Title

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Journal

Immunity, 45(3)

ISSN

1074-7613

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Publication Date

2016-09-01

DOI

10.1016/j.immuni.2016.07.012

Peer reviewed

Published in final edited form as:

Immunity. 2016 September 20; 45(3): 527–539. doi:10.1016/j.immuni.2016.07.012.

Distinct Genetic Networks Orchestrate the Emergence of Specific Waves of Fetal and Adult B-1 and B-2 Development

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SUMMARY

B cell development is often depicted as a linear process initiating in the fetus and continuing postnatally. Using a PU.1 hypomorphic mouse model, we found that B-1 and B-2 lymphopoiesis occurred in distinct fetal and adult waves differentially dependent on the *Sfpi1* 14 kB upstream regulatory element. The initial wave of fetal B-1 development was absent in PU.1 hypomorphic mice, while subsequent fetal and adult waves emerged. In contrast, B-2 lymphopoiesis occurred in distinct fetal and adult waves. Whole transcriptome profiling of fetal and adult B cell progenitors supported the existence of three waves of B-1 and two waves of B-2 development, and revealed that the network of transcription factors governing B lineage specification and commitment was highly divergent between B-1 and B-2 progenitors. These findings support the view that the B-1 and B-2 lineages are distinct and provide a genetic basis for layering of immune system development.

Graphical abstract

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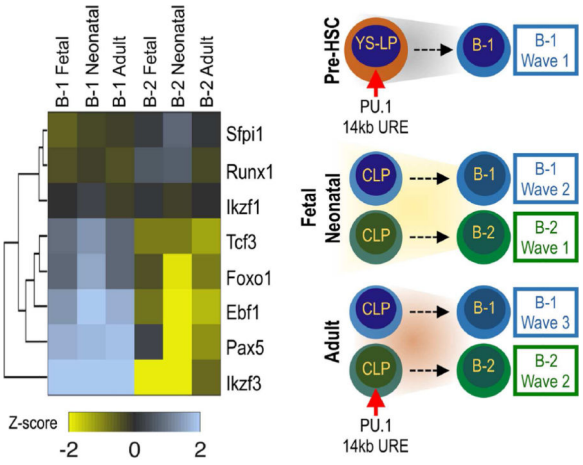
SUPPLEMENTAL INFORMATION

On line supplemental information includes 5 figures, 3 tables and 1 Experimental Procedures section.

AUTHOR CONTRIBUTIONS

EM-R and MF designed and performed experiments, DC performed the bioinformatics analyses, BB-M made cDNA libraries and conducted PCR reactions, CB and MF maintained the mouse colony, and EM-R and KD designed experiments and wrote the manuscript.



INTRODUCTION

The development of the different hematopoietic lineages is dependent on the expression of specific transcription factors in hematopoietic stem cells (HSCs) and immature, uncommitted progenitors at specific times during development (Orkin, 2008). The transcriptional regulation of B lymphopoiesis is well defined, and studies of genetically engineered mice have made it possible to identify the hierarchy of factors required for the emergence of lymphoid specified progenitors from HSCs and their commitment to the B cell lineage (Busslinger, 2004; Dakic et al., 2007; Hagman, 2006; Rothenberg, 2014). However, these studies often revealed that lack of expression of a particular transcription factor differentially affect fetal and adult B lymphopoiesis (Nutt, 1997; Reya, 2000; Ye et al., 2005). Such results are difficult to reconcile with traditional schemes of hematopoiesis depicting B cell development as a linear process that initiates in the fetus and continues in the bone marrow (BM) after birth. Instead, these observations are consistent with suggestions that the regulation of lymphocyte development is not uniform throughout development (Kincade et al., 2002; Rothenberg and Dionne, 2002).

Differences in the transcriptional regulation of fetal and adult B cell development are particularly well illustrated by studies of mice that do not express PU.1, a pioneer transcription factor expressed in HSCs and their progeny (Heinz et al., 2010). This Ets family member is expressed in hematopoietic cells and regulates B lymphopoiesis through its ability to induce the expression of other transcription factors, cytokine receptors, and various lineage specific cell surface determinants (Dakic et al., 2007; De Koter, 2002; DeKoter et al., 2007). It was originally thought that all B lymphopoiesis was blocked in mice with a deletion of *Sfpi1*, the gene that encodes PU.1, but further studies indicated that limited fetal B lymphopoiesis occurred in PU.1 deficient strains (Ye et al., 2005). The cells produced were B-1 B cells, a population of innate-like effectors generated most efficiently in the fetus, which respond to pathogens like encapsulated bacteria (Alugupalli et al., 2004; Baumgarth, 2011). In contrast, few if any B-2 B cells, which participate in adaptive immune responses, were produced. Similarly, a PU.1 hypomorphic strain, generated by deleting an upstream regulatory element (URE) located 14kb from the *Sfpi1* transcription start site,

exhibited normal numbers of B-1 B cells and a severe depletion of B-2 B cells (Rosenbauer et al., 2006). B-1 and B-2 B cells have been proposed to be distinct lineages (Herzenberg, 2000; Kantor and Herzenberg, 1993), and these observations provide preliminary, genetic evidence in support of this hypothesis.

Models hypothesizing the existence of multiple waves of B-1 B cell development have been proposed, with the initial wave initiating prior to the emergence of definitive HSCs (Montecino-Rodriguez and Dorshkind, 2012; Yoshimoto, 2015; Yoshimoto et al., 2011). In contrast, B-2 lymphopoiesis is depicted as occurring in a single wave that initiates in fetal liver (FL) subsequent to the emergence of HSCs and continuing in adult BM following their migration to that tissue (Hardy, 2007). We reasoned that dissecting B lymphopoiesis in the Sfpi1 14kb URE deficient (UREΔ/Δ) mice might provide evidence that B-1 B cells develop in multiple waves and further elucidate differences between the transcriptional regulation of fetal and adult B cell development. These mice are viable (Rosenbauer et al., 2006) in contrast to strains with a germ line deletion of PU.1 that die in utero or soon after birth (McKercher, 1996; Scott, 1994). While adult B cell development is attenuated in UREΔ/Δ mice (Rosenbauer et al., 2006), the status of fetal B lymphopoiesis in that strain has not been reported.

Here we show that this approach, combined with analysis of the transcriptomes of fetal and adult B-1 and B-2 progenitors, provided genetic evidence for the existence of three waves of B-1 and, surprisingly, two waves of B-2 B cell production and demonstrated a role for PU.1 dosage in regulating B lymphocyte production within and between these waves. In addition to establishing that the regulation of B lymphopoiesis is not uniform throughout development, our comprehensive analysis of the B-1 and B-2 progenitor transcriptomes showed that expression of the core network of transcription factors that orchestrate B-1 and B-2 B cell specification and commitment were distinct, supporting the hypothesis that these B cell subsets represent distinct lineages.

RESULTS

UREΔ/Δ Mice Have a Selective Defect in Fetal B-1 Lymphopoiesis

Mice with a deletion of the 14kb URE, hereafter referred to as UREΔ/Δ mice, were generated by breeding UREΔ/+ heterozygotes. In agreement with previous observations (Rosenbauer et al., 2006), we observed an 80% reduction in *Sfpi1* expression by UREΔ/Δ BM cells (Figure 1A).

The frequency of B-1a and B-1b B cells as determined by flow cytometry was similar in 6–8 week old UREΔ/Δ and URE+/+ mice (Figure 1B and 1C). Because the number of cells in the peritoneal cavity of UREΔ/Δ mice is higher than in URE+/+ controls, their total B-1a and B-1b B cell numbers were significantly increased (Figure 1C). Similar trends were observed when UREΔ/+ and URE+/+ mice were compared (Figure 1B and 1C). We also observed no difference in the frequency and number of B-1 B cells in the spleen (SPL) of UREΔ/Δ and control mice (Figure S1). These observations confirmed that B-1 B cell development occurs in UREΔ/Δ mice (Rosenbauer et al., 2006).

