notch signaling. IL-7R+ progenitors are rare (<2% of CD19–CD34+ cells) in circulating umbilical cord blood (CB), but comprise 20%-30% of CD19–CD34+ cells produced from CB CD34+ cells cultured *in vitro* or *in vivo* in the human-mouse xenograft model. These data suggest that IL-7R+ progenitors are common lymphoid progenitors in CB lymphopoiesis, previously undetected because of their low frequency in circulating CB. These committed lymphoid progenitors may provide a therapeutic alternative that have impact on clinical outcome such as immune reconstitution after CB stem cell transplant if proved to be expandable efficiently.

Introduction

Insufficient lymphopoiesis can lead to compromised, adaptive immune function with increased susceptibility to infection and death. Insufficient lymphopoiesis is a feature of the early stages of immune reconstitution following hematopoietic stem (HSC) transplant (Fry & Mackall, 2005). It can also be a consequence of the myeloid skewing of hematopoiesis that has been associated with chronic inflammation (Esplin et al., 2011; Nagai et al., 2006) and with aging of the immune system (Ergen, Boles, & Goodell, 2012; Ergen & Goodell, 2010). The lymphocytes that are present in these cases often result from clonal expansion of mature cells, and thus the diversity of the adaptive immune response is also limited (Blackman & Woodland, 2011; Fry & Mackall, 2005). Identifying the lymphoid-restricted progenitor populations that are intermediates in the differentiation of lymphoid lineage cells from HSCs will provide a foundation for developing therapies to augment the production of lymphocytes. These populations could potentially be targeted for *ex vivo* (Zakrzewski et al., 2006) or *in vivo* (Liuba, Pronk, Stott, & Jacobsen, 2009) expansion to increase the *de novo* production of lymphocytes and restore a robust and diverse polyclonal adaptive immune response.

Umbilical cord blood (CB) is a hematopoietic source used increasingly for HSC transplant, even in adults (Jacobson et al., 2012). As compared to adult BM and mobilized peripheral blood, CB transplant results in lower incidences of graft versus host disease (Broxmeyer, 2008). Thus, CB is an ideal source of stem/progenitors for augmenting lymphoid reconstitution. Previous studies aimed at identifying lymphoid-restricted progenitors in CB lymphopoiesis have focused on cells present in circulating CB. The CD19–CD34+ progenitors in those studies were identified based on expression CD7 and/or CD10 and required the use of multiple lineage markers and additional

surface markers to eliminate cells with myeloid lineage potential (Hao et al., 2001; Rossi et al., 2003)

In the mouse, expression of the IL-7 receptor α chain (IL-7R) is a distinguishing feature of the earliest lymphoid-restricted progenitors with B cell potential (Kondo, Weissman, & Akashi, 1997). Human B cell development has been thought to differ from that in mouse concerning the requirement for IL-7 (Lee, Namen, Gillis, Ellingsworth, & Kincade, 1989; Namen et al., 1988). However, in our previous studies we used a novel human-only co-culture system to show that IL-7 is critical for expanding human CD19+ B cell progenitors produced from HSCs in CB and for the production of human B lineage cells from adult BM. Here we show that IL-7R expression can be used alone to identify CD19–CD34+ progenitors that give rise to B/NK cells as well as T cells, but completely lack myeloid differentiation potential. These IL-7R+ progenitors are rare in circulating CB but comprise a substantial fraction of the CD19–CD34+ progenitors generated from HSCs during CB lymphopoiesis *in vitro* and *in vivo* in the xenograft model. Thus, IL-7R+ progenitors represent a potential target population for therapies aimed at augmenting lymphocyte production to increase adaptive immune responses.

Materials and Methods

Cell Sources

Cord blood samples were obtained by informed consent in accordance with approved Loma Linda University Institutional Review Board (IRB) protocols. Adult human BM was purchased from Poietics Cell Systems (Lonza, Walkersville, Inc., Gaithersburg, MD). The murine S17 stromal cell line was kindly provided by Dr. Kenneth Dorshkind (Cumano, Dorshkind, Gillis, & Paige, 1990). The murine

OP9/OP9Delta1 stromal cell lines were kind gifts from J. C. Zuniga-Pflucker (University of Toronto, Ontario, Canada).

Cell Cultures

Human CB CD34+ cells were seeded onto primary human BM stromal layers plated at 7000 cells/well in 96-well tissue culture plates. Cultures were maintained in B cell medium supplemented with IL-3 at 1 ng/ml (first week only) and Flt-3 ligand (FL) at 1 ng/ml (both from R&D Systems Inc., Minneapolis, MN), as described previously (Parrish et al., 2009). After 2 weeks of culture, CD34+CD19– cells were harvested and FACS-sorted into IL-7R– and IL-7R+ subpopulations, which were used for lineage specific differentiation both in vitro and in vivo. For clonal myelo-erythroid assays 500 to 1000 FACS sorted cells were plated into 1-mL of 1% methylcellulose media (MethoCultGFH4434; Stem Cell Technologies, Vancouver, BC). Colony Forming Units (CFUs) including CFU-GM, BFU-E/CFU-E and CFU-GEMM were counted after 14 days in culture.

To evaluate B and NK lineage potential, CB-derived cells cultured with the murine S17 stromal line for 2 weeks in B cell medium supplemented with IL-7 (5ng/mL), Flt-3 ligand (5ng/mL), and IL-15 (20 ng/mL), all from R&D Systems (Minneapolis, MN) (Hao et al., 2001). FACS Sorted CB cells (3000-5000 cells per well for bulk cultures or single cells per well for clonal assays) were seeded onto S17 stromal cells in 96-well plates. In vitro T-cell development cultures were carried out as described previously (La Motte-Mohs, Herer, & Zuniga-Pflucker, 2005). OP9-control and OP9-DL1 monolayers were prepared 1 day in advance by plating stromal cells at $2x10^5$ cells/well in 6-well

culture dishes, and progenitor cell populations were plated at $1x10^5$ cells per well onto stromal layer in α-MEM media (Cellgro) containing 20% fetal calf serum (FCS), supplemented with L-glutamine and penicillin/streptomycin. Culture medium was supplemented with 5 ng/mL of IL-7 and FL (R&D Systems, Minneapolis, MN). Media containing cytokines was replaced every 2-3 days and cells were transferred to new layers of OP9/OP9Delta1 stromal cells weekly. Cultures were evaluated weekly for T cell differentiation status.

Flow Cytometry and Cell Sorting

Cells were stained for flow cytometry analysis with standard protocols using the following mouse-anti-human antibodies: CD34 PerCP, CD19 Pacific Blue or APC, CD7 PE or PE-Cy5, CD45-PE-Cy7, CD56 FITC and CD1a efluor450 from eBioscience; IL-7Rα-PE from Miltenyi Biotec Inc; CD10 APC-Cy7, CD11b PE, CD5 APC from BD Pharmingen. Flow cytometry acquisition and analysis were performed using a MACSQuant® Analyzer (Miltenyi Biotec Inc. Auburn, CA) and FlowJo data analysis software v9 (FlowJo LLC, Ashland, OR). Cell sorting was performed using a FACSAria (BD Biosciences Immunocytometry Systems).

