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Fetal Hematopoietic Stem Cell Transplantation Fails to Fully Regenerate the B-Lymphocyte Compartment

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SUMMARY

B cells are key components of cellular and humoral immunity and, like all lymphocytes, are thought to originate and renew from hematopoietic stem cells (HSCs). However, our recent single-HSC transfer studies demonstrate that adult bone marrow HSCs do not regenerate B-1a, a subset of tissue B cells required for protection against pneumonia, influenza, and other infections. Since B-1a are regenerated by transfers of fetal liver, the question arises as to whether B-1a derive from fetal, but not adult, HSCs. Here we show that, similar to adult HSCs, fetal HSCs selectively fail to regenerate B-1a. We also show that, in humanized mice, human fetal liver regenerates tissue B cells that are phenotypically similar to murine B-1a, raising the question of whether human HSC transplantation, the mainstay of such models, is sufficient to regenerate human B-1a. Thus, our studies overtly challenge the current paradigm that HSCs give rise to all components of the immune system.

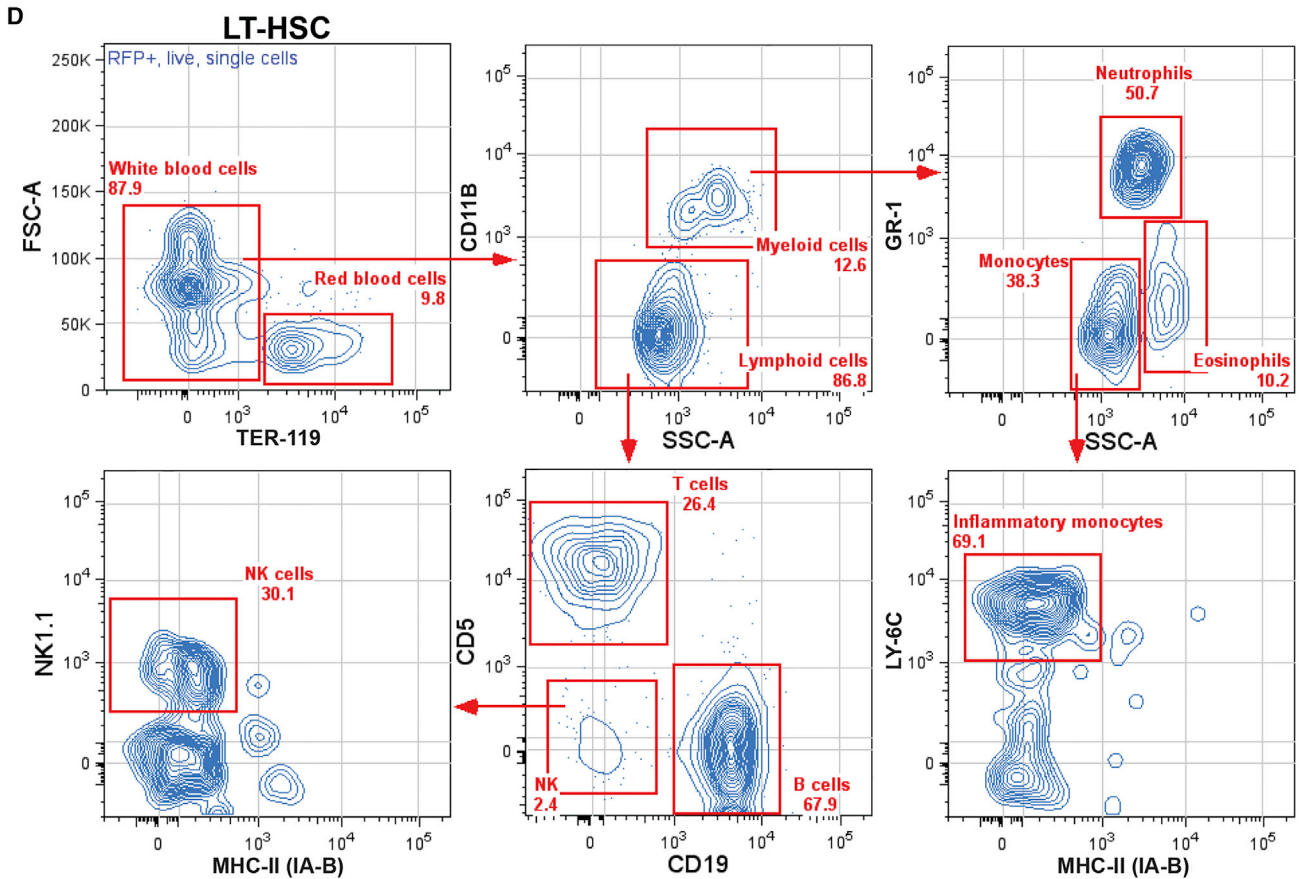
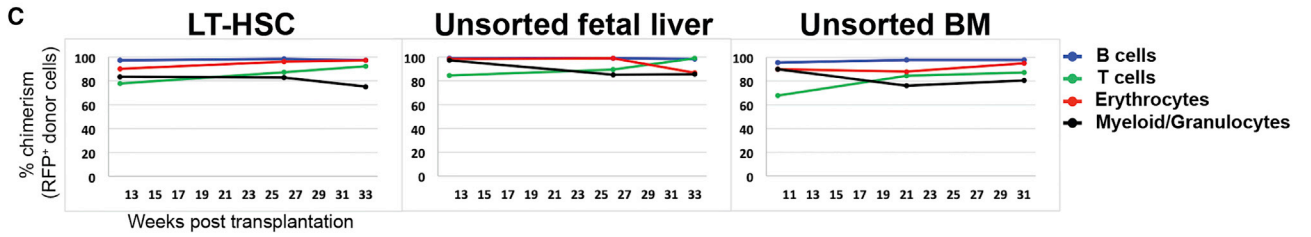
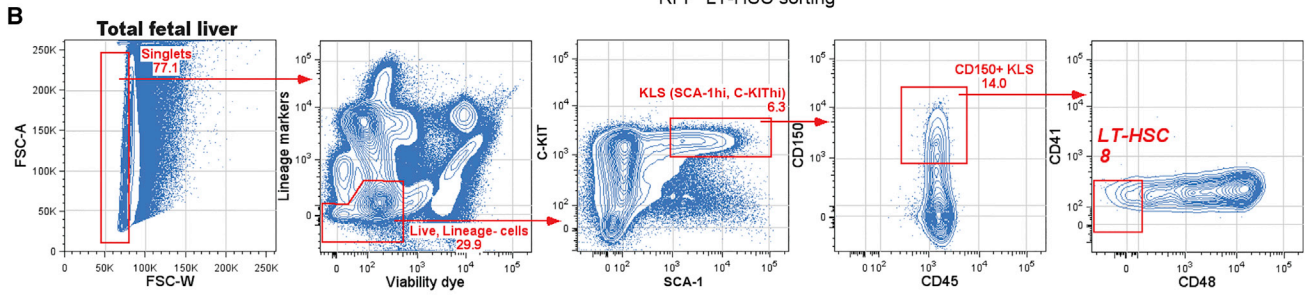
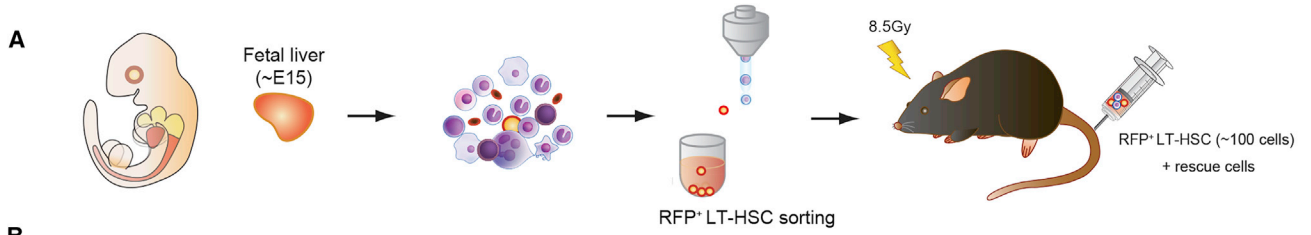
INTRODUCTION