We (Barber et al., 2011; Montecino-Rodriguez et al., 2006) and others (Esplin et al., 2009; Ghosn et al., 2011; Holodick et al., 2014; Kobayashi et al., 2014; Yoshimoto et al., 2011) have reported that lineage negative (Lin^-) $CD93^+CD19^+CD45R(B220)^{-/low}$ (hereafter $CD19^+CD45R^{-/low}$) B-1 specified progenitors are phenotypically distinct from $Lin^-CD93^+CD19^-CD45R(B220)^+CD43^+$ (hereafter $CD19^-CD45R^+$) B-2 progenitors (Figure S2). These populations are at a comparable pre-pro-B cell stage, as both have undergone heavy chain D-J, but not V-D-J gene rearrangement (Montecino-Rodriguez et al., 2006), and B-1 progenitors lack V_H-D-J_H transcripts (de Andres et al., 2002). B-1 progenitor number peaks during late gestation and declines after birth as B-2 development establishes in adult BM (Montecino-Rodriguez et al., 2006). The fact that B-1 B cells are observed in UREΔ/Δ mice suggested that B-1 development would be normal in that strain. We measured B-1 progenitor frequency and number by flow cytometry in UREΔ/+ and UREΔ/Δ mice at various stages of gestation in order to test this hypothesis. B-1 progenitors were detected in UREΔ/+ mice as early as E14.5. However, while $CD19^+CD45R^{-/low}$ B-1 progenitors were present in the UREΔ/Δ fetus, they were not reliably detected until E18.5 (Figure 1D and 1E). These results suggested that some, but not all, aspects of B-1 development are intact in UREΔ/Δ mice.

The Pre-HSC Wave of B-1 Development is Absent in UREΔ/Δ Mice

Several recent studies showing that lymphoid progenitors are present in the yolk sac (YS) and para-aortic splanchnopleura at least a day prior to the emergence of definitive HSCs support the existence of a pre-HSC wave of lymphocyte development (Böiers et al., 2013; Kobayashi et al., 2014; Yoshimoto et al., 2011; Yoshimoto et al., 2012). We considered the possibility that the B-1 progenitors present in E14.5 UREΔ/+ embryos are primarily derived from this pre-HSC wave that does not emerge in UREΔ/Δ mice. We developed a short-term assay in which progenitor cells are seeded in semisolid medium supplemented with B lymphopoietic cytokines and S17 stromal cells to test this possibility (Figure S3). As $CD19^+CD45R^{-/low}$ B-1 progenitors differentiate in this system, they exhibit a gradual increase in $CD45R^+$ expression, while $CD19^-CD45R^+$ B-2 progenitors gradually increase CD19 expression as both populations mature into $CD19^+CD45R^+$ B-1 or B-2 pro-B cells. These developmental transitions were evident upon immunostaining of cells harvested from the cultures (Figure 2A). This system also allowed multipotent progenitors (MPPs) and common lymphoid progenitors (CLPs) to differentiate into B-1 and B-2 progenitors and their maturation into $CD19^+CD45R^+$ pro-B cells (Figure S3).

Cells from E10.5 UREΔ/+ YS and FL generated B-1 lineage cells when cultured in this system (Figure 2B), in agreement with a previous report showing that YS cells at that age exhibit B-1 but not B-2 lineage potential (Yoshimoto, 2011). In contrast, cells from E10.5 UREΔ/Δ YS or FL failed to generate any CD19 or CD45R expressing cells (Figure 2C). Despite their lack of B progenitor activity, E10.5 UREΔ/Δ YS and FL cells generated $CD4^+$ and $CD8^+$ lymphocytes when seeded over OP9-Delta-like-1 stromal cells (Figure 2D and 2E) (Schmitt and Zuniga-Pflucker, 2002).

The lack of B-1 potential in hematopoietic tissues from E10.5 UREΔ/Δ mice suggested that the upstream precursors from which B-1 progenitors develop were either absent or that they

were present and their development was blocked. To distinguish between these possibilities, we compared the emergence of progenitor populations in FL at various gestational ages. B6 mice were used as controls because URE deficient mice are on a partial B6 background and URE+/+ and B6 mice have a similar hematological profile (Figure S4). This revealed that $Lin^{-}CD117^{high}Sca\text{-}1^{+}$ (LSK) progenitors in B6 mice included a $CD117^{high}CD127(IL\text{-}7R\alpha)^{+}$ population whose frequency declined with increasing embryonic age (Figure 2F and 2G). The latter cells, distinguished from CLPs by their high level of CD117, were also observed in E12.5 YS but were not present in adult BM (data not shown). $Lin^{-}CD117^{high}Sca\text{-}1^{+}CD127^{+}$ cells were not detected in the UREΔ/Δ YS (data not shown) or FL (Figure 2F and 2G). We cultured E12.5 $Lin^{-}CD117^{high}Sca\text{-}1^{+}CD127^{+}$ cells from B6 mice in the semisolid medium assay to assess their B cell potential. As shown in Figure 2H, they primarily generated B-1 lineage cells, indicating that the $Lin^{-}CD117^{high}Sca\text{-}1^{+}CD127^{+}$ population emerges in a pre-HSC wave and is the likely source of B-1 progenitors detected in the fetus by E14.5.

The adult wave of B-2 development is missing in UREΔ/Δ Mice

In contrast to elevated numbers of B-1 B cells in UREΔ/Δ mice, the frequency and number of follicular (FO) B cells, the major B-2 B cell population, were significantly reduced in their SPL compared to UREΔ/+ and URE+/+ littermates (Figure 3A and 3B). In contrast, Marginal Zone (MZ) and peritoneal B-2 B cell numbers were not reduced (Figures 3A, 3B and S1). We considered the possibility that this FO deficiency resulted from defects in primary B-2 cell development.

B-2 B cell development is dependent on an intact HSC compartment (Hardy, 2007), and low PU.1 expression can result in loss of HSCs (Iwasaki et al., 2005; Staber et al., 2013). However, HSCs were present in fetal and adult UREΔ/Δ mice (Figure S5) and exhibited myeloid and lymphoid potential when transplanted into lethally irradiated recipients (data not shown). We therefore assessed whether adult UREΔ/Δ mice harbored defects in down-stream progenitors. $Lin^{-}CD117^{+}Sca\text{-}1^{+}CD150^{-}$ cells and $Lin^{-}CD117^{low}Sca\text{-}1^{low}$ cells, which include MPPs and CLPs respectively, were detected in UREΔ/Δ BM (Figure S5). However, UREΔ/Δ MPPs expressed reduced levels of CD135 (data not shown) and few CD127 expressing CLPs were detected in the BM of young adult UREΔ/Δ mice (Figure S5F). These observations are consistent with the role of PU.1 in regulating expression of these two genes (De Koter, 2002; DeKoter et al., 2007). Consistent with these altered patterns of CD135 and CD127 expression (Jensen et al., 2008), primary B lymphopoiesis was depressed in UREΔ/Δ BM as shown by a significant reduction in the frequency and number of pro-B and pre-B cells (Figure 3C and 3D).

We considered the possibility that, despite their abnormal phenotype, the $Lin^{-}CD117^{+}Sca\text{-}1^{+}CD150^{-}$ and $Lin^{-}CD117^{low}Sca\text{-}1^{low}$ cells present in UREΔ/Δ BM were a source of the FO and Marginal Zone (MZ) B cells in the SPL and the $CD23^{+}sIgM^{+}$ B-2 B cells in the peritoneal cavity of UREΔ/Δ mice (Figure 3A, 3B and S1). We isolated these progenitors from 4 week old UREΔ/Δ and UREΔ/+ mice and tested them in our in vitro assay. Both UREΔ/+ progenitor populations, which included $CD127^{+}$ cells, primarily generated B-2 lineage cells (Figure 3E and 3F). This result is in agreement with the

observation that B-1 lymphopoiesis wanes in young adults and B-2 lineage cell production predominates. In contrast, UREΔ/Δ $Lin^{-}CD117^{+}$ $Sca\text{-}1^{+}CD150^{-}$ and $Lin^{-}CD117^{low}Sca\text{-}1^{low}$ cells, which lack $CD127^{+}$ expression, preferentially generated B-1 lineage cells (Figure 3E and 3F). These results indicated that the few B cell progenitors present in the BM of young adult UREΔ/Δ mice (Figure 3D) were B-1 specified and provide further evidence that IL-7Rα expression is essential for B-2 lymphopoiesis (Carvalho et al., 2001; Sitnicka et al., 2003; Vosshenrich et al., 2003). The data also raised the possibility that the FO, MZ, and peritoneal B-2 B cells present in adult UREΔ/Δ mice (Figure 3A and 3B) were generated during an earlier wave of development.