Transplantation Studies in NOD/SCID/-2mnull mice.

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NOD/SCID/-2mnull; NSG) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed and bred under pathogen-free conditions in the Loma Linda University animal facility and studied in accordance with the Institutional Animal Care and Use Committee (IACUC) approved

protocol. CB CD34+ HSCs ($2X10^4$) or sorted IL-7R+ and IL-7R- progenitors ($1.5X10^4$) were intravenously injected into sublethally irradiated (225 cGy) 4 to 6 week-old NSG mice. 5–7 weeks after transplantation, mice were sacrificed and cells were harvested from BM.

Results

IL-7R+ Progenitors are Produced In Vitro from CD34+ CB Cells, but are Rare in Circulating CB

Using a novel human-only co-culture system, our previous studies showed that IL-7 is essential for adult human B lymphopoiesis and critical for B cell production from HSCs in CB (Parrish et al., 2009). To identify the earliest CD19– lymphoid progenitors that are potentially targeted by IL-7 we used flow cytometry to evaluate expression of IL-7R on CD19–CD34+ progenitors produced from CB CD34+ co-cultured with primary human BM stroma (human-only co-cultures). As shown in Figures 1A (left panel) and 1C, approximately 20% of the CD19–CD34+ progenitors generated *in vitro* were comprised of IL-7R+ cells (designated IL-7R+ progenitors here). Surprisingly, the frequency of IL-7R+ progenitors in uncultured CB was significantly lower, making up only ~2% of the CD19–CD34+ cells present in circulating CB (Figure 1B, left panel, and Figure 1C).

This led us to ask whether progenitor populations that express CD7 or CD10, two markers previously used to identify lymphoid progenitors (Galy, Travis, Cen, & Chen, 1995; Hao et al., 2001; Rossi et al., 2003) were similarly under-represented among circulating CB cells as compared to their counterparts generated *in vitro.* As shown in Figures 1A–C, the CD19–CD34+ cord blood progenitors that express CD10 or CD7 are present at similar frequencies in the *in vitro*-generated progeny of CB CD34+ cells and in

uncultured CB. Taken together, these data show that 1) IL-7R+ progenitors are a major progenitor population produced from CB HSCs during *in vitro* culture and 2) unlike CD7+ and CD10+ progenitors, IL-7R+ progenitors are largely absent from circulating CB.

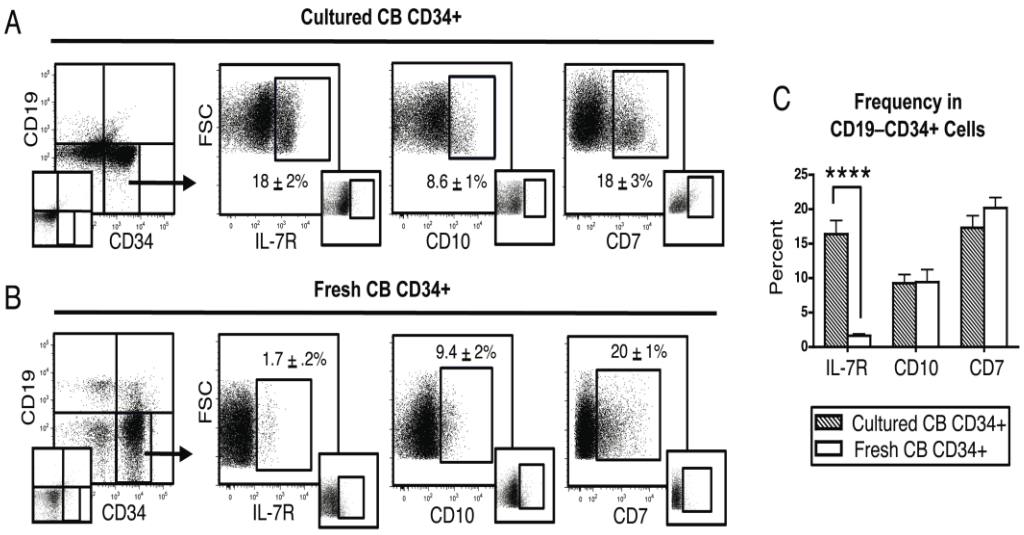


Figure 1 ***(Figure 31).*** IL-7R+ progenitors are produced in vitro from CD34+ CB cells, but are rare in circulating CB. Samples were harvested from a "human-only" culture based on co-culturing human CB CD34+ HSCs on primary human BM stroma with FL for 2 weeks, supplemented with IL-3 for the first week only. (A) The expression of IL-7Rα, CD10 and CD7 were evaluated on cultured CD34+CD19– cells. (B) The representative expressions of IL-7Rα, CD10 and CD7 in CD34+CD19– populations were also analyzed in unmanipulated CB samples. Data shown in each dot plot are the mean ± SEM of three to 12 independent experiments. Isotype controls are displayed in the lower left or right corner of each larger dot plot. Summarized in (C) are the frequencies of IL-7Rα, CD10 and CD7 cells in either cultured cells or unmanipulated CD19–CD34+ cells, where n = three to 12 independent experiments, **** $P < 0.0001$.

IL-7R Expression Identifies an In Vitro-Generated Progenitor Population with Lymphoid but not Myeloid Differentiation Potential

To determine the differentiation potential of the IL-7R+ progenitors, CFU assays were used to assess myeloid lineage potential and stromal cell co-cultures were used to determine B and NK lineage potential. CD19–CD34+ cells generated *in vitro* from cultures of CB CD34+ cells (Figure 2A) were FACS sorted into IL-7R– and IL-7R+ subsets and assessed for myeloid and lymphoid differentiation potential. For comparison, culture-generated CD19–CD34+ cells were also FACS sorted into CD10– and CD10+, as well as CD7– and CD7+ subsets.

CFU assays showed that the IL-7R– subset of CD19-CD34+ cells generated myeloid and erythroid colonies, whereas IL-7R+ progenitors were completely devoid of myeloid or erythroid differentiation potential (Figure 2B, top panel). In contrast, both CD10– and CD10+ subsets of CD19–CD34+ cells retained substantial myelo-erythroid potential, although CD10+ cells formed significantly lower numbers of colonies when compared to CD10– cells (Figure 2B, middle panel). No difference was observed between the ability of CD19– CD34+ cells that were CD7– or CD7+ to generate myeloid and erythroid colonies (Figure 2B, bottom panel). These data suggest that IL-7R+ progenitors generated *in vitro* from CD34+ CB cells have lost myelo-erythroid differentiation potential.