The hematopoietic stem cell (HSC) is currently defined by its ability to both self-renew and stably reconstitute all components of the immune system, including erythrocytes, myeloid cells, granulocytes, and lymphocytes. The current paradigm posits that a single HSC, which resides in the bone marrow (BM), gives rise to all components of the immune system (Osawa et al., 1996). However, we recently showed that HSC isolated from adult BM fail to fully reconstitute the lymphocyte compartment in a murine model of HSC transplantation (Ghosn et al., 2012). In essence, we showed that a single HSC purified from adult BM and transplanted into irradiated recipients selectively fails to reconstitute the subset of tissue B cells known as B-1a (Ghosn et al., 2012). Similarly, fate-mapping studies by others (Ginhoux et al., 2010) have shown that tissue macrophages, such as microglia and Langerhans cells, and some subsets of T cells (Yoshimoto et al., 2012), originate independently of HSCs.

Since the initial purification and characterization of murine HSCs in 1988 (Spangrude et al., 1988), the phenotype and reconstitution potential of HSCs from both mice and humans have been extensively studied. Currently HSCs are widely used in human regenerative therapies to restore immunity in irradiated or otherwise immune-compromised patients (Czechowicz and Weissman, 2011; Liang and Zuniga-Pflucker, 2015; Pasquini and Zhu, 2014). However, relatively little is known about the scope of this reconstitution, particularly with respect to subsets of myeloid cells and lymphocytes (i.e., tissue B cells) that are not readily detectable in blood.

Among lymphocytes, B cells are a key component of both cellular and humoral immunity, serving both as antigen-presenting cells and antibody-producing cells. In adult mice, B cells are commonly divided into five functionally distinct subsets: follicular (FO), marginal zone (MZ), transitional, B-1b, and B-1a B cells. These subsets specialize in the recognition of the various classes of antigens and differ in mechanisms by which they elicit or provide immune effector functions. For example, FO B (also known as B-2) produce the well-described germinal center, T-cell-dependent immune responses against protein antigens. In contrast, B-1a produce a rapid antibody response to T-cell-independent type 2 antigens (mainly lipids and polysaccharides) (Martin et al., 2001). B-1a are also known to produce most of the “natural” antibodies in serum that protect from influenza (Choi and Baumgarth, 2008), pneumonia (Haas et al., 2005; Weber et al., 2014), and other serious infectious diseases. Together, the various B-cell subsets orchestrate a multi-component antibody response that helps achieve broad protection against infections.

Since the initial purification and characterization of murine HSCs in 1988 (Spangrude et al., 1988), the phenotype and reconstitution potential of HSCs from both mice and humans have been extensively studied. Currently HSCs are widely used in human regenerative therapies to restore immunity in irradiated or otherwise immune-compromised patients (Czechowicz and Weissman, 2011; Liang and Zuniga-Pflucker, 2015; Pasquini and Zhu, 2014). However, relatively little is known about the scope of this reconstitution, particularly with respect to subsets of myeloid cells and lymphocytes (i.e., tissue B cells) that are not readily detectable in blood.



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Since B-1a largely develop in situ during fetal life and are readily reconstituted in adult recipients by transfers of fetal liver (Kantor and Herzenberg, 1993), the question arises as to whether B-1a are reconstituted by fetal liver LT-HSCs, which selectively lose this reconstitution capacity as animals mature to adults (Ghosn et al., 2012). Alternatively, B-1a are reconstituted from distinct HSC-independent progenitors that are predominant in fetal liver, but scarce in adult BM. Support for this latter hypothesis, which views B-1a and FO B as separately developing lineages, comes from recent studies of fetal hematopoiesis (Kobayashi et al., 2014). In essence, small but detectable numbers of B-1-restricted progenitors were found in the fetal liver of HSC-deficient transgenic mice (*Cbfb*^{-/-}:*Tek-GFP/Cbfb*) (Kobayashi et al., 2014), suggesting that, in this transgenic condition, B-1 can develop independently of HSCs. To date, however, the potential of purified fetal liver LT-HSCs transplants to regenerate B-1a in vivo has not been tested.

Here, we resolve this issue by showing that, similar to adult BM, fetal liver LT-HSCs effectively regenerate FO, MZ, transitional, B-1b, and peripheral blood B cells but still fail to regenerate B-1a. These findings provide further evidence that B-1a emerge as a separate B-cell lineage that develops independently of LT-HSCs. Consistent with these findings, we show that fetal liver LT-HSCs also fail to reconstitute a key component of the B-1a repertoire (i.e., VH11), which produce anti-phospholipid antibodies known to promote housekeeping activities and prevent autoimmunity (Behar and Scharff, 1988; Elkon and Silverman, 2012; Nguyen et al., 2015).

Together, these studies challenge the broadly accepted paradigm that LT-HSCs can give rise to all components of the immune system. We discuss these findings in light of recent studies showing that this HSC-independent developmental strategy may also apply in other hematopoietic lineages (Ginhoux et al., 2010; Schulz et al., 2012; Yoshimoto et al., 2012). Furthermore, from a medical standpoint, we call into question current human regenerative therapies in which HSC transplantation is used to restore immunity in immune-compromised individuals,

and consider the probable shortcomings of an immune system that lacks the B-1a cell subset.

RESULTS

Fetal Liver LT-HSCs Provide Long-Term Reconstitution of All Major Peripheral Blood Subsets

The fetal site at which the first long-term (LT)-HSC emerges is still greatly debated (Cumano et al., 2000; Lux et al., 2008; Medvinsky and Dzierzak, 1996; Palis et al., 1999). Nevertheless, most researchers agree that, regardless of their origin, LT-HSCs migrate to the fetal liver at approximately embryonic day 11.5 (~E11.5) (Kumaravelu et al., 2002), where they greatly increase in number and initiate hematopoiesis before finally migrating to the BM.

To purify the LT-HSCs from fetal liver for studies here, we designed a new high-dimensional (Hi-D) fluorescence-activated cell sorting (FACS) panel with which we sorted LT-HSCs as C-KIT^{hi}, SCA-1^{hi}, CD150⁺, CD48⁻, CD41⁻, CD45⁺, CD38⁺, CD34^{lo}, and lineage-negative (Lin⁻) cells negative or low for GR-1, NK1.1, CD11B, CD3, B220, IL-7R, and TER-119 (Figures 1B and S1). The LT-HSC phenotype defined here identifies all of the detectable LT-HSCs in the mouse, as demonstrated by several single-cell adoptive transfer studies (Ghosn et al., 2012; Inlay et al., 2014; Kiel et al., 2005; Kim et al., 2006; Osawa et al., 1996; Yamamoto et al., 2013). Hence, to date this phenotype represents the most pure and undifferentiated HSC capable of providing long-term multi-lineage reconstitution in irradiated mice.

