Differential roles for human fetal B lymphocytes in childhood and adult immunopathologies

Background and Significance: B lymphocytes, and their antibodies, are necessary to provide long-term protection against infections and to maintain homeostasis by clearing of apoptotic cells[1] and shaping of the mucosal microbiome through IgA and other effector molecules[2]. Dysregulation of B lymphocytes can lead to susceptibility to infections[3], dysbiosis of the gut microbiota[4], and other immune-related pathologies, including autoimmunity[5,6] (i.e. Lupus, Rheumatoid Arthritis, etc.) and blood malignancies, such as Leukemia and Lymphomas[7,8].

The B lymphocyte lineage develops progressively during ontogeny. In both mice and humans, the types of B cells that develop in fetuses and infants differ from those of adulthood[9-11]. Murine fetal B cells (aka B-1a), but not those of adult bone marrow (aka B-2), 1) produce most of the life-long protective natural antibodies (IgM[12] and IgA[2]), 2) recognize endogenous antigens such as those on apoptotic cells[1] and so have been implicated in the pathogenesis of autoimmune disorders[5], 3) differentiate into IL-10-producing B regulatory cells[13] , and 4) are prone to mutations that lead to B-cell malignancies such as Leukemia (cALL and CLL)[7,8]. However, although we[10,14-17] and others[13,18,19] have demonstrated that fetal B cells persist throughout adulthood to provide functions that are distinct from those of adult bone marrow (BM) B cells in mice, their counterparts in humans have not yet been fully investigated.

Based on recent supporting literature[8,9,20,21] and our preliminary data (see below), we hypothesize that, similar to mouse[10,14-17], human fetal liver B lymphocytes represent a separate lineage of lymphocytes that persists throughout adulthood. As such they would provide non-redundant life-long protective natural antibodies, including a biased antibody repertoire that recognizes auto-antigens (i.e. antigens on apoptotic cells) and participate in the pathogenesis of autoimmune disorders (i.e. Lupus) in both infants and adults. In addition, we hypothesize that the B lymphocytes that develop early in fetal liver, but not BM, are the likely origins of childhood and adult B-cell Leukemia such as cALL and CLL. To test the first hypothesis, we propose the following **Specific Aims**:

Aim 1. **Characterization and comparison of human fetal liver B lymphocytes at different gestational ages and human adult BM B lymphocytes.** *Phenotype: we will perform high-dimensional 19-parameter flow cytometry analysis to detect surface and intracellular proteins using our human B-cell panel that we have designed and validated previously. Gene expression: we will perform high-throughput single-cell RNA-sequencing to reveal the full gene expression and antibody clonotype/repertoire (heavy + light chain BCR-sequencing) of individual B cells using the 10x Genomics technology.*

Aim 2. **Generation of humanized mice reconstituted with either human fetal liver or adult bone marrow to test the functional differences between human fetal vs. adult B lymphocytes *in vivo*.** *We will transplant human hematopoietic stem/progenitor cells ($CD34^+$) from either fetal liver or adult BM into $CD47^{-/-}RAG^{-/-}\gamma chain^{-/-}$ triple knockout mice.*

We expect the results from these studies to provide new insights into the mechanisms of immune-mediated pathologies (i.e. B-cell malignancies, autoimmunity, etc.) that preferentially affect children. In addition, the insights we develop here into the functional differences between the B cells, and their antibody repertoire, that operate at different developmental ages can impact the development of novel vaccine strategies designed to trigger the types of B cells that develop and predominate in infants. The initial studies proposed here will provide additional preliminary data for the NIH Program Announcement PAR-18-333 (NIAID and NICHD), which calls for proposals aimed at *Understanding the Early Development of the Immune System*. Within one year, I will submit an NIH R01 grant proposal through NIH PAR-18-333.

Preliminary data

Aim 1. Previously, we have designed and validated a novel 19-parameter Hi-D FACS panel (see Experimental Design below) to identify and characterize subsets of human B lymphocytes from fetal liver and adult bone marrow (BM). Our preliminary phenotyping studies have revealed great heterogeneity within the B cell compartment in human adult peripheral blood (Fig. 1). Initial findings comparing fetal B cells isolated from human fetal liver (15 weeks of gestation) and adult BM (26-year-old healthy donor) point to the existence of a separate lineage of B cells that are present in fetal liver, but not adult BM (Fig. 2), similar to our previous reports in mouse[10,14,15].