Therefore we determined if IL-7Rα was expressed on fetal UREΔ/Δ cells at any time during gestation. While CD127 is expressed on $Lin^{-}CD117^{low}Sca\text{-}1^{low}CD127^{+}$ B6 CLPs as early as E13.5, $CD127^{+}$ CLPs were not detected in UREΔ/Δ mice at this age (Figure 4A, 4B, 4C and 4D). However, low numbers of $Lin^{-}CD117^{low}Sca\text{-}1^{low}CD127^{+}$ cells were transiently detected between E15.5 and birth in UREΔ/Δ FL (Figure 4B, 4C and 4D). This result raised the possibility that a fetal wave of B-2 lymphopoiesis was intact in UREΔ/Δ mice. To test this, we cultured $Lin^{-}CD117^{+}Sca\text{-}1^{+}CD150^{-}$ and $Lin^{-}CD117^{low}Sca\text{-}1^{low}CD127^{+}$ cells isolated from E15.5 UREΔ/Δ FL in our in vitro assay and observed that, in contrast to their adult BM counterparts, they generated B-2 lineage cells (Figure 4E).

The above results defined the existence of fetal and adult waves of B-2 B cell development and demonstrated that emergence of the latter wave was dependent on the 14kb URE. The results further suggested that the two waves were not equally dependent on PU.1. Subsequent qPCR analyses, which revealed that *Sfpi1* levels were extremely low in fetal and neonatal B-2 cell progenitors compared to their adult B-2 counterparts, provided support for this hypothesis (Figure 4F). We also measured *Sfpi1* levels in fetal and adult B-1 progenitors and observed that, while lower than those in adult B-2 progenitors, *Sfpi1* levels were higher in adult B-1 progenitors when compared to their fetal counterparts (Figure 4F).

B-1 and B-2 Progenitors Have Distinct Transcriptional Signatures

The above results indicated that the regulation of B-1 and B-2 development is not uniform throughout development. In order to understand these differences, we compared the transcriptomes of B-1 and B-2 progenitors isolated from E15.5 fetal, day 2 neonatal, and 9–11 week old B6 mice. The E15.5 time point would include B-1 progenitors primarily derived from the pre-HSC wave of development while progenitors present in neonates would primarily be derived from all fetal waves. We analyzed these populations because it is currently not possible to distinguish committed B-1 and B-2 progenitors at a more immature stage of development.

Principal component analysis of the gene expression data showed that differences between B-1 and B-2 progenitors, regardless of the ages tested, were greater than those within the respective B-1 or B-2 populations (Figure 5A). Hierarchical gene clustering indicated that 1548 genes were differentially expressed (fold-change>2, p-value<0.01) between B-1 and B-2 progenitors and that these included genes involved in developmental, metabolic, and signaling processes (hypergeometric p-values=8.7E-36; p=2.8E-33; and p=2.8E-33, respectively; Table S1). The data also showed that fetal, neonatal and adult B-1 progenitors

did not cluster together, indicating that they represent three developmental waves (Figure 5A). In contrast, fetal and neonatal B-2 progenitors clustered together away from adult B-2 progenitors, in agreement with the above observation that B-2 development took place in independent fetal and adult waves (Figure 5A).

We also observed that B-1 and B-2 progenitors exhibited differences in expression of genes that constitute the core of the molecular network that regulate the B cell differentiation process (Lin et al., 2012). For example, B-1 progenitors expressed lower levels of *Runx1* and *Sfpi1* and higher levels of *Ikzf3* than B-2 progenitors (Figure 5B). Analysis of *Sfpi1* expression on independent samples by qRT-PCR confirmed these RNA-Seq results (Figure 4F). In addition, the expression of the *Ebf1, Foxo1, Pax5*, and *Tcf3(E2A)* transcription factors were all higher in B-1 than in B-2 progenitors at all ages tested (Figure 5B). Further analyses showed that their target genes were, in general, expressed at elevated levels in B-1 progenitors. These included downstream transcription factors like *E2f2, Foxo1, Ets1, Lef1, Ebf1, Irf4*, and *Pou2af1*, as well as their B lineage specific downstream targets such as *Cd19, Cd79a, Cd79b, VpreB1, VpreB3*, and *Rag1* (Figure 5C–5E). In contrast, the *Tcf3(E2A)* target genes *Id2, Irf8, Runx2*, and *Runx3* were lower in B-1 compared to B-2 progenitors at all ages tested (Figure 5F). Together, these analyses provide evidence that the well-documented developmental differences between the B-1 and B-2 lineages (Herzenberg, 2000; Kantor and Herzenberg, 1993) are associated with major molecular differences in their B cell transcriptional programs.

Fetal and adult B cell developmental programs are distinct

The above data demonstrated clear differences in the expression of B lineage genes within and between the various waves of fetal and adult B-1 and B-2 lymphopoiesis. In addition, it is increasingly appreciated that the molecular pathways that regulate proliferation, survival, and self-renewal of fetal and adult HSCs and their progeny are different (Copley et al., 2013; Kim et al., 2007). To determine if we could identify genes, irrespective of those involved in B lineage specification, whose expression differed between fetal and adult B-1 and fetal and adult B-2 progenitors, we analyzed the B-1 and B-2 progenitor gene sets using Bayesian polytomous model selection. This analysis indicated that 1274 and 1449 genes varied with age within the B-1 and B-2 progenitor populations, respectively (Figure 6A and Table S2). The majority of these genes classified in *Model 1*, which included genes differentially expressed between fetal/neonatal and adult progenitors (*Model 1*, Fig 6A). Most of these genes were annotated in general cellular metabolic and signaling processes, and there was very little overlap between the B-1 and B-2 gene sets (Figure 6B and 6C and Table S2).

The genes classified in this analysis included *Lin28b* and *Hmg2a*. Both genes were expressed by E15.5 fetal and d2 neonatal B-1 and B-2 progenitors, and their expression was reduced in adult populations. *Lin28b* expression has been associated with production of B-1a B cells (Yuan et al., 2012), so its detection in B-2 progenitors was unexpected. In order to confirm the RNA-Seq expression estimates, we assessed expression of these genes by qPCR on independently isolated samples. The results showed that *Lin28b* was expressed in fetal and neonatal B-2 progenitors, although at consistently lower levels than fetal B-1 progenitors (Figure 6D).

DISCUSSION

Traditional models present B lymphopoiesis as a uniform process that initiates in FL subsequent to the emergence of HSCs and continues in BM after birth. The various molecular events, like expression of transcription factors that regulate B lineage specification and commitment, are often integrated into such linear schemes of development (Hagman, 2006; Rothenberg, 2014). However, these models are difficult to reconcile with studies showing that deficiencies in the expression of particular transcription factors differentially affect fetal and adult B lymphopoiesis and B-1 and B-2 development in particular (Kincade et al., 2002; Nutt, 1997; Reya, 2000; Rothenberg and Dionne, 2002; Ye et al., 2005). By demonstrating the existence of distinct, differentially regulated waves of fetal and adult B-1 and B-2 B cell development, we provide a mechanistic basis for the interpretation of these earlier studies. We also demonstrate that the molecular network of transcription factors that orchestrates early B cell development differs in B-1 and B-2 progenitors, thus providing genetic evidence that these are in fact distinct B cell lineages.

Analyses of PU.1 hypomorphic mice allowed us to establish when these different waves of B cell development emerge during development. In this regard, we and others have proposed the existence of pre-HSC, fetal, and adult waves of B-1 B cell development (Montecino-Rodriguez and Dorshkind, 2012; Yoshimoto et al., 2011), and our results provide evidence that these waves exist. Interestingly, the pre-HSC B-1 wave in E10.5 UREΔ/Δ YS and FL was ablated in the PU.1 hypomorphs, and this correlated with a severe deficiency in $Lin^{-}CD117^{high}Sca\text{-}1^{+}CD127^{+}$ progenitors, which we demonstrated can generate B-1 B progenitors. These cells are distinguished from CLPs by their high CD117 expression and may be similar to a recently described population of E11.5 $Lin^{-}Kit^{+}Flt3^{+}IL\text{-}7R\alpha^{+}$ progenitors with B cell developmental potential (Böiers et al., 2013). Whether these progenitors are the only source of B-1 cells that emerge in the pre-HSC wave remains to be determined. However, the fact that they are already present at a time when HSCs are emerging supports the premise that they develop in a stem cell independent manner. Our results also revealed that B-2 lymphopoiesis takes place in two waves that can be distinguished by their differential dependence on an intact *Sfpi1* 14 kb URE. This result was not anticipated, because B-2 B cell development is generally described as a single wave of development that initiates in the fetus and continues in post-natal BM (Hardy, 2007). However, we observed that the adult wave of B-2 lymphopoiesis was largely ablated in UREΔ/Δ mice and this correlated with a deficiency in $IL\text{-}7R\alpha^{+}$ CLPs in their BM.