To facilitate subsequent identification of LT-HSC progeny in adoptive recipients, we isolated LT-HSCs from transgenic mice expressing monomeric red fluorescent protein (RFP) knocked into the Rosa26 locus (Ueno and Weissman, 2006). The RFP reporter is stably and ubiquitously expressed in all (>99%) hematopoietic cells, including fetal liver LT-HSCs (Figures S2A and S2B). Since the phenotype, percentage, and absolute number of the various hematopoietic cells (including B-cell subsets) are virtually identical between TM7-RFP and C57BL/6 (Figures S2C and S2D), the TM7-RFP strain provides a robust and reliable model with

Figure 1. Fetal Liver LT-HSCs Provide Stable Long-Term Reconstitution of All Major Blood Subsets

- (A) Experimental design. ~E15 livers were processed into single-cell suspensions and stained as described in the Experimental Procedures. RFP⁺ LT-HSCs were sorted and injected intravenously along with rescue RFP⁻GFP⁺ BM cells into lethally irradiated C57BL/6.
- (B) Gating strategy to purify fetal liver LT-HSCs (Lin⁻, C-KIT^{hi}, SCA-1^{hi}, CD150⁺, CD48⁻, CD41⁻, CD45⁺) (see also Figure S1). KLS: C-KIT^{hi}, Lineage⁻, SCA-1^{hi} cells.
- (C) Levels of blood chimerism (% RFP⁺ donor cells) in mice that received ~100 sorted fetal liver LT-HSCs (~E15), ~1.5 × 10⁶ unsorted fetal liver (~E15), and ~1.5 × 10⁶ unsorted adult (>8 weeks old) BM. Recipient mice were bled several times to determine the percentage of donor-derived B cells, T cells, erythrocytes, and myeloid-granulocytes.
- (D) Gating strategy identifying all major peripheral blood subsets derived from fetal liver LT-HSCs (RFP⁺). Data are representative of six recipient mice from three independent experiments.



which to study the reconstitution of the various B-cell subsets by adoptively transferred LT-HSCs.

Six to 9 months after transplantation, more than 70% of total red and white blood cells (including erythrocytes, myeloid cells, granulocytes, T cells, and B cells) in the peripheral blood of recipient mice are derived from the donor-purified fetal liver LT-HSCs (Figure 1C). This high level of chimerism (>70%), obtained even 9 months after transplantation, confirms that the fetal liver LT-HSC studied here were fully multi-potent, since they demonstrated significant and stable reconstitution for all of the major peripheral blood subsets (Figure 1D). The levels of chimerism and long-term reconstitution for the various subsets of blood cells were similar between recipients of sorted fetal liver LT-HSCs and unsorted fetal liver and adult BM (>70%) (Figure 1C). These findings are consistent with previous studies demonstrating that the LT-HSC phenotype we use here marks all of the long-term multi-lineage HSCs in these tissues (Ghosh et al., 2012; Inlay et al., 2014; Kiel et al., 2005; Kim et al., 2006; Osawa et al., 1996; Yamamoto et al., 2013).

Fetal Liver LT-HSCs Efficiently Reconstitute B-2 and B-1b, but Selectively Fail to Reconstitute B-1a in Periphery

To determine the potential of fetal liver LT-HSCs to reconstitute B-cell subsets in the periphery, particularly their ability to reconstitute B cells that are not readily detectable in blood, we harvested peritoneal cavity, spleen, and BM of recipient mice 6–9 months after transplantation. To simultaneously identify several functionally distinct subsets of B cells (i.e., FO, MZ, transitional, B-1a, and B-1b), we used new 18-parameter FACS panels designed for each of the tissues we analyzed.

Surprisingly, fetal liver LT-HSC transplantation failed to reconstitute the resident peritoneal B-1a (CD5⁺ B cells). In contrast, B-2 and B-1b cells were readily reconstituted in all recipients (Figures 2A–2C). Importantly, even 6–9 months after transplantation, when all hematopoietic cells had stably developed and renewed in the peripheral blood of recipient mice (Figure 2D), there were still no B-1a detectable in the reconstituted mice.

Despite being unable to reconstitute B-1a, fetal liver LT-HSCs reconstituted a substantial proportion of B-1b (Figures 2A–2C). This finding is quite surprising in light of the presumed close lineage relationship between B-1a and B-1b cells. In essence, our findings place B-1b and B-2 cells in the same developmental lineage, which derives from LT-HSCs. Thus, B-1a is placed in a separate developmental pathway that originates in fetal life independently of the fetal (~E15) liver LT-HSCs sorted and transferred in our studies.

To determine whether the fetal liver CD150⁻ fraction, unlike CD150⁺CD48⁻ LT-HSCs, can reconstitute B-1a cells, we purified and transferred fetal liver KLS CD150⁻ progenitor

cells and compared their B-1a reconstitution potential with that of total unsorted fetal liver. We show that KLS CD150⁻ progenitors reconstitute B-1a cells to the same extent as unsorted fetal liver, suggesting that most B-1a progenitors are contained within the CD150⁻ fraction (Figures 2A–2C). Hence, the failure of LT-HSCs to reconstitute B-1a is not due to a lack of support for B-1a development in the adult recipient environment, as peritoneal B-1a are readily reconstituted by both sorted CD150⁻ fraction and unsorted fetal liver, albeit at about half the normal level (i.e., this reconstitution is statistically significantly less compared with the numbers—absolute and relative—of B-1a cells obtained from adult unmanipulated mice) (Figures 2A–2C). In addition, transfers of LT-HSCs purified from neonatal (day 0) or adult (>8 weeks) BM into neonatal (day 0) recipients fail to reconstitute B-1a (Figures S3A and S3B), indicating that the selective inability of LT-HSCs to reconstitute B-1a reflects an intrinsic genetic program that excludes their development to B-1a (rather than a response that is dictated by the fetal/neonatal environment).

Similarly, when LT-HSCs from adult BM are co-transferred with unsorted fetal liver cells from congenic donors, the BM LT-HSC do not reconstitute B-1a, even though the co-transferred fetal liver readily reconstitutes B-1a (Figure S3C). Finally, earlier transfer studies have clearly shown that B-1a are readily reconstituted when early progenitors (fetal liver, neonatal spleen, and neonatal BM) are transferred into lethally irradiated adult recipients (Herzenberg et al., 1992; Kantor and Herzenberg, 1993). Together, these findings demonstrate that in an environment conducive to B-1a development, fetal liver LT-HSC transplantation gives rise to B-2 and B-1b cells, but do not give rise to B-1a.

Analysis of the BM of mice reconstituted with LT-HSCs 9 months after transplantation shows that fetal liver LT-HSCs reconstitute all major hematopoietic lineages, including KLS cells (C-KIT^{hi}, Lin⁻, SCA-1^{hi}) (Inlay et al., 2014), myeloid progenitors (Lin⁻, SCA-1⁻, C-KIT^{hi}, which includes CMPs, GMPs, and MEPs) (Inlay et al., 2014), lymphoid progenitors (Lin⁻, SCA-1^{+hi}, and C-KIT^{-lo}, which includes IL-7R⁺ common lymphoid progenitors [CLPs]) (Welner et al., 2008), and their respective differentiated lineages (Lin⁺) (Figure 2E). Consistent with the high levels of donor LT-HSC chimerism in peripheral blood in these mice (Figure 2D), >90% of total BM cells, including LT-HSCs, are derived from the transferred RFP⁺ fetal liver transplants (Figure 2E).

Hence, although fetal liver RFP⁺ LT-HSC transplants fail to reconstitute B-1a cells in the periphery, they are fully capable of reconstituting and stably renewing all other B-cell subsets (as well as other hematopoietic lineages). Thus, we conclude that these latter BM-derived cells all belong to a single, predominant, LT-HSC-derived hematopoietic lineage to which B-2 and B-1b, but not B-1a, belong.