To determine the B and NK potential of IL-7R+ progenitors, we seeded these cells into murine S17 stromal cell co-cultures under lymphoid conditions (FL, IL-15 and/or IL-7) as previously described (Hao et al., 2001). As a control, CD19–CD34+ progenitors that were IL-7R– were also evaluated. Distinct populations of CD19+ and

CD56+ cells were identified in bulk cultures from both the IL-7R– and IL-7R+ progenitors (Figures 2C and D). These data provide evidence that IL-7R, but not CD10 or CD7, can be used as a single marker to identify an early lymphoid progenitor population in CB lymphopoiesis that is completely devoid of myelo-erythroid potential. These results also demonstrate that that IL-7R+ progenitors can give rise to both B and NK cells.

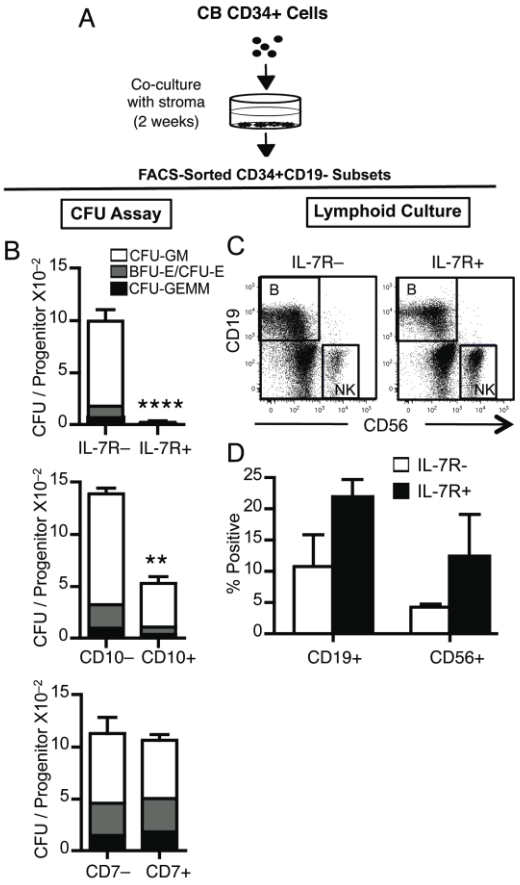


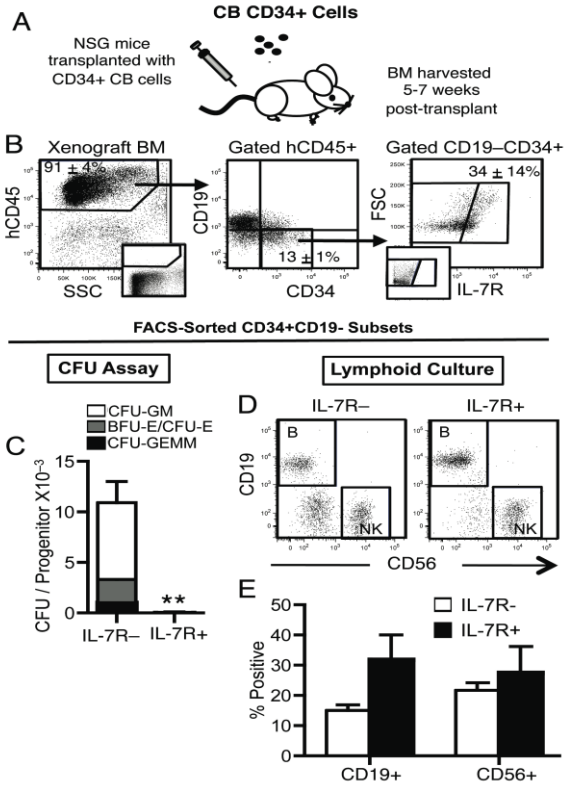
Figure 2 ***(Figure 32)*.** Expression of IL7R, but not CD10 or CD7 identifies CB-derived progenitors with B-NK but not myeloid differentiation potential. (A) Schema of study design. "Human-only" co-cultures were used to generate progenitor cells as described in Figure 1. CD34+CD19– cells were sorted based on expression of IL-7R, CD10 and CD7 and assayed in lineage-specific *in vitro* assays. (B) CD34+CD19–IL-7R+/IL-7R– , CD34+CD19–CD10+/CD10– and CD34+CD19–CD7+/CD7– cells were seeded in triplicate in methylcellulose-based myeloid-erythroid cultures (CFU-Assay). CFUs were counted on day 14. Data are summarized as the number of CFU per progenitor plated. Data are presented as mean values ± SEM of triplicate determinations from three to seven individual experiments, ** $p < 0.01$, **** $p < 0.0001$. CD34+CD19–IL-7R+/IL-7R– cells were also cultured in B and NK lineage differentiation cultures, supplemented with FL, IL-7 and IL-15. Cells were harvested on day 14 and analyzed by flow cytometry. Displayed in (C) are representative dot plots of CD19 versus CD56 staining from cultures initiated with sorted CD34+CD19–IL-7R+/IL-7R– cells. (D) Graphed is the percent (mean ± SEM) of CD19+ and CD56+ cells generated from cultured IL-7R– and IL-7R+ cells in three – five experiments.

CB CD34+ Cells Produce Lymphoid-Restricted IL-7R+ Progenitors In Vivo in the Human-Mouse Xenograft Model

To determine if IL-7R+ progenitors are generated during *in vivo* differentiation from CB CD34+ cells we used a human-mouse xenograft model. Xenograft mice were produced by transplanting human CB CD34+ cells into sublethally irradiated, immune deficient NSG mice. BM was harvested 5–7 weeks post transplant and stained for flow cytometry to detect human cells generated *in vivo* (Figure 3A). Flow cytometry showed that human cells (hCD45+) comprised ~90% of harvested BM (Figure 3B, left panel) and that CD19–CD34+ cells made up ~13% of the human cells in BM (Figure 3B, middle panel). Approximately one third of the CD19–CD34+ cells generated *in vivo* expressed the IL-7R (Figure 3B, right panel). These data show that IL-7R+ progenitors are produced from CB CD34+ cells *in vivo* in the human-mouse xenograft model and suggest that IL-7R+ progenitors represent a major intermediate during *in vivo* hematopoiesis from CB HSCs.

Next we evaluated the lymphoid and myeloid differentiation potential of IL-7R+ progenitors produced *in vivo*. hCD45+CD19–CD34+ cells harvested from BM of human mouse xenografts were FACS-sorted into IL-7R– and IL-7R+ subsets and evaluated for myeloid potential in CFU assays and for lymphoid potential in stromal cell co-cultures. Data obtained from human progenitors generated *in vivo* in the xenograft model was similar to that from culture-generated cells: the IL-7R– progenitor population gave rise to myeloid and erythroid colonies in CFU assays, however, no CFU colonies were produced from IL-7R+ progenitors (Figure 3C). In stromal cell co-cultures grown under lymphoid conditions, both IL-7R– and IL-7R+ subpopulations gave rise to CD19+ and CD56+ cells

(Figures 3D and E). These data show that IL-7R+ progenitors generated *in vivo* from CB HSCs have B and NK, but lack myeloid differentiation potential.