In addition to characterizing the phenotypic differences between the human fetal and adult B-cell lineages, we aim to characterize their functions by revealing their unique global gene expression profile at a single-cell level (full transcriptome and B-cell repertoire analysis), using our recently acquired 10x Genomics Chromium technology[22]. The results from the global gene expression (total RNA-seq) and B-cell repertoire (BCR-seq) analysis from the same cell will inform our next steps towards a more targeted approach to characterize the functional differences between the fetal and adult B-cell lineages.

Aim 2. Our initial transplantation studies surprisingly show that, in humanized mice, human fetal liver regenerates tissue-resident (peritoneal) B lymphocytes that are phenotypically similar to murine fetal B-1a[14]. Here, we propose to continue and expand these studies by comparing the regenerative potential of human fetal liver (12-20 weeks) to adult BM (30-40 years old), and reveal whether human fetal liver cells, similar to mouse[10,14,15], give rise to different types of B lymphocytes upon transplantation into humanized mice.

Experimental Design and Methods

Aim 1. **Characterization and comparison of human fetal liver B lymphocytes at different gestational ages and human adult BM B lymphocytes.** Comprehensive 19-paramenter Hi-D FACS assays and analyses will be carried out on single cell suspensions obtained from human fetal liver and adult BM. Total of 6 fetal tissue specimens will be obtained from the first and second trimester fetal cadavers (from 12-20 gestational weeks). These developmental ages were chosen based on previous studies showing that early lymphopoiesis can be detected in human fetal tissues in as early as 12 weeks of gestation[9]. Total of 6 BM samples will be obtained from adult healthy donors ages 30-40 years old.

Human specimens will be obtained from University of Washington (Department of Pediatrics) and All Cells LLC. (Quincy, MA). All human specimens obtained for these studies will be tested for human diseases (including HIV/HBsAG/HC) and only the uninfected material (tested negative) will be aseptically processed in our facilities using established protocols. Fetal liver will be digested at 37°C for 30min. with 0.25% collagenase-I, then again using enzyme-free cell dissociation buffer (Gibco). Both fetal liver and adult BM samples will be mechanically pushed through 40µm nylon filter to obtain single cell suspensions and re-suspended using custom RPMI-1640 (deficient in biotin, L-glutamine, phenol red, riboflavin, and sodium bicarbonate) containing 3% new born calf serum (defRPMI).

19-parameter Hi-D FACS: cells will be resuspended at $100x10^6$ cells/mL in defRPMI and stained on ice for 30 min. with a 17-color, 19-parameter panel containing the following fluorochrome-conjugated mAbs (BD and BioLegend) to identify different subsets of **human B cells**: CD45, B220, CD5, IgD, IgM, CD38, CD43, CD23, CD27, Dump (CD3/CD14/CD16), MTK (mitotracker), CD9, CD95, CD80/86, CD20, CD30, CD24, CD19, and Zombie amine-reactive dye (viability). After washing, cells will be stained and incubated on ice for 15min. with Qdot605- or BV711-conjugated streptavidin to reveal biotin-coupled antibodies and subsequently fixed using

BD FACS Lysing solution. To distinguish auto-fluorescent cells from cells expressing low levels of a particular surface marker, we will establish upper thresholds for auto-fluorescence by staining samples with fluorescence-minus-one (FMO) control stain sets in which a reagent for a channel of interest is omitted. My extensive background in hi-dimensional flow cytometry technology (Hi-D FACS)[23-27] will be pivotal for this part of the project.