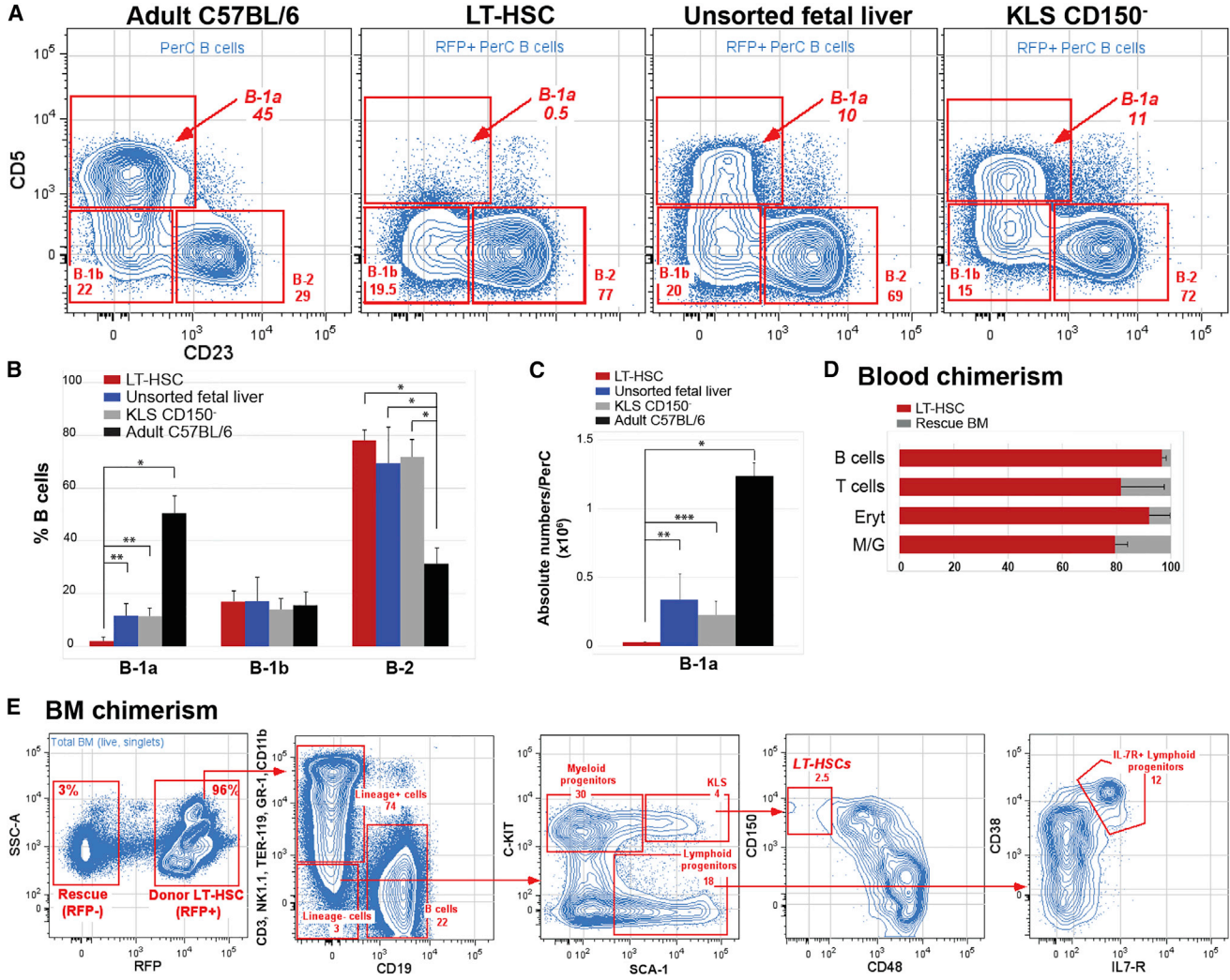


Figure 2. Fetal Liver LT-HSCs Selectively Fail to Reconstitute B-1a in Peritoneal Cavity

(A) B-cell subsets from adult intact (unmanipulated) C57BL/6 were compared with B cells from mice that received RFP⁺ sorted fetal liver LT-HSCs, unsorted fetal liver, and sorted fetal liver KLS CD150⁻ transplants (~E15 embryos). Total donor B cells (RFP⁺, CD19⁺) were analyzed for the expression of CD5 and CD23 to identify B-1a (CD5⁺CD23⁻), B-1b (CD5⁻CD23⁻), and B-2 (CD5⁻CD23⁺).

(B) Percentage of B-cell subsets in PerC of mice that received sorted or unsorted fetal liver transplants compared with adult unmanipulated C57BL/6. *p < 0.0001, **p = 0.001.

(C) Absolute numbers of B-1a cells in PerC of mice that received sorted or unsorted fetal liver transplants. *p < 0.0001, **p = 0.0009, ***p = 0.03. Error bars represent the mean and SD for B-cell numbers obtained from the fully chimeric recipient mice that showed the highest levels of B-cell chimerism (six mice for LT-HSCs, seven mice for unsorted fetal liver, and four mice for KLS CD150⁻) from three independent experiments. p Value for means comparison using Student's t test, $\alpha = 0.05$ (JMP; SAS Institute).

(D) Percentage of blood cell subsets (RFP⁺) detected in recipients ~7.5 months after LT-HSC transplantation, when recipient PerC cells were harvested.

(E) BM from recipient mouse ~7.5 months after transplantation. RFP⁻ cells are derived from rescue BM co-transplanted with RFP⁺ LT-HSCs. KLS: C-KIT^{hi}, Lineage⁻, SCA-1^{hi} population enriched for LT-HSCs.

Human Fetal Liver Transplants Reconstitute Peritoneal B Cells that Resemble Murine B-1a

Important from a medical standpoint, we show that transfers of human fetal liver (~E20–24 gestational weeks) into BLT (BM, liver, thymus) humanized mice readily gives

rise to peritoneal B cells expressing a phenotype similar to B-1a cells that are derived from mouse fetal liver transplants (Figure S4). Thus, similar to murine fetal liver (Figures 2 and S4), human fetal liver contains progenitors for phenotypically distinct B-cell subsets, one of which