Our data showed that the pre-HSC wave of B-1 development and the adult wave of B-2 development were absent in UREΔ/Δ mice and that these deficiencies correlated with the absence of IL-7Rα expressing progenitors. These results are consistent with the known role of PU.1 in regulating the expression of this cell surface determinant (Carotta et al., 2010; De Koter, 2002). It is thought that B-1 B cell development is not dependent on IL-7 (Carvalho et al., 2001; Vosshenrich et al., 2004). However, the absence of the pre-HSC wave would not have been detected in *IL-7* or *Il7r* deficient mice because the fetal and adult B-1 waves would be present. Our results further indicated that the 14kb URE is required for the development of the post-natal wave of B-2 development, which is consistent with the observation that PU.1 expression in adult CLPs is dependent on this regulatory element

(Rosenbauer et al., 2006). That B-2 lymphopoiesis was blocked in adult UREΔ/Δ mice is consistent with observations that B cell development is suppressed in *IL-7* or *Il7r* deficient mice. However, we detected a transient wave of $CD127^+$ progenitors with B-2 B cell potential in fetal UREΔ/Δ mice, suggesting that fetal and adult B-2 progenitors are not equally dependent on PU.1 for IL-7Rα expression. Taken together, the results demonstrate an unappreciated role for PU.1 dosage in regulating B lymphocyte production within and between the various waves of B-1 and B-2 development.

A requirement for precise regulation of PU.1 expression during hematopoietic development has been extensively discussed (Dakic et al., 2007; Rosenbauer et al., 2006; Staber et al., 2013). This is achieved through the binding of specific combinations of transcription factors that include *Runx1, Sfpi1, Ikzf* and/or *Foxo1* to multiple *Sfpi1* cis-regulatory elements (Leddin et al., 2011; Okuno et al., 2005; Zarnegar et al., 2010). Of relevance to this point is our observation that the expression of these factors was not uniform between fetal and adult B-1 and B-2 progenitors arising in the different waves. Therefore, a different combination of factors (Heinz et al., 2010), along with the possible utilization of different regulatory elements to which they bind, likely underlies differences in *Sfpi1* expression between B-1 and B-2 progenitors arising in the various waves. However, additional mechanisms may also be operative (Wei et al., 2014). For example, B-1 progenitors are much more proliferative than B-2 progenitors (Montecino-Rodriguez et al., 2014). Because, PU.1 intracellular concentrations can be regulated through lengthening of the cell cycle, the *Sfpi1* levels in B-1 progenitors may be influenced by their high proliferation (Kueh et al., 2013).

The existence of multiple waves of B-1 and B-2 B cell development was supported by principal component analysis of the transcriptomes of fetal and adult B-1 and B-2 progenitors. The data showed that the B-1 progenitors arising in each of these waves had distinct transcriptional signatures while B-2 progenitors segregated into fetal and adult populations according to their global patterns of gene expression. The latter result was consistent with the functional studies demonstrating that fetal, but not adult, progenitors from UREΔ/Δ mice could generate B-2 B cells. The analysis of the genetic data sets also provided a possible explanation for why the fetal and adult B-1 and fetal B-2 waves are intact in UREΔ/Δ mice. It is relevant in this regard that expression of pioneer transcription factors like *Tcf3(E2A), Ikzf1* and *Ikzf3* that participate in B cell lineage priming in HSCs and early hematopoietic progenitors differs between progenitors arising in the different waves (Choukrallah and Matthias, 2014). These results suggested that in UREΔ/Δ mice, the PU.1 deficiency resulting from deletion of the 14kb URE is likely compensated for by a combination of other pioneer transcription factors, which could include related Ets family members (Hagman, 2006), that allow B cell development to progress within selected developmental windows. In this context, the observation that *Ikzf3* is elevated in B-1 progenitors is particularly interesting since this subunit has been shown to boost the transcriptional activity of Ikaros complexes (Morgan et al., 1997).

In addition to these differences within the respective waves of B-1 and B-2 development, our analyses also demonstrated that the transcriptional signatures of B-1 and B-2 progenitors, regardless of whether they were derived from the fetus or adult, were highly divergent. Specifically, major differences in expression levels of the transcription factors that regulate

B cell specification and commitment were observed. B lymphopoiesis requires the concerted expression of a set of transcription factors that include *Runx1, Sfpi1, Ikzf, Tcf3(E2A)* and *Foxo1* which prime subsequent expression of B lineage specific transcription factors like *Ebf1* and *Pax5* (Choukrallah and Matthias, 2014; Lin et al., 2012). All these genes are linked in feed-forward and feed-backward regulatory loops that establish a B cell-specific transcription profile and repress alternative hematopoietic cell fates. Our analyses showed that except for *Runx1* and *Sfpi1*, B-1 progenitors expressed higher levels of these transcriptional regulators than B-2 progenitors. In addition, B-1 and B-2 progenitors exhibited major differences in expression of their B cell specific gene targets. These data demonstrated that the developmental programs in B-1 and B-2 B cell progenitors were distinct and that they were established early during ontogeny.

Finally, we analyzed our transcriptome data using model selection techniques in order to provide a stringent classification of genes involved in proliferation, survival, and/or self-renewal of fetal and adult B-1 and B-2 progenitors, irrespective of their role in B lineage specification. This was undertaken in view of the increasing literature indicating that fundamental cellular processes differ between fetal and adult hematopoietic stem cells (Copley et al., 2013; Kim et al., 2007). Consistent with these observations, this analysis revealed profound differences in the expression of genes between fetal/neonatal and adult B-1 and fetal/neonatal and adult B-2 progenitors, and these included *Hmga2* and *Lin28b*. *Hmga2* expression has been associated with fetal hematopoiesis (Berent-Maoz et al., 2015; Copley et al., 2013), but our results showing that fetal/neonatal B-2 progenitors expressed higher levels than fetal/neonatal B-1 progenitors indicated that its expression is not uniform across all B cell lineages.

Our results also revealed that fetal and adult B cell progenitors were further distinguished by their differential expression of *Lin28b*. These observations are in agreement with (Yuan et al. 2012), who reported that *Lin28b* is expressed in fetal but not adult B cell progenitors. However, we extended their observations by showing the pattern of *Lin28b* in B-1 versus B-2 progenitors. Our data demonstrated that fetal, but not adult, B-1 B cell progenitors express *Lin28b*. This was consistent with the finding that *Lin28b* is associated with the production of B-1a B cells (Yuan et al. 2012), and the fact that B-1a B cells are efficiently generated from fetal but not adult BM progenitors (Barber et al., 2011; Ghosn et al., 2012). We also showed that *Lin28b* is expressed in fetal but not adult B-2 progenitors. It has been proposed that a fraction of MZ B cells, which exhibit functional responses similar to that of B-1 B cells (Kearney, 2005), are derived from fetal progenitors (Yoshimoto, 2015). Therefore, our results suggest that some MZ B cells may derive from these *Lin28b* expressing B-2 progenitors. This may also be the case for peritoneal B-2 B cells, which were present in UREΔ/Δ mice.

In summary, our observations establish the existence of multiple, differentially regulated waves of fetal and adult B cell development and provide evidence that the network of transcription factors that orchestrate B lymphopoiesis is dynamic during fetal and adult B-1 and B-2 development. These results provide a new framework for the analyses of lymphoid development in general and a basis for understanding why studies of various knockout strains of mice often show differential effects on fetal versus adult B cell development.

EXPERIMENTAL PROCEDURES

Mice and genotyping

Sfpi1$^{tm1.3Dgt}$/J mice were obtained from and the Jackson Laboratory (Bar Harbor, ME) and were genotyped according to their recommendations. B6 mice were obtained from the Jackson Laboratory or the UCLA Division of Laboratory Animal Medicine. All protocols were approved by the UCLA Institutional Animal Care and Use Committee.

Flow Cytometry

YS, FL, SPL, BM, and peritoneal cavity cell suspensions were prepared as previously described (Min et al., 2006; Montecino-Rodriguez et al., 2006), and HSCs, CLPs, B-1 and B-2 progenitors, and B lineage cells were resolved with specific combinations of FITC, PE, PercP Cy5.5, PECy7, APC efluor780, Pacific Blue, and 605NC conjugated antibodies (Table S3). Progenitors were purified using Aria Cell sorters (BD Biosciences) located in the Jonsson Cancer Center flow cytometry core and analyses were performed on an LSRII (BD Biosciences) located in the Broad Stem Cell Research Center flow cytometry core at UCLA. FL or BM controls were included in the experiments to account for instrumental variations during analyses.