Clonal Analysis of IL-7R+ Progenitors in a Single Cell Assay of

Figure 3 ***(Figure 33).*** CD34+CD19-IL7R+ progenitors generated in the human-mouse xenograft model possess only B-NK potential. (A) Schema of study design. $2x10^4$ CD34+ CB CD34+ stem cells were transplanted into sublethally irradiated (225 cGy) NOD/SCID/-2mnull (NSG) mice, BM cells were harvested five to seven weeks later and analyzed by flow cytometry. A representative diagram of IL-7R expression gated on human (h) CD45+CD34+CD19– cells is shown in (B), where isotype controls are displayed in either the lower right corner or the lower left corner of the respective plots. Data are presented as mean values ± SEM of three separate experiments. Cells were sorted into hCD45+CD34+CD19–IL-7R– and hCD45+CD34+CD19–IL-7R+ populations and evaluated in various assays. (C) CFU assays were performed on these sorted cells as described in Figure 2. Data are presented as mean ± SEM of CFU per progenitor cell from three separate experiments performed in triplicate, ** $p < 0.01$. Cells were also analyzed after being cultured in B cell and NK cell differentiation conditions for 2 weeks. The expressions of CD19 and CD56 on the cultured cells were analyzed by flow cytometry. Representative plots of CD19 and CD56 expression are shown in (D) and data presented as mean values ± SEM of three separate experiments are shown in (E).

B and NK Potential

To determine whether the IL-7R+ progenitor population is comprised of multi-lineage lymphoid progenitors or a mixture of B- and NK-committed cells, we evaluated *in vitro*- and *in vivo*-generated IL-7R+ progenitors in single cell assays. IL-7R– and IL-7R+ cells generated in human only co-cultures or in human-mouse xenografts from CD19–CD34+ progenitors, were FACS-sorted as described in Figures 2 and 3. Single cells were FACS sorted into co-culture with murine S17 stroma in individual wells of 96-well plates and grown under lymphoid conditions. After two weeks of culture, each well was harvested and evaluated by flow cytometry to detect the presence of B (CD19+) and/or NK (CD56+) lineage cells.

Consistent with enrichment of lymphoid progenitors, the total cloning frequency in lymphoid conditions was ~3 fold higher for IL-7R+ progenitors as compared to cells that were IL-7R– (Table 1). Bi-potential, B-NK progenitors gave rise to one in 25 of the total clones produced from *in vitro*-generated IL-7R+ progenitors and one in 18 of the total clones produced from IL-7R+ progenitors generated *in vivo.* Single lineage, B- or NK-committed cells comprised the majority of *in vitro* and *in vivo*-generated IL-7R+ progenitors. Taken together with CFU data these results provide evidence that IL-7R+ progenitors are a lymphoid committed progenitor population that includes bi-potential B-NK progenitors, as well as more differentiated B- and NK-committed progenitors.

Table 1 (*Table 4*). In vitro Analysis of the Lymphoid Lineage Potential of Single CD19– CD34+ IL-7R– and CD19- CD34+ IL-7R+ Progenitors

	In Vitro-Generated Progenitors		Xenograft-Generated Progenitors	
Progenitor Subset	IL-7R–	IL-7R+	IL-7R–	IL-7R+
Total Cloning Frequency (#Total clones detected)	3.7% (403)	12.4% (404)	1.4% (479)	4.71% (576)
B-NK Cloning Frequency (# B-NK Cell Clones)	0% (0)	.5% (2)	0% (0)	.2% (1)
B-Only Cloning Frequency (# B Cell-Only Clones)	1.7% (7)	5.9% (24)	1.3% (6)	3.3% (15)
NK-Only Cloning Frequency (# NK Cell-Only Clones)	2.0% (8)	5.9% (24)	0% (0)	.5% (3)

In Vitro Exposure of IL-7R+ Progenitors to OP9-Delta1 Co-Culture Induces Phenotypic Changes that are Consistent with Early T-Cell Development

The initiation of commitment to the T cell lineage by human HSC/progenitor cells is marked by expression of CD7 (Hoebeke et al., 2007) as well as CD1a (Haddad et al., 2006; La Motte-Mohs et al., 2005) and CD5 (Awong et al., 2009). Specifically, CD34+CD7+ cells are the earliest T progenitors detected in cultures induced by the Notch signaling (Awong et al., 2009) . To determine the T lineage potential of IL-7R+ progenitors generated in vitro and in vivo, IL-7R+ cells were cultured on OP9-DL1 or OP9-control cells with FL and IL-7 as previously described (La Motte-Mohs et al., 2005). As controls, purified CD34+ stem cells and IL-7R– progenitors were also evaluated. Purified $CD34^+$ CB cells and IL-7R– progenitors sorted from *in vitro* cultures and in vivo xenograft animals cultured on OP9-DL1, produced a small population of cells

expressing CD7 within the first week of culture (data not shown). CD7 expression continued to increase between weeks 1 to 2 and corresponded with the emergence of a $CD34^{-}CD7^{+}$ population (Figure 4A, Rows 1–2). The $CD34^{-}CD7^{+}$ population continued to increase through week 6 of culture. A T-cell committed progenitor subset expressing CD5 and CD1a emerged from within the $CD34^{-}/CD7^{+}$ population between weeks 2 and 3 and peaked between weeks 4 and 5 (Supplemental Figure 1A-B). A higher percentage of CD5+CD1a+ cells was generated from IL-7R– sorted cells than purified CD34+ cells (Supplemental Figure 1A-B). In contrast, IL-7R+ progenitors, gave rise to a lower percentage of CD34+CD7+ early T cell progenitors but higher percentage of CD34–CD7+ in the first two weeks of culture (Figures 4A–B, third row), suggesting that at the beginning of the culture, this population is more differentiated than IL-7R– cells. Consistently, IL-7R+ progenitors generated a higher percentage of CD7+CD5+CD1a+ T lineage committed cells during the first two weeks of culture in contrast to 3–4 week from IL-7R– cells (Figure 4B). Robust, early T cell differentiation continued through week 6. $CD34+CD7^{+}$ early T cell progenitors and CD7+CD5+CD1a+ T lineage committed cells were not detected in the negative control of CD34+ stem cells on OP9-control . This data show that IL-7R+ progenitors are able to produce early T cell commited progenitors.