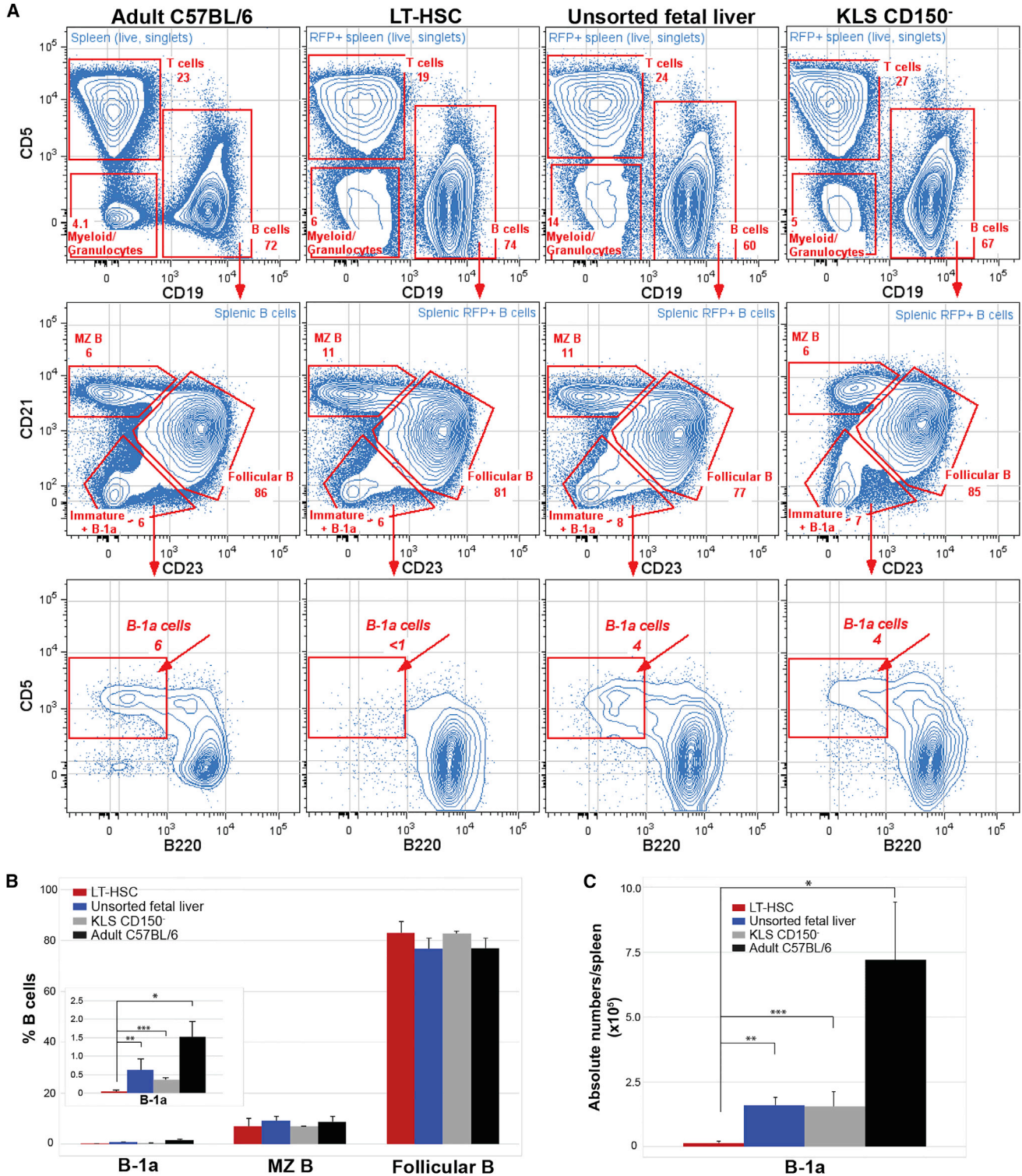


Figure 3. Fetal Liver LT-HSCs Effectively Reconstitute FO and MZ B Cells, but Fail to Reconstitute B-1a in Spleen

(A) Splenic B-cell subsets in adult intact (unmanipulated) C57BL/6 compared with B cells that were reconstituted in mice that received RFP⁺ sorted fetal liver LT-HSCs, unsorted fetal liver, and sorted fetal liver KLS CD150⁻ transplants (~E15 embryos). Live, single cells were analyzed for the expression of CD5 and CD19 to identify T and B cells, respectively. Spleen from recipient mice were first gated on RFP⁺ to

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expresses a phenotype similar to murine peritoneal B-1a and specifically migrates to mouse peritoneal cavity in a humanized mouse model (Figure S4). Although the definitive phenotype and characterization for human B-1a cells is yet to be determined, our data clearly show that human fetal liver transplants give rise to a subset of B cells that migrate into the mouse peritoneal cavity and express a phenotype similar to mouse peritoneal B-1a, raising the likelihood that human B-1a develop independently of human fetal liver LT-HSCs.

Fetal Liver LT-HSCs Efficiently Reconstitute FO, MZ, and Transitional B Cells, but Fail to Reconstitute B-1a in Spleen

Similar to the results from the peritoneal cavity (Figure 2), fetal liver LT-HSCs failed to reconstitute splenic B-1a (Figure 3). All of the other major B-cell subsets are reconstituted and stably renewed at normal proportions, comparable with unmanipulated mice (Figure 3). Hence, except for B-1a, B-cell development is essentially normal in mice that received purified fetal liver LT-HSCs (Figure 3).

Fetal Liver LT-HSC Transplantation Does Not Restore the VH11 Anti-Phospholipid Repertoire, a Well-Known Functional Component of B-1a

To further confirm the selective inability of the LT-HSC to give rise to B-1a, we investigated the expression of the *IgHV_H11* gene family (Figure 4), which is known to be uniquely expressed by B-1a (Hardy et al., 2004). The VH11 antibody repertoire in wild-type mice is largely encoded by germ-line sequences and shows little or no N-insertion and/or mutation. B-1a expressing VH11 produce “natural” antibodies that react with the endogenous trimethylammonium group expressed on phosphatidylcholine and sphingomyelin, phospholipids found in or on erythrocytes, platelets, stomach, intestine, and circulating low-density lipoprotein (Hardy et al., 2004; Poncet et al., 1990). Collectively, these well-known B-1a autoantibodies are thought to participate in “housekeeping” activities (Elkon and Silverman, 2012) that include the recognition and/or neutralization of potentially deleterious autoantigens, some of which have been implicated in autoimmune disease (Behar and Scharff, 1988; Gronwall et al., 2012; Nguyen et al., 2015).

Consistent with the inability of fetal liver LT-HSCs to give rise to B-1a, we find that VH11-encoded B cells are virtually absent in mice reconstituted with fetal liver LT-HSC even 7 months after transplantation (Figure 4). Thus, the absence of phenotypically identifiable B-1a in the reconstituted mice is supported by the absence of one of the key functional B-1a subsets present in wild-type mice, indicating that *IgHV_H11* variable region gene expression by B cells is restricted to those generated by a separate developmental pathway that gives rise to B-1a during late fetal and early neonatal life.

DISCUSSION

Here, we define a lineage of tissue B cells that originates independently of the fetal liver LT-HSC. In essence we show that, in a transplantation setting, fetal liver LT-HSCs selectively fail to regenerate B-1a cells in otherwise fully reconstituted hosts. These findings call into question the current view that HSCs regenerate all components of the immune system, a view of particular importance to modern medicine, since HSC transplantation is broadly used to restore immune function in patients in whom the immune system has been compromised (Czechowicz and Weissman, 2011; Liang and Zuniga-Pflucker, 2015; Pasquini and Zhu, 2014). Our data demonstrate that at least two B-cell lineages overlap in development during ontogeny and are independently renewed throughout life. The B-2 lineage, which mainly gives rise to FO, MZ, transitional, and B-1b B cells, originates and is continually renewed by HSCs located in both fetal liver and adult BM. In contrast, the B-1a lineage, which gives rise to B cells located mainly in pleural and peritoneal cavities, omentum, and spleen, originates in the fetus independently of liver HSCs and persists independently via self-renewal throughout life.

These developmental distinctions between the lineages to which B-1a and B-2 belong, i.e., HSC-independent versus HSC-dependent, likely reflect a deeper development schism that transcends the development of all mammalian hematopoietic lineages. In both mice and humans, the first source of hematopoietic cells found during ontogeny is located extra-embryonically in the yolk sac, in an anatomic

include all progeny derived from the donor transplants. CD21 and CD23 expression levels on B cells (CD19⁺) distinguished MZ (CD21^{hi}CD23⁻), FO (CD23^{hi}CD21^{lo}), and immature + B-1a (CD21^{lo/-}CD23^{lo/-}). B-1a were identified as CD5⁺B220^{lo/-}.

(B) Percentage of B-cell subsets in spleen of mice that received sorted or unsorted fetal liver transplants compared to adult unmanipulated C57BL/6. *p < 0.0001, **p = 0.001, ***p = 0.05.

(C) Absolute numbers of B-1a cells in spleen of mice that received sorted or unsorted fetal liver transplants. *p < 0.0001, **p = 0.03, ***p = 0.05. Error bars represent the mean and SD for the B-cell numbers obtained from the fully chimeric recipient mice that showed the highest levels of B-cell chimerism (six mice for LT-HSCs, seven mice for unsorted fetal liver, and four mice for KLS CD150⁻) from three independent experiments. p Value for means comparison using Student's t test, $\alpha = 0.05$.