In vitro cultures

T cell potential was measured by culturing cells on OP9-Delta-like-1 stroma for 29 days. Cultures were initiated in 12 well plates in complete media (Schmitt and Zuniga-Pflucker, 2002) supplemented with 10ng/ml Flt-3 and 10ng/ml IL-7 (Biosource) and 30nM L-ascorbic acid (1-ascorbic acid-2-phosphate sesquimagnesium salt; SIGMA) (Manning et al., 2013). YS and FL from E10.5 embryos were seeded at 2 embryos equivalent per well.

A semi-solid medium assay was developed to assess the B-1 and B-2 potential of hematopoietic progenitors (Figure S3). Hematopoietic cells were mixed with 5×10^4 S17 stromal cells in 1% complete methylcellulose medium (Collins and Dorshkind, 1987) supplemented with 10ng/mL Flt-3L and 10ng/mL IL-7 (Biosource). Two hundred purified progenitors or 2 embryos equivalents of E10.5 YS or FL were seeded per dish. B lineage cell production was assessed following 9–13 days of culture.

qPCR

RNA was extracted with the RNeasy Plus microkit and cDNA was synthesized with the RT^2 First Strand kit as recommended by QIAGEN. qPCR was run and analyzed as described (Berent-Maoz et al., 2015) using Taqman primers: *mGapdh* (Mn99999915-g1), *mSfpi1* (Mn00488142-m1) and *mLin28b* (Mn01190673-m1; Applied Biosystems).

Whole transcriptome profiling (RNA-Seq) and data processing

Whole transcriptome profiling of purified Lin$^-$CD93$^+$CD19$^+$CD45R$^{-/low}$ B-1 and Lin$^-$CD93$^+$CD19$^-$CD45R$^+$CD43$^+$ B-2 progenitors was performed. RNA-Seq libraries were built and pre-processed as described (Berent-Maoz et al., 2015). Adult average gene expression data are based on two independent pools of BM cells prepared from two groups of 9–11 week old B6 mice of 8 and 5 animals each. Day 2 neonatal data were obtained from

progenitors purified from a pool of liver cells harvested from 46 neonates from 6 separate litters. The E15.5 fetal data were obtained from progenitors isolated from a pool of fetal liver cells harvested from 25 embryos pooled from 3 separate litters. Raw sequence files are available at NCBI's Gene Expression Omnibus (GSE81411). The average number of paired-end reads (100bp) was 23 million per sample. RNA-Seq data analysis (alignment, summarization, normalization, differential analysis and functional annotation) was performed as described (Casero et al., 2015) and in Supplemental Experimental Procedures using the GRCm38.71 primary assembly and annotation of the mouse genome. On average, unique alignment rates (using a similarity score of 66%) were around 85% (std=2.8%) for all samples. Pair-wise differential expression was performed to classify genes as differentially expressed (p-value<0.01, moderate log2 fold-change>1) between B-1 and B-2 progenitor samples. Expression heatmaps represent the Z-scores for each gene using variance-stabilized data. Gene classification in stage-specific models for B-1 or B-2 progenitors was obtained from MMSEQ (Turro et al., 2011) expression estimates. *mmdiff* (Turro et al., 2014) was used to test five different models (Figure 6A): a *null model* (similar average expression in the three stages), and four *alternative models*: three *single-stage models* (similar average expression at two stages but different in one), and a model where the average expression is *different in all three stages*. Bayesian polytomous model selection was applied with an 80% prior probability for the *null model* and equal probabilities for the others. For each gene, this provides the model with the highest posterior probability. Genes classified in each alternative model are provided in Table S2.

Statistical analysis

Data are expressed as a mean ± SEM as indicated in the figure legends. Differences between groups were tested by a two-tailed, unpaired Student's t test ($\alpha = 0.05$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant AI021256 (KD). The JCCC flow core is supported by NIH grants CA16042 and AI28697.

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Highlights

- B-1 B cell development occurs in two fetal and one adult wave
- B-2 B cell development occurs in a fetal and an adult wave
- The transcription factor network that governs B-1 and B-2 specification is distinct
- Genetic analyses indicate that B-1 and B-2 B cells arise as distinct lineages

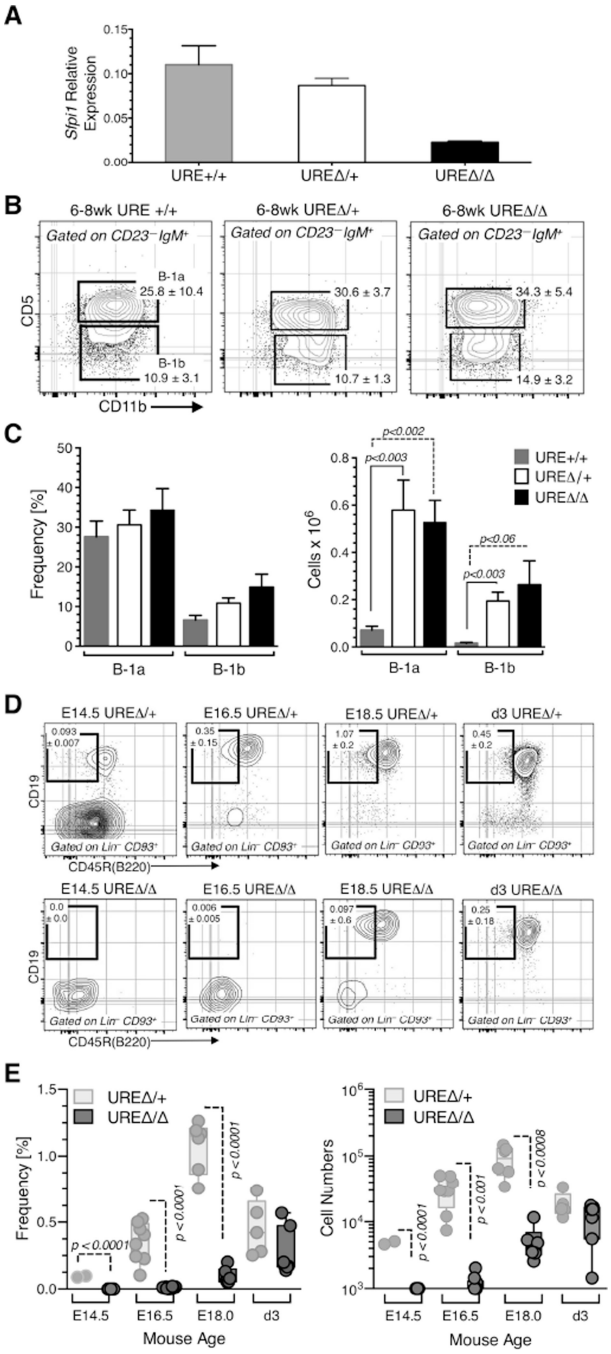

Figure 1. The emergence of B-1 B cell progenitors is delayed in UREΔ/Δ embryos

(A) Expression ± SEM of *Sfpi1* relative to *Gapdh* in URE+/+, UREΔ/+, and UREΔ/Δ BM cells from 6–8 week old mice was determined by qPCR. (B) Representative plots depicting the immunostaining strategy used to determine the frequency of B-1a and B-1b B cells in the peritoneal cavity (PerC) of URE+/+, UREΔ/+, and UREΔ/Δ. Values within gates indicate mean ± SEM of these populations. (C) Frequency and total number ± SEM of B-1a and B-1b B cells in the PerC of individual URE+/+ (n=6), UREΔ/+ (n=10), and UREΔ/Δ (n=7) mice. p values are indicated. (D) Representative plots depicting the immunostaining strategy

used to determine the frequency of $Lin^{-}CD93^{+}CD19^{+}CD45R^{-/low}$ B-1 progenitors in UREΔ/+ and UREΔ/Δ FL. Values within gates indicate B-1 progenitor mean ± SEM at the indicated gestational ages (E) Frequency and total number ± SEM of B-1 progenitors in UREΔ/+ and UREΔ/Δ FL at the indicated gestational ages. Symbols represent individual embryos except for E14.5 where pools of 4–12 fetuses are indicated. p values are indicated.