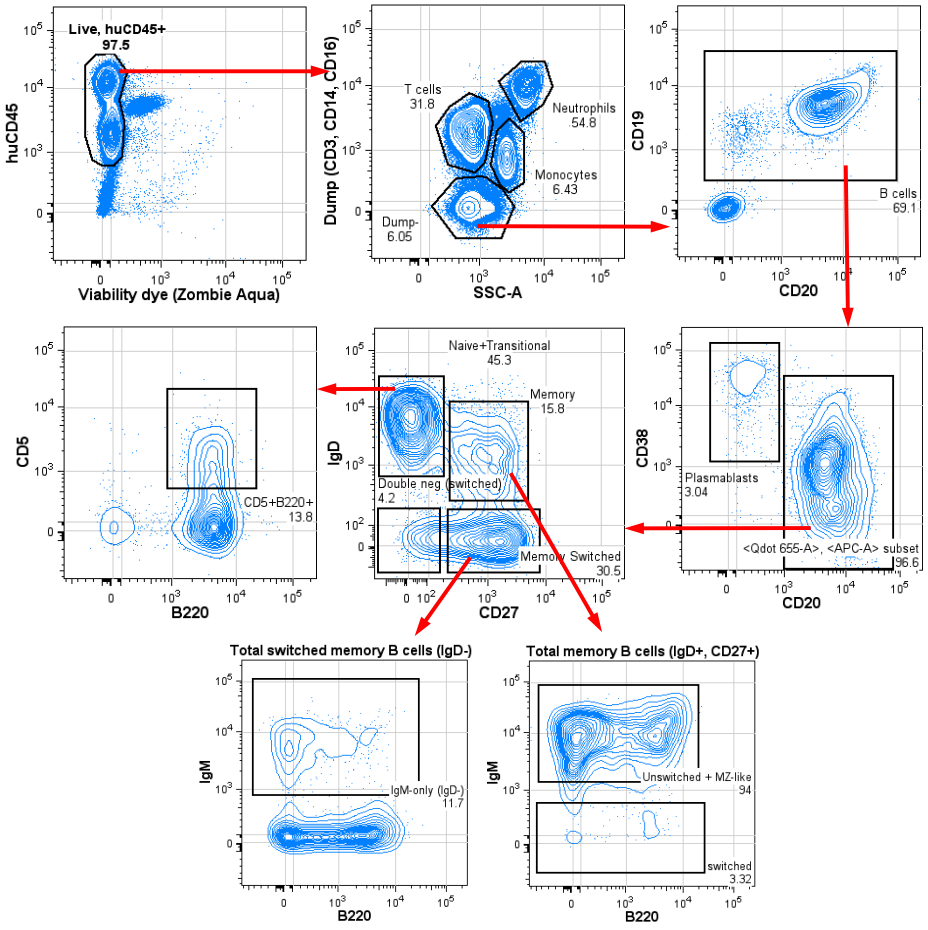


Figure 1. 19-parameter Hi-D FACS panel to identify the several human B cell subsets as well as other lymphoid, myeloid and granuloid lineages. Data show total peripheral blood of healthy adult female.

Single-cell RNA- and BCR-sequencing: we will perform single-cell both RNA- and BCR-sequencing of the various human B cell subsets in fetal liver and adult BM. These studies will provide biological insights into the functional differences (global gene expression) between the B cells that are developed from fetal and adult progenitors and the BCR repertoire they are programmed to express in fetal versus adult life. These gene expression studies (full transcriptome) can also be expected to inform and greatly broaden the set of markers (both intra- and extra-cellular) we can use in our Hi-D flow cytometry panels to further identify and purify functionally distinct subsets of B lymphocytes. We will use our recently acquired 10x Genomics GemCode™ system, a droplet-based technology that enables encapsulation of individual cells into 100,000s of uniquely barcode-containing partitions within minutes using the Chromium instrument[22]. The 10x technology platform enables high-throughput single cell RNA-seq for gene expression analysis combined with immune repertoire profiling of T and B cell receptors (TCRs and BCRs) from the same cell. For data analysis, we will use Loupe software (10x Genomics) and the SeqGeq application (Flowjo, LLC), which leverages an intuitive interface similar to the

Flow Cytometry Flowjo software we have been using for many years. Statistical analysis will be performed using JMP Genomics software (SAS Institute) and GraphPad Prism. For these studies, we will collaborate with Dr. Iñaki Sanz and his group, who have extensive experience on human B cell biology, including B-cell repertoire analysis (BCR-seq) and autoimmune diseases[28].