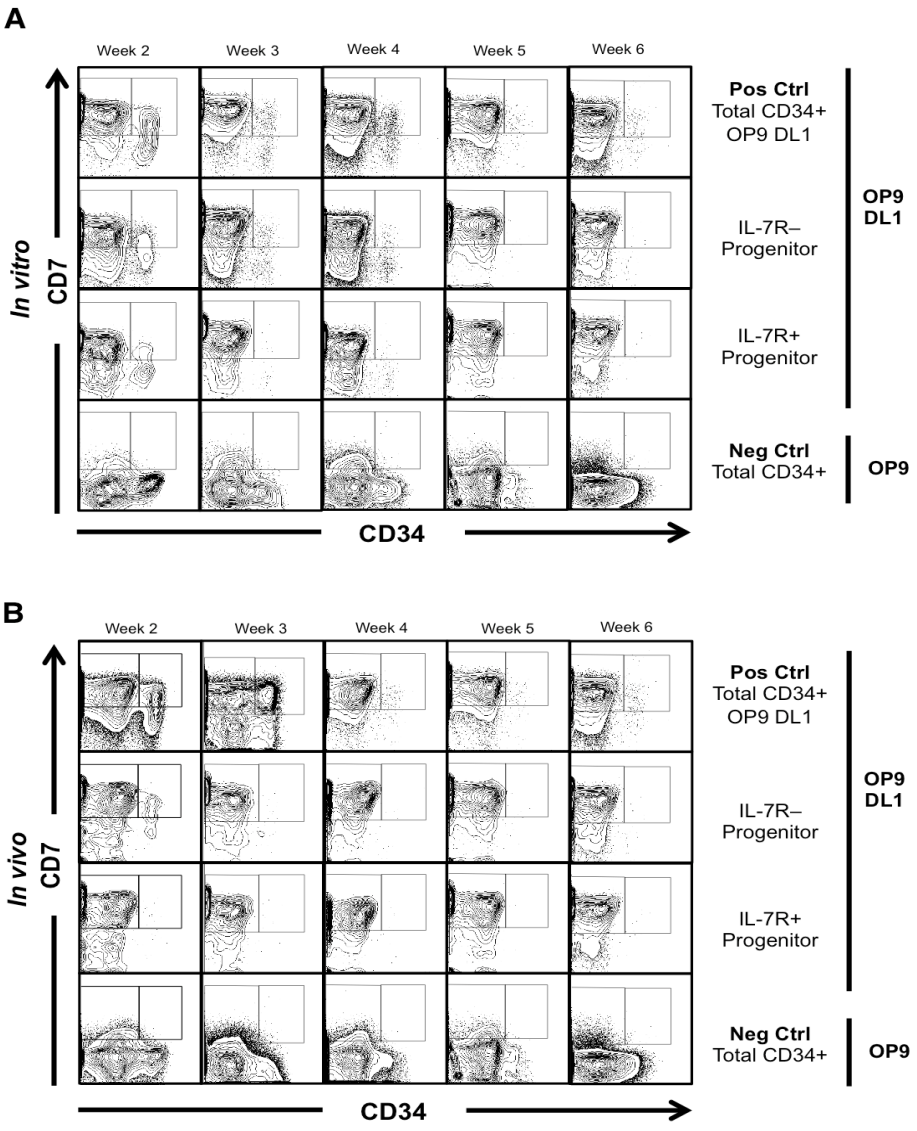


Figure 4 (*Figure 34*). IL-7R+ progenitors generated in vitro and in vivo, produce CD7+ T cell progenitors. CD34+CD19-IL-7R– and CD34+CD19-IL-7R+ progenitors generated (A) in vitro as described in Figure 2 or (B) in vivo as described in Figure 3 were FACS-sorted and cultured on OP-9DL-1 cells. Total CD34+ cells were cultured on OP9–DL1 cells or OP9 cells for positive or negative controls, respectively. Cultures were harvested at the intervals shown and cells stained for flow cytometry to identify CD34– CD7+ T cell progenitors

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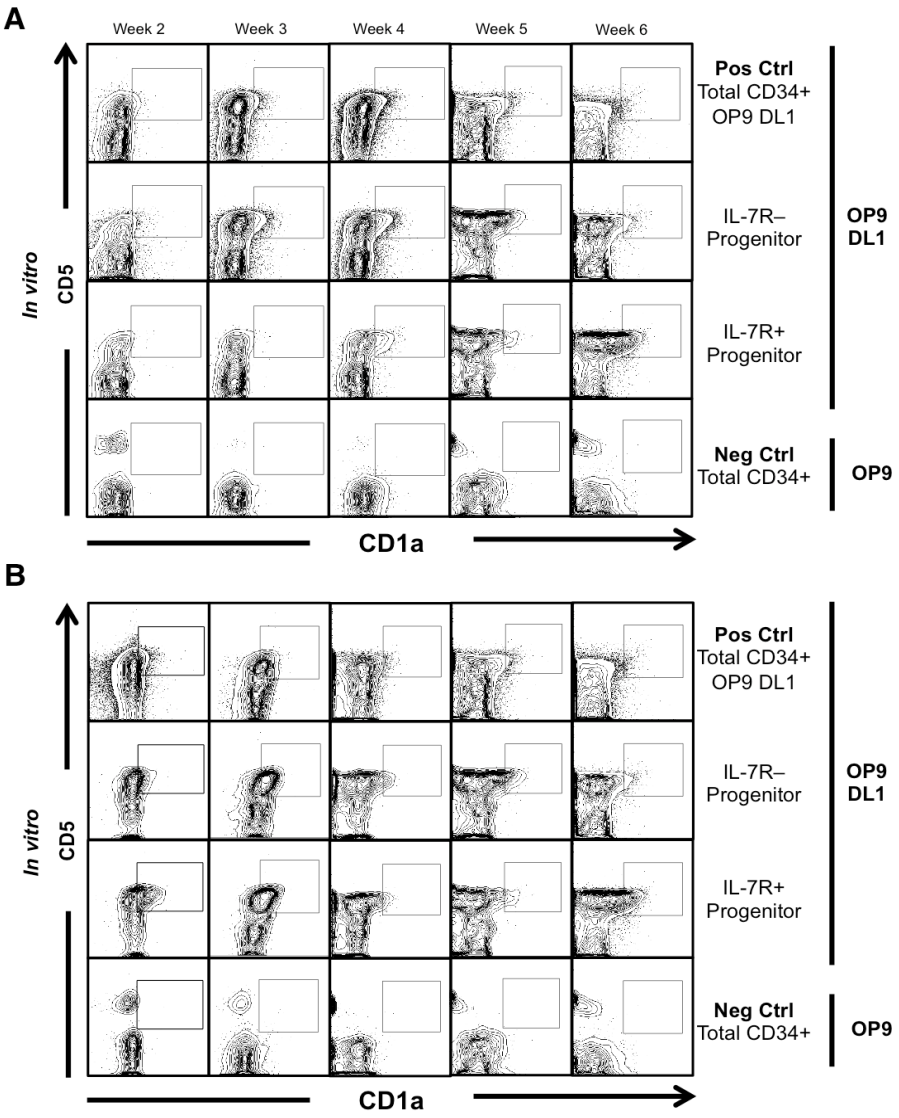

Supplemental Figure 1 ***(Figure 35).*** CD5+CD1a+ T cell committed progenitors are generated from IL-7R+ progenitors. Cells were generated and cultured as in Figure 4 CD5 and CD1a expression were evaluated on CD7+ CD34+/– cells shown in Figure 4.