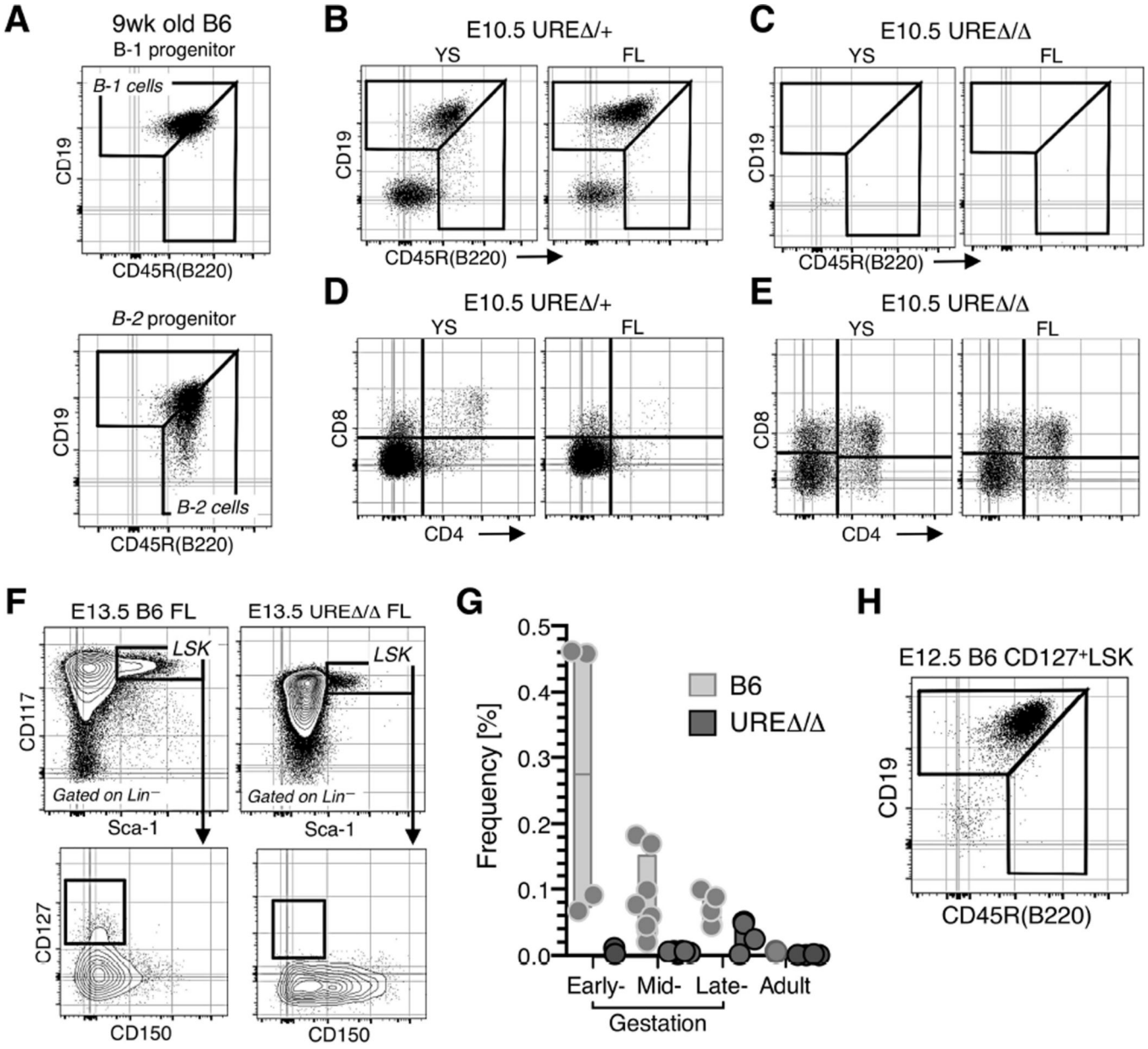

Figure 2. The pre-HSC wave of B-1 B cell development is absent in UREΔ/Δ mice

(A) B-1 and B-2 progenitors were purified from 9 week old B6 BM and cultured for 9 days in semi-solid medium. Representative plots showing expression of CD19 and CD45R on cells harvested from the cultures are shown. A detailed protocol for this semisolid culture system is provided in Figure S3. (B) and (C) Representative plots depicting B lineage cell production from total YS or FL cells harvested from E10.5 UREΔ/+ and E10.5 UREΔ/Δ mice following 13 days in culture. $CD19^{-}CD45R^{-}$ cells in the plots represent surviving non-B lineage cells. (D) and (E) Representative plots depicting T cell production from total YS or FL from E10.5 UREΔ/+ and E10.5 UREΔ/Δ mice following 29 days of culture over OP9-Delta-like-1 stromal cells. Data in panels A–D are representative of 2 independent experiments for each strain. YS and FL cells were pooled from 6–12 embryos in each

experiment. (F) Representative plots depicting the immunostaining strategy used to determine the frequency of $Lin^{-}CD117^{high}Sca\text{-}1^{+}$ (LSK) $CD127^{+}$ cells in E13.5 B6 and UREΔ/Δ FL. (G) Frequency of $Lin^{-}CD117^{high}Sca\text{-}1^{+}$ (LSK) $CD127^{+}$ cells in B6 and UREΔ/Δ FL at early (E12–E15), mid (E15–17), and late (E17–19) gestation and in 4–8 week old mice. Each symbol represents an individual animal except for the early gestation time points where each symbol represents pools of 4–12 fetuses. (H) Representative plots showing the B-1 potential of $Lin^{-}CD117^{high}Sca\text{-}1^{+}CD127^{+}$ ($CD127^{+}$ LSK) cells from E12.5 B6 FL following 13 of culture in semisolid media. Data representative of 2 independent experiments.