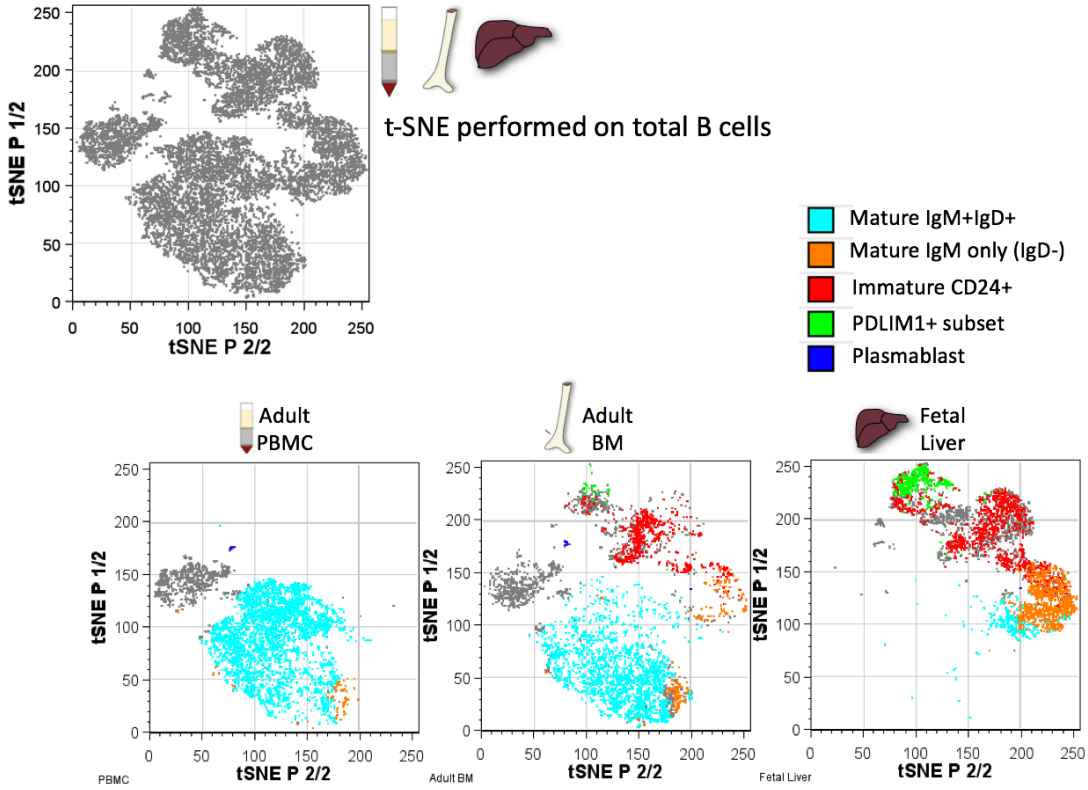


Figure 2. t-SNE plots performed on the 19-parameter Hi-D FACS panels used to identify the several human B cell subsets. Data show unique subsets of human B cells that develop in fetal, but not adult, tissues.

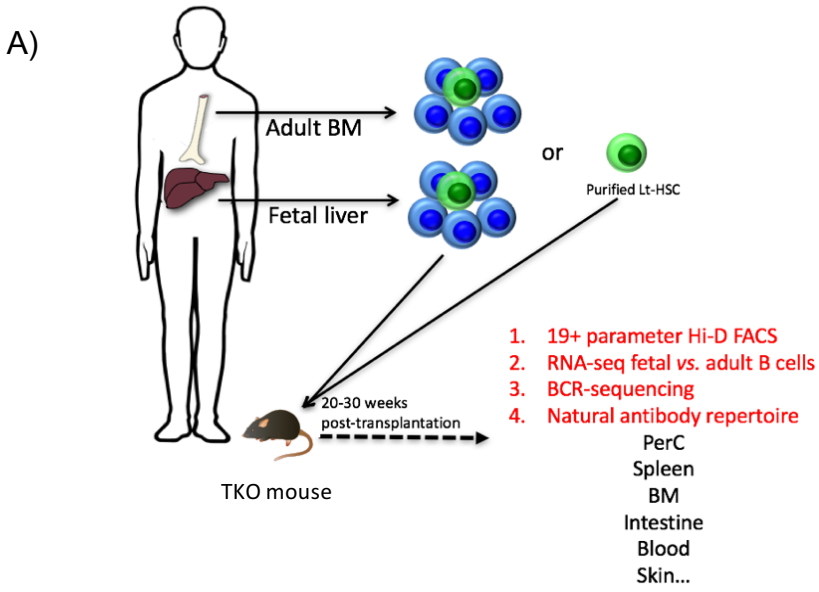
Aim 2. **Generation of humanized mice reconstituted with either human fetal liver or adult bone marrow to test the functional differences between human fetal vs. adult B lymphocytes *in vivo*.** We will generate 2 cohorts of humanized mice to characterize the different subsets of human B cells that develop in infants (i.e. from fetal liver transplants) and in adults (i.e. from adult BM transplants). The research funding requested here will be utilized to generate the 2 cohorts, but this funding will not be sufficient to perform the actual functional studies comparing fetal vs. adult B lymphocytes *in vivo*. However, this pilot project will allow us to characterize this novel humanized model by assessing the percentage of B cell chimerism and the B-cell phenotype (Hi-D FACS) that develop in each cohort (i.e. fetal liver vs adult BM cohorts). We then expect to submit an R01 proposal (NIH PAR-18-333) using these 2 cohorts of mice to investigate 1) whether fetal B cells (fetal liver cohort), unlike adult B cells (adult BM cohort), are more susceptible to develop lupus-like autoimmune pathologies (we will perform the humanized Lupus model as described in [29]), and 2) whether fetal B cells, unlike adult B cells, are more susceptible to become malignant (leukemia/lymphoma) cells that affect children, such as cALL and CLL, using similar approaches described in [8,30].