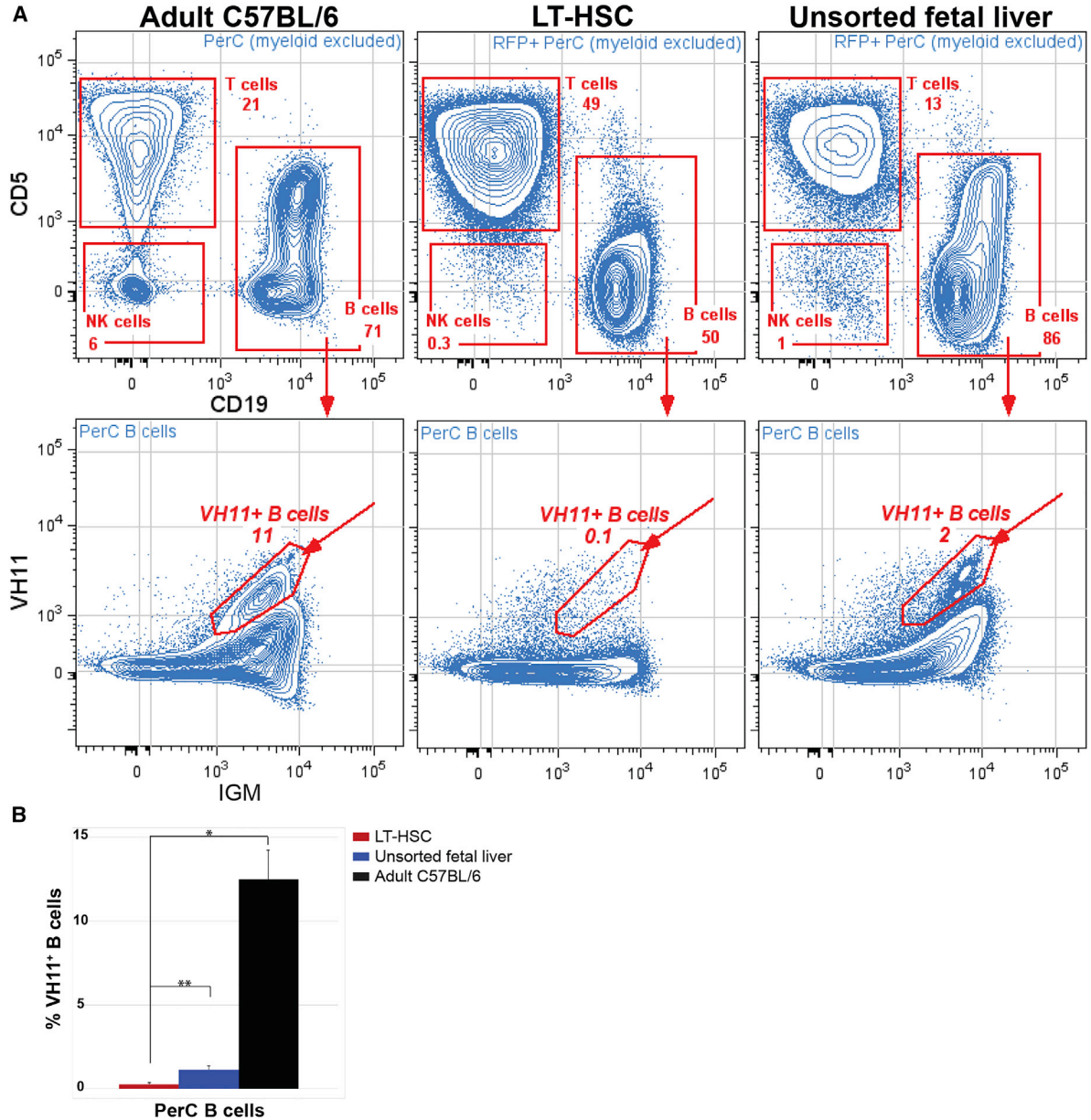


Figure 4. Anti-Phospholipid VH11 Antibody Repertoire Is Absent in Mice that Received LT-HSC Transplantation

(A) PerC cells from adult (unmanipulated) C57BL/6, and mice that received RFP⁺ sorted LT-HSCs and unsorted fetal liver (~E15) were stained for anti-VH11 in an 18-par Hi-D FACS panel. Cells were first gated on RFP⁺ to include all progeny derived from donor transplants. Donor-derived B cells (RFP⁺, CD19⁺) were analyzed for the expression of surface VH11 and IGM. B cells expressing IGM VH11 repertoire form a diagonal cluster in the FACS plot. Recipient mice were analyzed ~7.5 months after transplantation.

(B) Percentage of VH11⁺IGM⁺ B cells in PerC of mice that received sorted (LT-HSCs) or unsorted fetal liver transplants compared to adult unmanipulated C57BL/6. *p < 0.0001, **p = 0.0007. Error bars represent the mean and SD. p Value for means comparison using Student's t test, $\alpha = 0.05$. Data are representative of the six recipient mice (in each group) that showed the highest levels of B-cell chimerism in three independent experiments.

region known as the blood islands (Lux et al., 2008; Moore and Metcalf, 1970; Ueno and Weissman, 2006). Fetal nucleated erythrocytes are the first hematopoietic lineage to emerge within the blood islands at ~E7 (Palis

et al., 1999), prior to and independent of the development of HSCs. Similarly, tissue macrophages, such as brain microglia, epidermal Langerhans cells, and some subsets of liver Kupffer cells, originate in the yolk sac at ~E8, prior



to the development of HSCs (Ginhoux et al., 2010; Schulz et al., 2012). Animals deficient in HSCs normally generate these tissue macrophages, despite their inability to reconstitute HSC-dependent macrophages, such as those derived from LY6-C^{hi} monocytes (Hashimoto et al., 2013; Schulz et al., 2012).

This initial wave of HSC-independent erythrocyte-myeloid development in the yolk sac (Lux et al., 2008; Palis et al., 1999) is immediately followed by an initial wave of lymphopoiesis (Boiers et al., 2013; Yoshimoto et al., 2011, 2012). Recent studies, using Rag1-GFP reporter mice, show that the earliest lymphomyeloid-primed progenitors (Lin⁻C-KIT⁺RAG1GFP⁺IL7Ra⁺) can be detected in the yolk sac at ~E9, prior to HSC development (Boiers et al., 2013). Consistent with this finding, E9 yolk sac endothelium can differentiate into B-1-restricted progenitors in vitro, and reconstitute both B-1 and MZ B, but not B-2, in adoptive transfer recipients (Yoshimoto et al., 2011). Similarly, fetal T-cell progenitors have been reported to originate autonomously in E9 yolk sac prior to the development of HSCs (Yoshimoto et al., 2012). Taken together, these studies suggest that HSC-independent hematopoiesis occurs for the lymphoid, myeloid, and erythroid lineages. We therefore propose that the development of HSC-independent hematopoietic cells in early embryos reflects an overall developmental strategy of the immune system, which initiates the first waves of “primitive” hematopoietic cells carrying evolutionarily conserved functions that are distinct from the HSC-derived cell and functions of the adaptive immune system operating in adults.