Culture-Generated IL-7R+ Progenitors are Enriched for Cells with B and NK Differentiation Potential In Vivo

To determine the *in vivo* differentiation capacity of *in vitro*-generated IL-7R+ progenitors, we evaluated hematopoietic differentiation in NSG mice transplanted with IL-7R+ progenitors and IL-7R– progenitors for comparison, produced in human-only co-cultures (Figure 5A). Human CD45+ cells were detected in all recipients (Figures 5B and C). Recipients of IL-7R+ cells showed BM chimerism that was ~10 fold lower than that observed for recipients of IL-7R– cells (0.18 % and 10.85% respectively). As shown in Figure 5B, the majority of the human cells produced from transplanted IL-7R+ progenitors, fell within lymphoid light scatter gates, while those from recipients of IL-7R– progenitors include cells with lymphoid and myeloid light scatter (Figure 5B, left panel). *In vivo* differentiation of IL-7R+ progenitors produced primarily B lineage cells with limited NK cell (~5% of hCD45+ cells) and virtually no myeloid cells (Figures 5 D-F, right panels and G). In contrast, IL-7R– progenitors showed multi-lineage engraftment that include approximately equal proportions of B and myeloid lineage cells and NK cell production similar to that observed for IL-7R+ progenitors (Figures 5 D-F, left panels and G). These data show that IL-7R+ progenitors generated in vitro from CB CD34+ cells retain *in vivo* B and NK differentiation but lack myeloid differentiation potential.

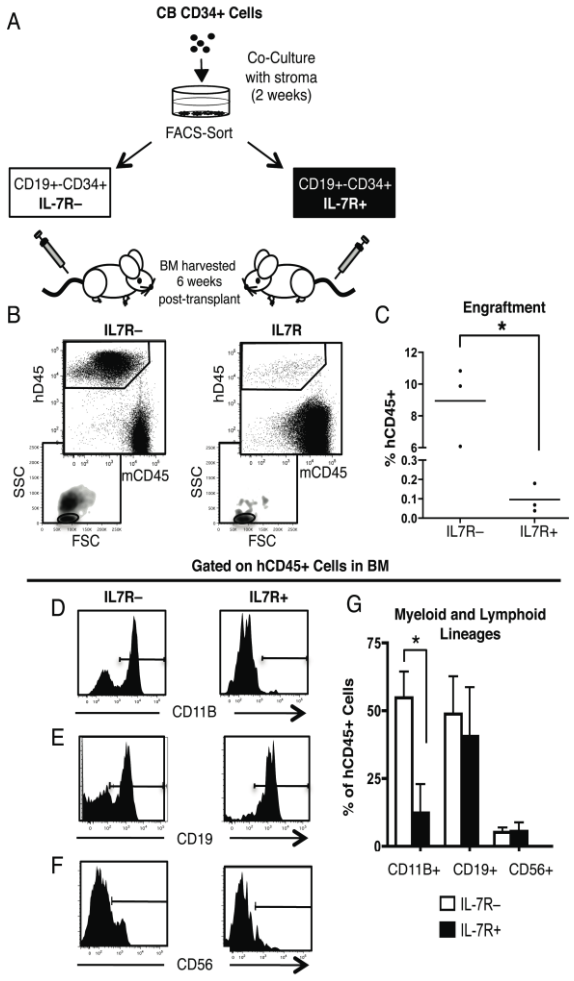

Figure 5 (*Figure 36*). CD34+CD19-IL7R+ progenitors generated *in vitro* culture are enriched for B-NK but lack myeloid differentiation potential in the human-mouse xenograft model. (A) Schema of study design. Cells generated in "Human-only" co-cultures described in Figure 2 were FACS-sorted into CD34+CD19-IL-7R– and CD34+CD19-IL-7R+ populations. Sorted CD34+CD19-IL-7R– or CD34+CD19-IL-7R+ cells, were transplanted into sublethally irradiated (225 cGy) NSG mouse at $1.5x10^4$. Six weeks later, animals were sacrificed and BM harvested where lymphoid and myeloid lineage markers were evaluated by flow cytometry. Representative dot plots of human CD45 (hCD45) versus mouse CD45 (mCD45) engraftment in animals transplanted with either IL-7R– or IL-7R+ cells are shown in (B). The forward scatter (FSC) and side scatter (SSC) properties of engrafted hCD45+ cells are shown in the lower left dot plots.
Displayed in (C) is the engraftment of hCD45 cells from IL-7R– and IL-7R+ mice, * $p < 0.05$. Representative diagrams of the myeloid marker, CD11b; B cell marker, CD19 and NK cell marker CD56 are shown in (D), (E) and (F), respectively. (G) Data shown are the frequencies (mean ± SEM) of CD11b, CD19 and CD56 in total hCD45+ produced in the BM of xenograft mice from CD34+CD19-IL-7R+ and CD34+CD19-IL-7R– cells. n = 3, * $p < 0.05$.

Discussion

Our studies identify a lymphoid progenitor population with B–, NK– and T– lineage potential, that suggest a transitional phase between the primitive hematopoietic stem cell and early lymphoid commitment in cord blood lymphopoiesis. While these IL-7R+ progenitors are relatively rare in circulating CB, they can be efficiently cultured in vitro and in vivo (Figure 1A). Furthermore, IL-7R+ progenitors when generated in vitro (Figure 2), then transplanted into xenograft mice (Figure 5) or in vivo (Figure 3), retain their ability to produce B and NK lineages. These progenitors are also capable of producing early T cell progenitors (Figure 5 and Supplemental Figure 1).

The expression of IL-7Rα (IL-7R) by Lin–Thy-1–Sca-1^{lo}c-Kit^{lo} murine bone marrow (BM) progenitors, has been used to identify lymphoid-restricted progenitors in a primary pathway of mouse B cell development (Kondo et al., 1997). However the utility of IL-7R expression in defining the human CLP counterpart has not previously been evaluated. In attempts to define a CLP in humans, other groups have used expression of CD10 and CD7 by primitive CD34+CD38– progenitors from CB and BM, as markers of a multi-lymphoid lineage progenitor (Galy et al., 1995; Hao et al., 2001; Six et al., 2007). We found that CD34+ progenitors that expressed CD10+ or CD7+ retained the ability to generate myeloid and erythroid progenitors as compared to IL-7R+ progenitors that did not (Figure 2).