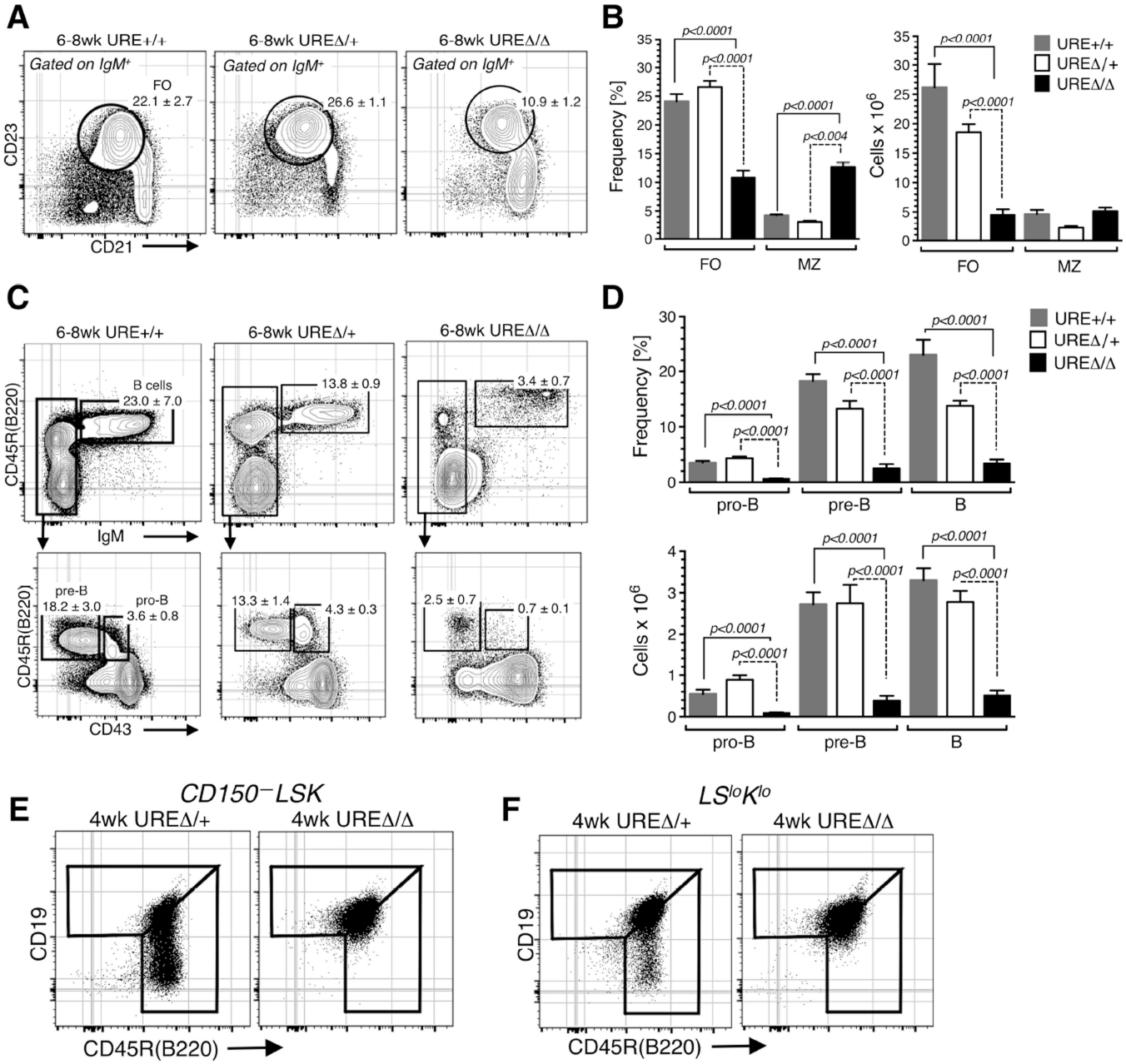

Figure 3. Adult B-2 lymphopoiesis is blocked in UREΔ/Δ mice

(A) Representative plots depicting the immunostaining strategy used to determine the frequency of Follicular (FO) B cells in the SPL of URE+/+, UREΔ/+, and UREΔ/Δ mice. The gates indicate mean ± SEM of these populations. (B) Frequency and total number ± SEM of FO and Marginal Zone (MZ) B cells in the SPL of URE+/+, UREΔ/+, and UREΔ/Δ mice. p values are indicated. (C) Representative plots depicting the immunostaining strategy used to determine the frequency of pro-B, pre-B, and B cells in the BM of URE+/+, UREΔ/+, and UREΔ/Δ mice. Values within gates indicate mean ± SEM of these populations. (D) Frequency and number ± SEM of pro-B, pre-B and B cells in the BM of URE+/+, UREΔ/+, and UREΔ/Δ mice. p values are indicated. The data in panels A–D are based on

analysis of individual URE+/+ (n=6), UREΔ/+ (n=10), and UREΔ/Δ (n=7) mice. (E) and (F) Representative plots showing the potential of $CD150^{-}$ LSK and $LS^{lo}K^{lo}$ cells, purified from 4 week old UREΔ/+ (n=6) and UREΔ/Δ n=6) BM, to develop into CD19 and/or CD45R expressing cells following 13 days of culture in semisolid medium. Data representative of 2 independent experiments

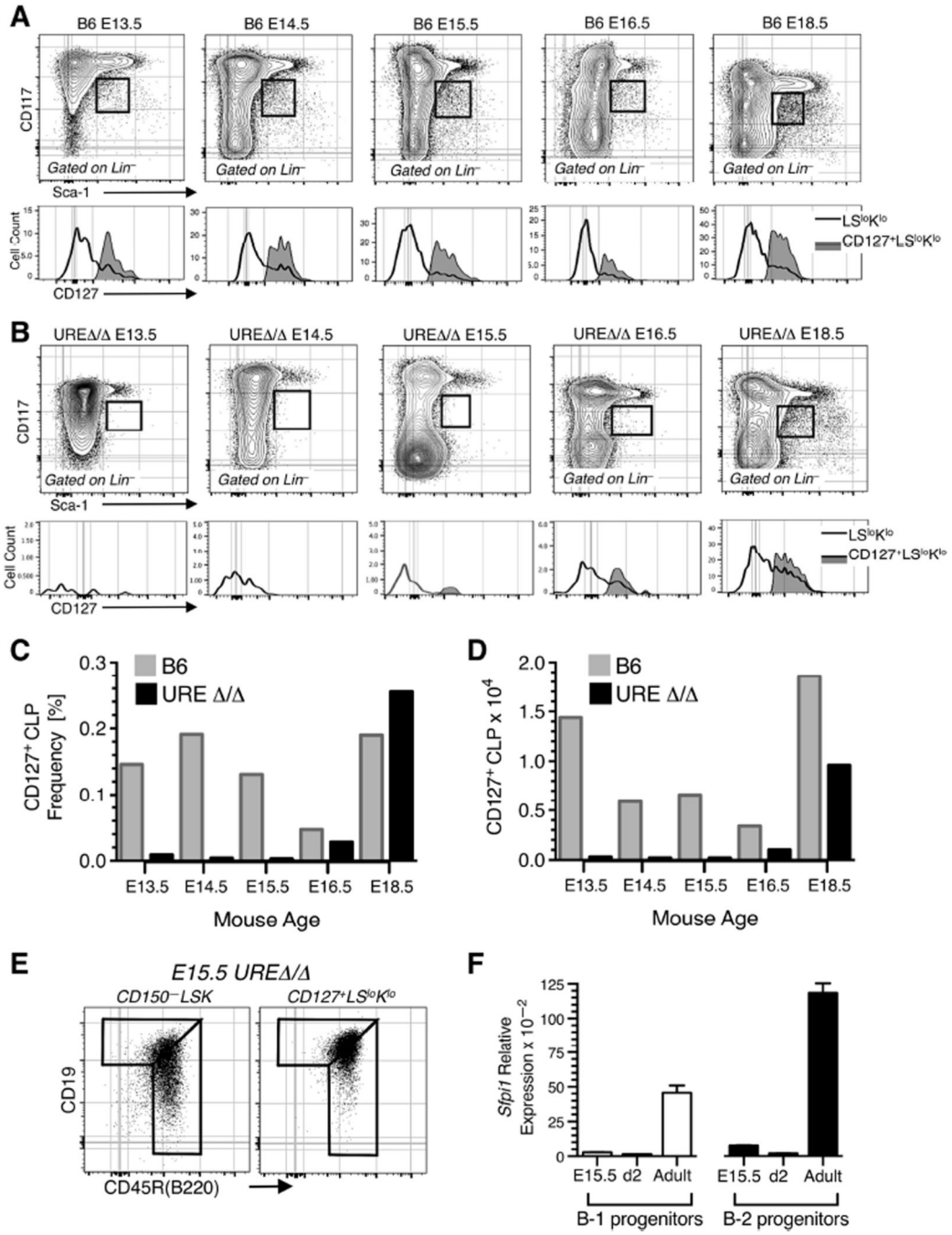

Figure 4. The emergence of CD127$^+$ LS^{lo}K^{lo} CLPs in fetal liver is transient

(A) and (B) Representative plots depicting the immunostaining strategy used to determine the frequency of Lin$^-$Sca-1^{lo}CD117^{lo} (LS^{lo}K^{lo}) CD127 expressing CLPs in FL of B6 and UREΔ/Δ mice at the indicated ages. Histograms show the frequency of CD127$^+$ cells (▬) within the gated LS^{lo}K^{lo} CPL cells (▬) in the corresponding upper panels. C) Frequency and (D) number ± SEM of CD127$^+$ CLPs in FL of B6 and UREΔ/Δ embryos. The data in panels A–D are based on two independent analyses in which FL cells were cells pooled from 6–12 embryos at the indicated gestational ages. (E) Representative plots showing the B-2

lineage potential of $Lin^{-}CD117^{+}Sca\text{-}1^{+}CD150^{-}$ ($CD150^{-}$ LSK) and $Lin^{-}CD117^{low}Sca\text{-}1^{low}CD127^{+}$ ($CD127^{+}LS^{lo}K^{lo}$) cells, isolated from FL pooled from 6–12 E15.5 UREΔ/Δ embryos, following 13 days of culture in semisolid medium. Data representative of 2 independent experiments. (F) Expression ± SEM of *Sfpi1* relative to *Gapdh* in purified fetal (E15.5; n=12–16), neonatal (d2; n=10–12) and adult (12 weeks; n=4–6) B-1 and B-2 progenitors of B6 mice as determined by qPCR Data representative of 2 independent experiments.