In this construction, B-1a are located in the initial lymphoid development wave, alongside fetal T cells (mostly $\gamma\delta$ T), microglia, Langerhans cells, and fetal nucleated erythrocytes. At least a subset of HSC-independent B-1a, we suggest, carries a pre-defined genetic program that has been refined throughout evolution to ensure the development of germ-line-encoded B-cell receptor (BCR) repertoires that are likely indispensable for basic immune functions and to promote homeostasis. The development of these germ-line-encoded repertoires, such as the VH11 gene family described here, are strictly regulated, as they show little to no mutation in their CDR3 sequences and are represented in the B-1a compartment in all mouse strains (Hardy et al., 2004; Kantor et al., 1995a). Ultimately, the ability of these HSC-independent immune cells to self-renew locally throughout adulthood (Hashimoto et al., 2013; Kantor et al., 1995b) enables them to participate in immune responses alongside HSC-dependent immune cells. Thus, to fully understand immune responses in children and adults, it is necessary to explore the mechanisms by which primitive (HSC-independent) immune cells that develop in fetal life contribute to the function of the (HSC-dependent) immune system operating in adults.

Although most B-1a cells largely develop de novo in fetal life and are maintained thereafter by self-renewal, some develop de novo in neonatal and adult mice (Ghosn et al., 2011; Gu et al., 1992; Holodick et al., 2009; Kantor et al., 1995b; Montecino-Rodriguez et al., 2006). We and others have shown that adult spleen (Ghosn et al., 2011) and BM (Montecino-Rodriguez et al., 2006) contain B-1-restricted progenitors (CD19⁺B220^{-/lo}) that give rise to B-1a and B-1b, but not B-2, in adoptive transfer recipients. However, the developmental origin of these B-1-restricted progenitors is largely unknown. A simple hypothesis is that B-1-restricted progenitors emerge in fetal life prior to and independent of LT-HSCs and are then maintained throughout adulthood by self-renewal. Alternatively, B-1-restricted progenitors emerge de novo in adults via an unknown developmental pathway(s) that is independent of LT-HSCs. In any event, the small number of B-1a that are usually reconstituted by transfers of adult BM, as shown in previous studies (Duber et al., 2009; Esplin et al., 2009; Holodick et al., 2009; Kantor and Herzenberg, 1993), must derive from “passenger” progenitor cells that accompany the actual LT-HSC.

Previous study by Dorshkind’s group (Barber et al., 2011) has tested the B-1 reconstitution potential for CLPs sorted from BM of neonatal (<2.5 weeks) and adult (>8 weeks) mice. The authors concluded that neonatal, but not adult, BM CLPs have the potential to give rise to B-1a. Because most CLPs are contained within the CD150⁻ fraction, the results from this early study corroborate our findings that fetal liver CD150⁻, but not CD150⁺ LT-HSCs, has the potential to reconstitute B-1a cells. However, Barber et al. (2011) also show that KLS CD150⁺ progenitors (enriched for LT-HSCs) sorted from neonatal BM have the potential to reconstitute B-1a, albeit at very low numbers. Given that the study was performed by transferring large numbers of heterogeneous KLS CD150⁺ progenitors, it is possible that the small number of B-1a cells detected in the recipient mice were derived from progenitor cells that accompanied the actual LT-HSCs.

Therefore, our findings that highly purified LT-HSCs fail to reconstitute B-1a cells, together with recent studies showing that HSC-deficient mice can develop B-1a cells (Kobayashi et al., 2014), suggest that at least some B-1a cells are a separate B-cell lineage that develops from progenitors expressing a phenotype similar to neonatal CLP and fetal KLS population (i.e., CD150⁻), but independent of the fetal liver LT-HSC pathway. In any event, the true origin and developmental pathway of B-1a cells is yet to be determined.

These insights into the HSC-independent versus HSC-dependent immune cell reconstitution pathway in mice can be expected to affect protocols used for HSC transplantation in clinical settings. Over the past 45 years, HSC



transplantation has been widely used to treat patients with cancers such as leukemias, lymphomas, and myelomas, and to treat several immune-deficiency disorders, among other diseases (Liang and Zuniga-Pflucker, 2015; Pasquini and Zhu, 2014). In the United States alone, approximately 20,000 HSC transplantations are performed yearly (Pasquini and Zhu, 2014). The survival rate for most HSC transplantation used to treat malignant diseases is only ~50% during the first few years after transplantation, and about 20% of all deaths after HSC transplantation are due to infectious diseases post transplant (Pasquini and Zhu, 2014). Thus, our data here call for further investigation on whether humans, like mice, generate immune cells independently of HSCs and whether these cells play critical roles that help protect HSC-transplanted patients from subsequent infectious diseases.

As we have suggested, the failure to reconstitute B-1a may be a serious impediment, since B-1a were shown to be necessary, for example, to protect against viral, bacterial, and fungal pathogens, such as influenza virus (Choi and Baumgarth, 2008), *Streptococcus pneumoniae* (Haas et al., 2005; Weber et al., 2014), and *Francisella tularensis* (Yang et al., 2012), among others. Moreover, B-1a secrete most of the natural antibodies detected in serum of unimmunized mice (Baumgarth et al., 2005; Gronwall et al., 2012), which is known to provide a first line of defense against viral (Ochsenbein et al., 1999) and bacterial (Haas et al., 2005; Ochsenbein et al., 1999) infections. A role for natural antibodies in protection from pneumonia has been appreciated for some time now (Briles et al., 1981). Knockout mice deficient in B-1a cells (*CD19^{-/-}*) lack natural antibodies and, hence, are more susceptible to infections caused by *S. pneumoniae* (Haas et al., 2005). Natural antibodies are also implicated in protection against pneumococci in humans (Carsetti et al., 2005). B-1a cells also generate innate response activator (IRA) B cells that produce both granulocyte-macrophage colony-stimulating factor and immunoglobulin M (Rauch et al., 2012). IRA B cells can protect against both microbial sepsis (Rauch et al., 2012) and pneumonia (Weber et al., 2014). Therefore, in therapeutic settings such as BM transplantation using highly purified HSCs (Czechowicz and Weissman, 2011), the lack of B-1a and natural antibodies may be detrimental.

The human equivalent of B-1 has recently been proposed for human peripheral blood (Rothstein et al., 2013), but the definitive phenotype of human tissue B-1a is yet to be fully defined. In any event, it is reasonable to expect that the functions provided by B-1a in mice are performed by the human equivalent. In fact, as we show here, human fetal liver transplants readily reconstitute B cells in the peritoneal cavity of humanized mice. These human-derived peritoneal B cells are phenotypically similar to murine peritoneal B-1a. Importantly, given that B-1a is rarely detectable

in blood, our findings here suggest that the human equivalent of B-1a are likely to be found to reside in peripheral tissues, rather than circulating in human blood. It remains to be determined whether, similar to mouse, LT-HSCs purified from human fetal liver fail to reconstitute the peritoneal (rather than blood) B cells we identified here. If so, the human equivalent of B-1a may also originate from a development pathway that is independent of the LT-HSC. Support for this hypothesis comes from several human B-cell development (Rechavi et al., 2015; Sanz et al., 2010), phenotyping (Rothstein et al., 2013; Sanz et al., 2010) and BCR sequencing (Rechavi et al., 2015) studies, which distinguish the B-cell development pathway in embryos and young children from the well-known pathway in adults.

Collectively, the findings we have presented here lead us to propose that the development of HSC-independent lineages early in ontogeny represents an evolutionary strategy that ensures the development of an immune system carrying unique functions that are evolutionarily conserved and distinct from the functions of the adaptive immune system operating in adults. In adults, HSC-independent and HSC-dependent immune systems co-exist and must collaborate to maintain homeostasis or resist infections.