The IL-7–IL-7R interaction has been seen as unimportant for human lymphoid development for the following reasons. Firstly, IL-7R is expressed at low levels on the human hematopoietic stem cell (HSC) (Hao et al., 2001; Ryan et al., 1997). Secondly, in vitro studies of human progenitors cultured on murine stromal cell lines, showed minimal

or reduced IL-7 effects on B cell production, as compared to mice (Rawlings, Quan, Kato, & Witte, 1995; Taguchi et al., 2007). Thirdly, early clinical observations of patients with severe combined immunodeficiency disease (SCID) due to defects in IL-7R signaling, had peripheral B cells, but lacked T cells (Puel, Ziegler, Buckley, & Leonard, 1998). A previous study from our lab, using a novel "human-only" culture model showed that human B cell development is no different from that of mouse, with respect to the requirement of IL-7 (Parrish et al., 2009). This study demonstrated that IL-7/IL-7R signaling is also important in human B cell development. Recent clinical reports highlight the relationship between IL-7/IL-7R signaling and B cells. A correlation between B-cell dysregulation and IL-7/IL-7R expression aberration has been described in active HIV-infected children and adult patients or HIV-exposed neonates (Borges-Almeida et al., 2011; Cagigi et al.; Malaspina, Moir, Chaitt, et al., 2006; Malaspina, Moir, Ho, et al., 2006). Therefore, the implications of IL-7R signaling in human lymphoid progenitor development, previously ignored, needs to be investigated.

CB CD34+ cells are a hematopoietic source used increasingly for stem cell transplant in a clinical setting. Slow immune cell reconstitution, especially T cells, is a major concern after transplantation. The first few weeks after transplant are characterized by a period of lymphopenia followed by rapid recovery of NK cells but a slower recovery of T cells (Charrier et al., 2013; Jacobson et al., 2012; Thomson et al., 2000). The slow pace of T cell recovery increases opportunistic infections in the patient (Charrier et al., 2013). B cell recovery may begin as early as 3 to 6 months post-CB transplantation (Nakatani et al., 2014) but can take up to 2 years (Seggewiss & Einsele, 2010). Therefore,

co-transplantation of CLPs with HSCs may be an important strategy to accelerate immune reconstitution, especially for B and T cells.

In summary, this is the first report to use IL-7R for characterizing a human lymphoid progenitor population. These committed lymphoid progenitors may prove useful as targets for expansion and genetic manipulation for clinical purposes, thus providing a therapeutic alternative that can impact clinical outcome such as immune reconstitution after CB stem cell transplant and also reduction of incidences of acute GVHD and transplant related mortality. Moreover, as CD10 and CD7, the previously described lymphoid markers do not possess discriminative power for lymphoid restriction, at least in CB, further studies on the relationship between IL-7R, CD10 and CD7 expressions are warranted.

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CHAPTER SEVEN

DISCUSSION

The interactions of hematopoietic progenitors with the bone marrow microenvironment; age and immune status of the individual; along with genetic and environmental factors, all contribute to creating an adaptive, complex process (Thomas, 2015). Although an increasing body of data suggests that IL-7Rα ligands can impact human B cell development (S. E. Johnson, Shah, Panoskaltsis-Mortari, & LeBien, 2005; Malaspina, Moir, Chaitt, et al., 2006; Malaspina, Moir, Ho, et al., 2006; Scheeren et al., 2010; Wolf, Weng, Stieglbauer, Shah, & LeBien, 1993), their roles remain controversial.

Using a unique, in vitro model of B lymphopoiesis, we demonstrate that IL-7 is required for the development of B cells in adulthood (Chapter 2), while TSLP may be dispensable at this later point in ontogeny (Chapter 5). We also investigated the contribution of TSLP and/or IL-7 in an innovative xenograft model with continuous human TSLP, and found that in the bone marrow, the increases were specific to the B cells and were maintained in the spleen (Chapter 3). Furthermore, in both in vitro and in vivo models of B cell differentiation, B cell precursors expanded from post-fetal HSC sources in response to TSLP or IL-7 stimulation (Chapter 4). Using CB HSCs, we identified a lymphoid progenitor-intermediate, the IL-7R+ progenitor. Although present at low frequency in CB, IL-7R+ progenitors were generated both in vitro and in vivo (Chapter 6). Collectively, we show that the IL-7Rα ligands, TSLP and/or IL-7, are important for the expansion of early B cell precursors from postnatal sources.

The cytokines that drive human B cell development remain elusive while those in the mouse have been extensively delineated both in vitro and in vivo. Together these data

show that the requirement for IL-7 and TSLP during B lymphopoiesis, change during the life of the mouse. During fetal life, B cells undergo IL-7 independent development, while in adulthood IL-7 is required for maturation of B cell progenitors beyond the pro-B stage of differentiation (Carvalho, Mota-Santos, Cumano, Demengeot, & Vieira, 2001; Kikuchi & Kondo, 2006; von Freeden-Jeffry et al., 1995). Conversely, in the absence of IL-7, TSLP stimulation supported the proliferation of fetal but not adult pro-B cells (Vosshenrich, Cumano, Muller, Di Santo, & Vieira, 2003, 2004). In opposition to the previous finding, Jensen et al demonstrated that Flt-3 ligand is a primary cytokine responsible for IL-7 independent B lymphopoiesis (Jensen et al., 2008). The importance of IL-7R signaling in developing B cells is further reinforced in adult IL-7R knockout mice, which suffer from a more severe B lymphopenia, at an earlier developmental stage than IL-7 knockout mice (Kikuchi, Lai, Hsu, & Kondo, 2005; Peschon et al., 1994). Collectively, these data establish that IL-7R and Flt-3 ligand induced signals are essential to mouse B lymphopoiesis.

Practical limitations of procuring healthy human bone marrow have made it experimentally difficult to evaluate developing B cell precursors in their native microenvironment. To address this limitation, in vitro models were developed by culturing hematopoietic CD34+ progenitors on murine stromal cells lines (Barker & Verfaillie, 2000; Fluckiger et al., 1998; Rawlings, Quan, Kato, & Witte, 1995). While this model produces a substantial pool of B cell precursors, the effects of uncharacterized murine proteins cannot be eliminated. To counter the effects of these as yet defined factors, others evaluated the process of human B cell differentiation in stromal cell free conditions (Kraus et al., 2014); on human bone marrow mesenchymal stem cells (MSC)

(Briquet et al., 2010; Ichii et al., 2008) or in conditioned media from MSC (Ichii et al., 2010), with or without the addition of exogenous cytokines. Another variable shown to influence the in vitro models of lymphopoiesis is the lot of FSC or FBS selected for media-supplementation (Ichii et al., 2010). By substituting human serum for FBS or FCS in our selective cytokine cultures, we removed the influence of unknown growth factors present in FCS while maintaining the stromal cell contact important for B cell development (Torlakovic, Tenstad, Funderud, & Rian, 2005).

While in vitro models provide important information about B lymphopoiesis, they are somewhat limiting, as they do not fully recapitulate the native, interactive multi-cellular bone marrow microenvironment. Humanized mouse models have been developed to overcome this limitation, but there are considerations for their use/selection. Firstly, the selected mouse strain matters, as each strain differs in its ability to engraft human cells. NOD-SCID IL2RG^{null} (NSG) mice show increased engraftment of human cells compared to other strains (Legrand, Weijer, & Spits, 2006; McDermott, Eppert, Lechman, Doedens, & Dick, 2010). Secondly, the source of stem cells can affect engraftment. HSCs from CB engraft at higher percentages compared to those from mobilized PB (Lepus et al., 2009). Thirdly, some cytokines are species specific, e.g. TSLP, IL-15 and BAFF. To overcome this limitation some have transgenically expressed human cytokines in mice, for example, IL-15 (Huntington et al., 2009), which has been a successful strategy. In our humanized model, based on injecting human stromal cells that express human TSLP at concentrations similar to that observed in non-diseased children (Lee et al., 2010), we saw functional effects of human TSLP on the differentiation of B

cell precursors. The utility of this approach is reinforced by studies in mice where systemic TSLP levels increased B cell production (Astrakhan et al., 2007).