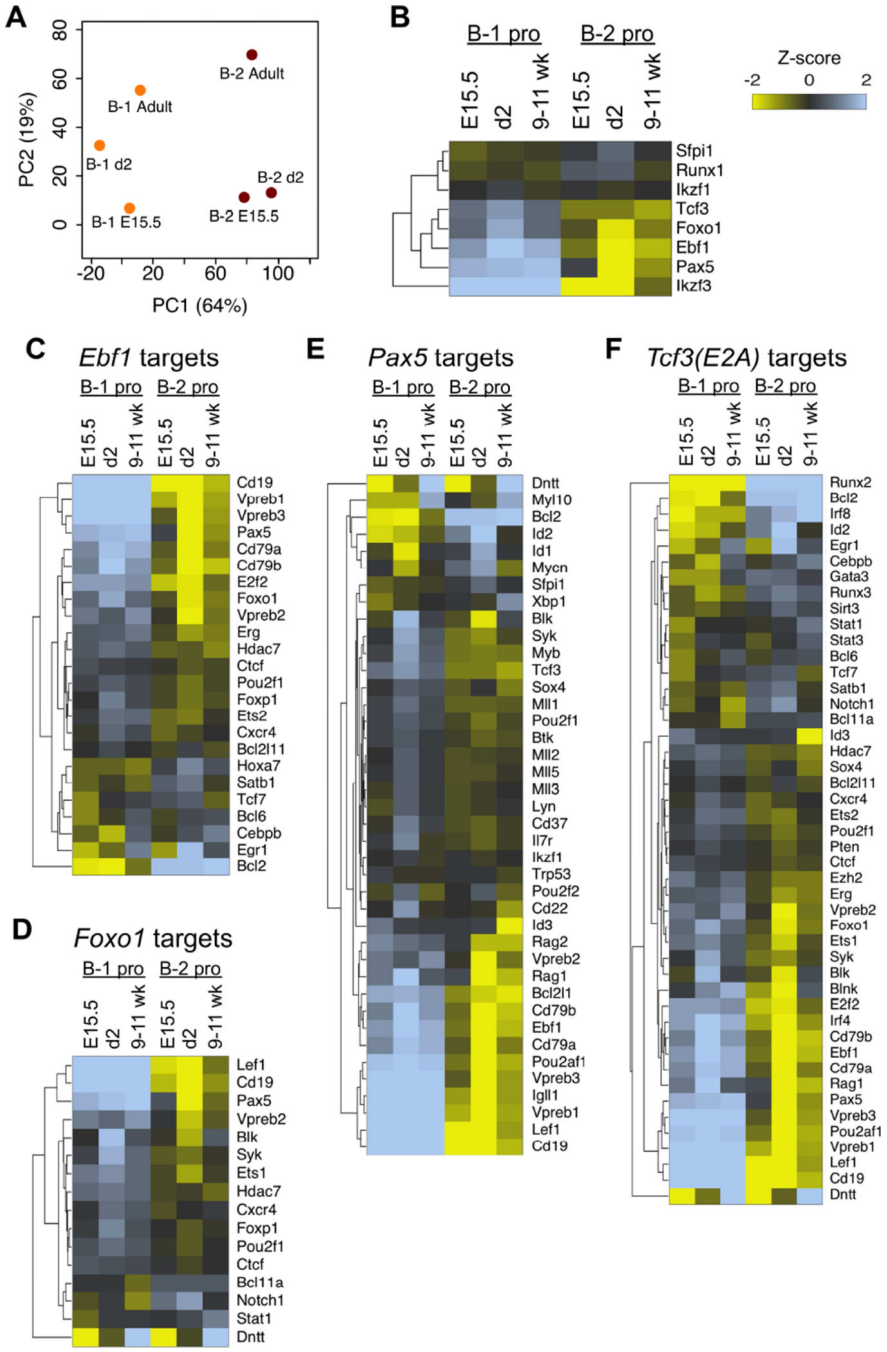

Figure 5. Whole transcriptome profiling reveals differences in the genetic signatures of B-1 and B-2 progenitors

(A) Principal component analysis of Lin^{-}CD93^{+}CD19^{+}CD45R$^{-/low}$ B-1 (B-1 pro) and Lin^{-}CD93^{+}CD19^{-}CD45R^{+}CD43^{+} B-2 (B-2 pro) progenitors from fetal (E15.5; pool of n=25, 3 litters), neonatal (d2; pool of n=46, 6 litters), and adult (9–11 wk; 2 independent groups, n=8 and n=5) B6 mice. (B) Expression heatmaps of core B cell transcription factors in fetal, neonatal, and adult B-1 and B-2 progenitors. Expression heatmaps for the target genes of: (C) *Ebf1*; (D) *Foxo1*; (E) *Pax5*; and (F) *Tcf3(E2a)* in fetal, neonatal, and adult B-1 and B-2 progenitors (log2 fold-change>2). The color scale indicates Z-scores. The complete

gene lists are included in Table S1. B-1 and B-2 progenitors were purified from fetal or adult mice as indicated in (A).

A

	Classified genes	Model 1 Fetal ≠ Adult, Neonate	Model 2 Fetal ≠ Adult, Neonate	Model 3 Fetal = Adult, Neonate	Model 4 Fetal ≠ Adult, Neonate
B-1 progenitor	1274	440 (346↑ 94↓)	322 (191↑ 131↓)	247 (98↑ 149↓)	265
B-2 progenitor	1449	778 (459↑ 319↓)	160 (108↑ 52↓)	317 (147↑ 170↓)	194

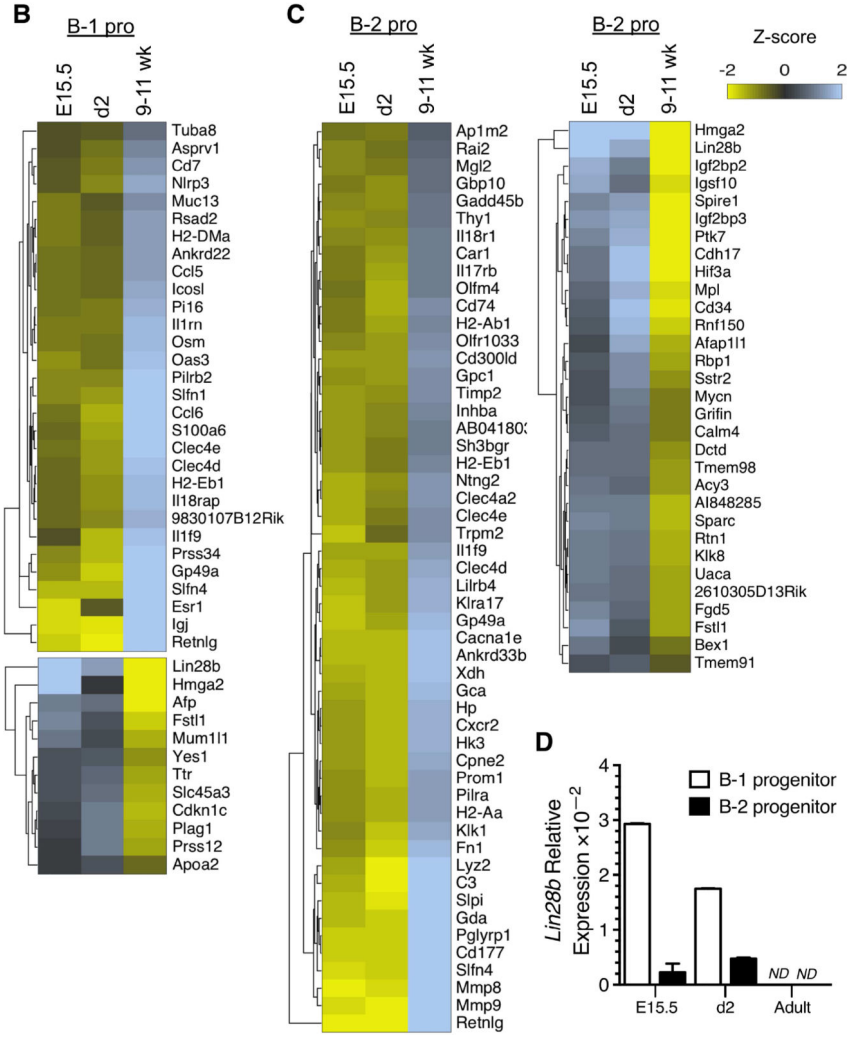

Figure 6. Fetal/neonatal and adult progenitors exhibit differences in gene expression

(A) Bayesian polytomous model selection results for B-1 and B-2 progenitors showing the total number of genes classified in four possible models as well as the proportion of genes classified in each model. (B) Expression heatmap of genes classified in *Model 1* (fetal=neonate≠adult) for B-1 progenitors (B-1 pro; log2 fold-change>1.5). (C) Expression heatmap of genes classified in *Model 1* (fetal=neonate≠adult) for B-2 progenitors (B-2 pro; log2 fold-change>1.5). The color scale indicates Z-scores. Complete gene lists are included in Table S2. (D) Expression of *Lin28b* relative to *Gapdh* in fetal (E15.5; n=12–16), neonatal

(d2; n=10–12), and adult (12 weeks; n=4–6) B-1 and B-2 progenitors determined by qPCR. Data representative of 2 independent experiments.