EXPERIMENTAL PROCEDURES

Mice and Tissue Preparation

CS7BL/6 and B6.IgHa mice (8–10 weeks old) were purchased from Jackson Labs or bred at Stanford. Transgenic mice expressing enhanced green (pCx-eGFP) (Wright et al., 2001), or red (TM7-RFP) (Ueno and Weissman, 2006) fluorescent protein were kindly provided by the Weissman laboratory (Stanford). NSG mice (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) were purchased from Jackson Labs and used to generate BLT humanized mice. Tissues from recipient mice were processed, filtered, and resuspended using custom RPMI-1640 deficient in biotin, L-glutamine, phenol red, riboflavin, and sodium bicarbonate, and containing 3% newborn calf serum (defRPMI).

Blood (~200 μ l) was harvested via tail vein into EDTA-containing tubes. Peritoneal cavity (PerC) cells were harvested by injecting 6 ml of defRPMI into PerC. Spleens were mechanically disrupted to obtain single-cell suspension. BM from femurs and tibias were flushed with defRPMI using a 28-gauge syringe. Cells were filtered over a 70- μ m nylon filter and erythrocytes were lysed using ACK buffer. Fetal livers were harvested from ~E15 timed pregnant mice, digested at 37°C for 30 min with 0.25% collagenase (Stem Cell Technologies), then again using enzyme-free cell dissociation buffer (Gibco). Liver cells were mechanically pushed through a 70- μ m nylon filter to obtain single-cell suspensions.

Timed pregnancies were confirmed via post-coital plug and embryonic ages were confirmed via microscopy. All the procedures were approved by Stanford Animal Care and Use Committee and are in compliance with Administrative Panel on Laboratory Animal Care guidelines.



18-Parameter High-Dimensional Flow Cytometry

Cells were resuspended at 100×10^6 cells/ml in defRPMI and stained on ice for 30 min (or 60 min when staining for CD34) with the following fluorochrome-conjugated monoclonal antibodies (mAbs) in a 16-color, 18-parameter staining combination (see [Table S1](#) for clones and sources). In brief, for recipient spleen: anti-CD23, CD5, CD19, CD93, NK1.1, GR-1, CD11B, CD21, CD43, TCR $\delta\gamma$, IGK (kappa), and IGL (lambda) light chains, and B220; PerC: anti-V δ 6.3/2, CD5, CD19, F4/80, NK1.1, IGM, IGK, VH11, CD23, IA/IE (or IA-B), CD11B, and B220; BM: anti-SCA-1, CD11A, CD38, CD41, CD150, CD3, GR-1, TER-119, NK1.1, B220, CD34, CD48, CD117, IL7R, CD19; and blood: anti-TER-119, CD5, B220, LY6C, GR-1, CD11B, NK1.1, and CD19.

Cells were stained on ice for 15 min with Qdot605- or BV711-conjugated streptavidin to reveal biotin-coupled antibodies. Stained cells were resuspended in 10 μ g/ml propidium iodide (PI) to exclude dead cells. When cells were fixed, amine-reactive dyes were used to exclude dead cells.

Both GFP and RFP were detected concomitantly with the reagents described above for a total of 18-parameter Hi-D FACS. Cells were analyzed on Stanford Shared FACS Facility instruments (BD LSRII). Data were collected for $0.2\text{--}3 \times 10^6$ cells and analyzed with FlowJo (TreeStar). To distinguish autofluorescent cells from cells expressing low levels of a particular surface marker, we established upper thresholds for autofluorescence by staining samples with fluorescence-minus-one control stain sets in which a reagent for a channel of interest is omitted.

Sorting and Transfer

Fetal liver from \sim E15 pCx-eGFP or TM7-RFP were processed as described above and stained with the following mAbs in a 15-color, 17-parameter staining combination: anti-SCA-1, CD38, CD150, CD34, CD48, CD117, CD41, CD45, IL7R, CD135, CD19, and lineage markers (Lin) anti-CD3, B220, NK1.1, GR-1, CD11B, and TER-119 ([Table S1](#)).

Cells were stained on ice for 15 min with BV711-conjugated streptavidin to reveal biotin-coupled mAbs and resuspended in PI, to exclude dead cells. RFP⁺ or GFP⁺ LT-HSCs were identified as Lin⁻, C-KIT^{hi}, SCA-1^{hi}, CD150⁺, CD48⁻, CD41⁻, CD34^{low}, CD45⁺, CD38⁺, IL-7R⁻, and CD135⁻ ([Figures 1](#) and [S1](#)). Sorted LT-HSCs were resuspended in serum-free defRPMI and about 100 cells were transferred intravenously into lethally irradiated (two doses of 4.25 Gy delivered 4 hr apart) C57BL/6 along with $\sim 2 \times 10^5$ BM rescue cells from 8-week-old congenic mice (i.e., pCx-eGFP or B6.IgHa). After 30+ weeks, recipient PerC, spleen, BM, and blood cells were harvested and LT-HSC-derived B cells (RFP⁺ or GFP⁺) were analyzed as described above. To determine B-cell reconstitution potential for neonatal or adult BM LT-HSCs, BM from RFP⁺ or GFP⁺ mice were processed and stained as described for fetal liver. LT-HSCs were sorted on Stanford Shared FACS Facility BD FACSARIAIII.

Chimerism

Lethally irradiated recipients were injected with one of the following donor cells: unsorted fetal liver ($\sim 1.5 \times 10^6$ cells), sorted fetal liver LT-HSCs (~ 100 cells), and sorted fetal liver KLS CD150⁺ (~ 6000 cells) from \sim E15 embryos. Donor cells were harvested from TM7-RFP mice and transferred intravenously into irradiated C57BL/6 along with

$\sim 2 \times 10^5$ adult BM rescue cells from congenic B6.IgHa or pCx-eGFP. Co-transfer of the rescue cells is necessary to prevent the lethally irradiated mice from succumbing to anemia during the first weeks after irradiation. It readily provides red and white blood cells until the transferred LT-HSCs reconstitute all major blood cells.

Recipient mice were bled weekly to determine the level of chimerism, which we defined as the percentage of cells derived from the donor fetal liver LT-HSCs (RFP⁺) found among total blood cells recovered from recipient mice. Here, we examined the recipient mice that showed full, long-term stable chimerism (i.e., development of all major hematopoietic lineages, including erythrocytes, myeloid cells, granulocytes, T cells, and B cells) and had the highest chimerism in the B-cell compartment (>50%–99% of total B cells in blood derived from sorted RFP⁺ or GFP⁺ fetal liver LT-HSCs). All of the reconstituted hematopoietic lineages from donor cells were still readily detectable in recipient blood when the mice were euthanized and tissues harvested at ≥ 33 weeks post transplantation. Eighteen mice received ~ 100 purified RFP⁺ fetal liver LT-HSC transplants, and six became fully chimeric and were analyzed for the presence of B-1a cells. The data shown in the various figures represent the six fully chimeric mice that received purified LT-HSCs, and the FACS plots shown in each figure represent the analysis from the same recipient mouse.

Humanized Mice

Humanized BLT mice were generated as described by [Stoddart et al. \(2011\)](#). In brief, two 1-mm³ pieces of human fetal liver and one 1-mm³ piece of thymus (Thy/Liv) were implanted together under the kidney capsule of 10-week-old female NSG mice. Three weeks after Thy/Liv implantation, NSG mice were irradiated (225 cGy) and injected 30 hr later with $2\text{--}10 \times 10^5$ CD34⁺ autologous human fetal liver cells (20–24 gestational weeks) containing heterogeneous populations of hematopoietic progenitors, including HSCs. 20–24 weeks after CD34⁺ injection, recipient PerC was processed and stained with the following fluorochrome-conjugated mAbs in a 16-color, 18-parameter staining combination: anti-CD45, IGM, CD20, CD14, CD16, CD80, CD24, CD5, CD19, B220, IGD, CD27, CD43, CD10, CD3, and CD38. Amine-reactive dye was used to exclude dead cells ([Table S1](#)). Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2015.11.011>.

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