Consistent with previous evaluations of fetal-derived and neonatal-derived HSCs (S. E. Johnson et al., 2005; Pribyl & LeBien, 1996), we demonstrate that CB HSCs are capable of IL-7R ligand independent B cell production in vitro. Unlike an earlier study (Pribyl & LeBien, 1996), we found that in vitro supplementation with IL-7 consistently augmented the number of B cell progenitors produced from HSC derived from CB. TSLP, a known expander of B cells during fetal development in humans and mice (Scheeren et al., 2010; Vosshenrich et al., 2003, 2004) also increased the production of B cells from CB, but not postnatal sources in our selective cytokine culture model. In stromal cell free conditions, Ichii et al, also found that TSLP was unable to expand postnatal B cell production (Ichii et al., 2010).

Understanding the potential role of TSLP in human B cell production is important because environmental pollutants, for example, diesel particles, can modulate TSLP expression (Bleck, Tse, Gordon, Ahsan, & Reibman, 2010). Significantly higher TSLP levels are seen in Th2-mediated inflammatory conditions like asthma (Ying et al., 2008) and some (Lee et al., 2010) but not all (Nakamura, Tsuchida, Tsunemi, Saeki, & Tamaki, 2008) cases of atopic dermatitis. As an assessment of BM is not required for the diagnosis of these conditions, it is not known if BM B cell progenitor numbers are expanded in these patients in response to increased circulating TSLP. Neither have there been reports of increased numbers of immature B cells in the circulation of patients with these conditions. Higher TSLP concentrations are also found in the synovial fluid (Koyama et al., 2007) and serum (Koyama, Ohba, Haro, & Nakao, 2015) of rheumatoid

arthritis patients compared to healthy controls but did not correlate with disease severity. Hence, a direct association of TSLP to the initiation or progression of atopic dermatitis and rheumatoid arthritis requires further studies.

The persistence of normal to increased numbers of B cells with decreased T cells in the peripheral blood of patients with SCID, due to mutations in either IL-7Rα or common gamma chain, has contributed to the conclusion that IL-7 is dispensable for human B lymphopoiesis (Giliani et al., 2005; Puel, Ziegler, Buckley, & Leonard, 1998). These studies evaluated patients, who at the time of diagnosis were less than one year old. In mice, HSCs switch from IL-7R independence to dependence one to two weeks after birth (Kikuchi & Kondo, 2006), while the characteristics of human HSCs, change between one to three years of age (Sidorov, Kimura, Yashin, & Aviv, 2009). It is therefore conceivable that the HSCs evaluated in those SCID patients (Giliani et al., 2005; Puel et al., 1998) were at a point in development where they did not require IL-7R signals for B cell development.

TSLP or TSLPR signaling has been implicated in several cancers including precursor B cell lymphoblastic leukemia (pre-B ALL). In an early study, TSLP increased the proliferation of and protected primary pre-BALL cells from apoptosis (Brown et al., 2007). Later studies identified aberrant expression of the TSLP receptor component, CRLF2, in pre-B-ALL (Mullighan et al., 2009; Russell et al., 2009; Yoda et al., 2010). Mutations in the tyrosine kinase, JAK2, were found to strongly associate with CRLF2 overexpression (Hertzberg et al., 2010). Not only do CRLF2 and mutant JAK2 interact (Mullighan et al., 2009) but when expressed in BaF3 cells, together these aberrations yielded phosphorylation of downstream kinases, in the absence of IL-7R and independent

of cytokine stimulation (Yoda et al., 2010), suggesting that the cytokine, TSLP, may not be an important contributor to the initiation or progression of the disease. We compared the gene expression profiles of cells from primary patients with those from PDX with or without TSLP, and found that the gene expression profiles of cells expanded with TSLP bore a profile more similar to primary patient cells than those expanded without. These findings are important because they demonstrate that along with independent aberrant CRLF2/JAK2 signaling, physiological concentrations of TSLP also modulate this signaling axis. They further underscore the importance of including TSLP in the development of treatments for pre-B ALL. A recent study demonstrated improved survival of CRLF2-PDX mice treated with a JAK inhibitor (Wu et al., 2015). As these PDX may not have been exposed to physiological concentrations of TSLP, an important future consideration will be, does TSLP change the response of CRLF2-PDX to current and future JAK inhibitors.

Transplantation of CB or BM-derived HSCs has been used in the treatment of leukemia for many years. While both sources have proven beneficial, they also have limitations. As compared to BM, transplantation with CB HSCs, results in a lower incidence of graft-versus-host-disease (GvHD) (Broxmeyer, 2008); faster reconstitution of B lymphocytes (Nakatani et al., 2014), although the frequency of memory B cells is delayed (Charrier et al., 2013); and slower reconstitution of neutrophils and platelets (Broxmeyer, 2008). While CB is more accessible, HSCs are present at a lower frequency (Broxmeyer, 2008) and cell dose is an important factor in immune reconstitution after transplantation (Rocha, Gluckman, Eurocord-Netcord, European, & Marrow Transplant, 2009). To address this limitation, some physicians have utilized the approach of

transplanting more than one CB unit from different donors (Scaradavou et al., 2013). By evaluating patient mortality and hematopoietic recovery i.e neutrophils and platelets, this strategy proved to be just as successful as transplanting a single CB unit (Scaradavou et al., 2013). Ex vivo expansion of IL-7R+ progenitors and co-transplantation with stem cells may be an important future step, as a means of boosting lymphoid recovery after BM transplantation.

Human and mouse studies reveal that with increasing age, B cell development is impaired (Arakawa-Hoyt et al., 1999). Both cell intrinsic and micro-environmental factors contribute to this decline. Micro-environmental changes in older mice include decreased IL-7 secretion by BM stromal cells (Stephan, Reilly, & Witte, 1998; Tsuboi et al., 2004) and decreased pro-B cell response to IL-7 (Stephan, Lill-Elghanian, & Witte, 1997); declining pre-B cell numbers in the bone marrow (Kline, Hayden, & Klinman, 1999) and increased inflammation (Riley, 2013). Subsequent studies suggest that pre-B cell numbers remain constant with age, but in older mice, pools of transitional and mature B cells are not efficiently generated (K. M. Johnson, Owen, & Witte, 2002). Studies of normal, BM B cell precursor subsets in elderly patients have been more limited. Our in vivo model with selective TSLP or IL-7 may be an important tool to evaluate the response of aging, human adult HSCs to the changing cytokine microenvironment in the bone marrow.

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