To generate the humanized cohorts, we will use a new type of recipient mice, developed recently by Dr. Hasenkrug at NIH[31], and available from The Jackson laboratory. These mice are generated in a C57BL/6 background with a triple gene knockout (TKO) (CD47-/-RAG-/-γchain-/-)[31]. A unique advantage of this transgenic TKO strain is sustained long-term engraftment of human

cells without the development of graft-versus-host disease (GVHD), commonly developed in standard models of NSG humanized mice. Our preliminary human fetal transplants, performed at Stanford, successfully regenerated and sustained peripheral blood B, T, myeloid, and NK cells, seven months after transplantation, and without signs of GVHD (Fig. 3B).

Generation of chimeric humanized mice: newborn (day 1-4) and adult (>8wks) mice will be irradiated with 100 rad and 600 rad, respectively, in two split doses. These sub-lethal irradiation doses were shown necessary to increase the human chimerism in TKO recipients[31]. Fetal liver and adult BM tissues will be processed into single cell suspensions and about 0.5-10 x 10^5 cells will be injected intravenously into newborn and adult irradiated TKO mice. Fetal liver and adult BM tissues will be stained with a 19-parameter FACS panel to identify and purify (FACS-sort) Hematopoietic Stem Cells (HSCs) using the Emory Pediatric Flow Cytometry Core. About 500 purified HSCs (identified as GPI-80^+, $CD133^+$, $CD34^+$, $CD90^+$, C-Kit^+, $CD38^-$, $CD3^-$, $CD14^-$, $CD16^-$, $CD19^-$) from either fetal or adult tissues will be injected intravenously into newborn (via facial vein) and adult (via tail vein) recipients. 20-30 weeks after transplantation, when the recipient mice show stable human chimerism in blood (tested weekly), all mice will be sacrificed and blood, spleen, bone marrow, lymph nodes, intestines and peritoneal cavity will be harvested for data analysis (Fig. 3A). The frequency of each immune cell (particularly B cells) will be identified by FACS Hi-D analyses (see Aim 1) and absolute cell numbers will be determined with TRUcount beads (BD).

The insights we develop here into the developmental and functional differences between the fetal and adult immune cells will shed light on mechanisms leading to hematological cancers and other (auto-) immune disorders that preferentially affect children and in which B cells play a major role. In addition, the results from our studies here can be expected to inform the development of novel vaccine strategies that are targeted to infants, particularly for immunization to pathogens against which the human fetal B lymphocytes (B-1a counterpart) is likely to play a key protective role.



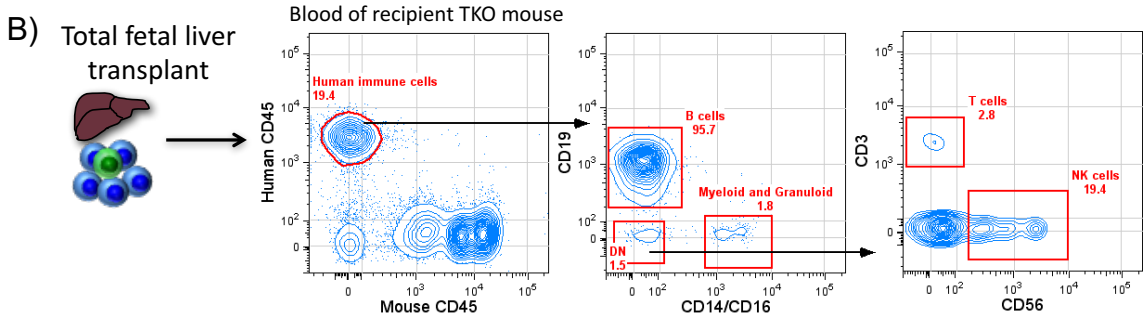


Figure 3. A) Experimental design to generate humanized mice. **B)** Human chimerism (human $CD45^+$) in peripheral blood of TKO recipient mice 7-months after transplantation of total human fetal liver cells from 14 weeks of gestation.

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