Materials and Methods

Supplementary Online Materials and Methods

Preparation of human tissues. All human tissue that was obtained for evaluation in this study was obtained with approval from and under the guidelines of the UCSF Committee on Human Research. Fetal tissues (mesenteric lymph node (mLN), liver, and thymus) from 18-22 gestational week specimens were obtained from Advanced Bioscience Resources and San Francisco General Hospital. Adult peripheral blood mononuclear cells (PBMCs) were isolated from healthy adults by intravenous blood draws using Vacutainer tubes (Na/EDTA Green Top BD) and density centrifugation over a ficoll-hypaque (Amersham Biosciences, Piscataway, NJ) gradient. Adult bone marrow samples ($400_{10}6$ mononuclear cells/donor) were obtained from healthy donors (ages: 19, 24(x2), 25, 43) (AllCells, LLC. Emeryville, CA). Isolation of fetal lymphocytes from mLN and was accomplished by mechanical dispersion in sterile phosphate buffered saline (PBS). For studies performed on peripheral cells, to account for potential differences due to differences in the tissue source of fetal and adult samples (mLN vs peripheral blood) we incubated fetal mLN cells and adult PBMC overnight in RPMI (2% FBS) prior to sorting the cells for *in vitro* incubation or microarray analysis.

Flow cytometry and Fluorescence Activated Cell Sorting (FACS). Surface staining for naïve T cell markers was performed as previously described (S1). In brief, mononuclear cell preparations were incubated in FACS staining buffer (PBS with 2% FBS and 2mM EDTA) with surface antibodies conjugated to fluorochromes. Antibodies used for phenotyping included: CD3 Alexa Fluor 700 (SP34-2; BD Pharmingen, San Diego, CA),

CD4 PE-Texas Red (GK1.5, Caltag/Invitrogen, Carlsbad, CA), CD8 PE-Cy5.5 (Clone 3B5; Caltag/Invitrogen), CD25 FITC (MA-251, BD Pharmingen), CD45RA PE (OX-33, BD Pharmingen), CCR7 PE-Cy7 (3D12, BD Pharmingen), CD95 APC (DX2, BD Pharmingen). All cells were stained with a live/dead marker (Amine-Aqua/Amcyan; Invitrogen) to exclude dead cells from the analysis. Intracellular staining for Foxp3 (PCH101; eBioscience, San Diego, CA) was performed according to the manufacturers protocol (eBioscience). To determine HLA type of cells in SCID-hu Thy/Liv experiments, cells were stained with a panel of biotin-conjugated antibodies against HLA markers (HLA-A2, A3, A9, and A11; One Lambda, Canoga Park, CA). Cells were subsequently stained with streptavidin conjugated to quantum dot 655 (Qdot655) (Invitrogen) for analysis by flow cytometry. Data was acquired with an LSRII flow cytometer (BD Biosciences, San Jose, CA). For cell sorting, mononuclear cells were stained with the appropriate antibodies and filtered through 70μM mesh filters (Falcon). The stained cells were subsequently sorted by FACS (FACS Aria, BD Biosciences), and their purity was determined by reanalyzing sorted cells. Sorted cells were washed and resuspended in sterile serum free cell culture media (SF X-VIVO 20, Biowhitakker, Walkersville, MD).

Proliferation Assays. For measurement of cell proliferation, cells were incubated with 5μM carboxy-fluorescein diacetate succinimidyl ester (CFSE; Invitrogen) in PBS with 5% FBS for 10 minutes at room temperature. The labeled cells were washed 3x in sterile RPMI containing 10% FBS to remove the excess CFSE. The cells were then resuspended in SF X-VIVO 20 media and incubated at the appropriate concentration in 96-well U-

bottom plates (Becton Dickinson, BD Labware, Franklin Lakes, NJ). For all mixed leukocyte reactions (MLR) responder cells were incubated at a ratio of 3 responders:1 allogeneic stimulator for 6 days under typical tissue culture conditions (37°C, 5% CO_2). Sources of allogeneic cells were irradiated PBMCs (~6000 cGy from a cesium source) from healthy adult donors.

Isolation of CD34+ hematopoietic stem/progenitor cells (HSPC). For fetal liver, mononuclear cells were isolated as previously described (S2). In brief, fetal liver was gently dissected into small pieces and suspended in complete RPMI media (10% fetal bovine serum (FBS, Hyclone), Penicillin/Streptomycin (Pen/Strep; Sigma, St Louis, MO), 2mM glutamine (Sigma)) supplemented with 1mg/mL collagenase/dispase (Roche, Mannheim), and 0.5 U/mL DNase I (Roche) and incubated at 37°C for 45 minutes. Fetal liver pieces were gently pipetted every 10-15 minutes to aid in tissue disruption. After 45 minutes, the cells were further dispersed by running the solution through a series of 70μM mesh filters (Falcon). The resultant suspension was then subjected to density centrifugation over a ficoll-hypaque gradient to obtain mononuclear cells. For fetal bone marrow, fetal leg bones (femurs and tibias) were obtained from 18-22 g.w. specimens and thoroughly washed in sterile PBS. The bones were then dissected and bone marrow was obtained by scraping the interior cavity with a sterile scalpel. After scraping out the majority of visible marrow, the fetal bones were subsequently incubated at 37°C for 45 minutes in complete RPMI supplemented with collagenase/dispase and DNase I as described for fetal liver CD34 isolation. After 45 minutes, all suspended bone marrow cells were combined, washed in sterile RPMI, and mononuclear cells were obtained by

density centrifugation over a ficoll-hypaque gradient. For adult bone marrow, the mononuclear cell fraction was provided by the supplier (AllCells, LLC.). For all samples, CD34 isolation was performed on the mononuclear cell fraction by labeling cells with anti-CD34 antibodies conjugated to magnetic beads and positive selection over a magnetic column (LS columns; Miltenyi Biotec) in accordance with the manufacturer's protocol (Human CD34 isolation kit, Miltenyi Biotec, Germany). In all cases, Purity of $CD34^+$ HSPC was determined by staining the positively selected fraction ($CD34^+$ fraction) with a panel of antibodies against lineage markers (CD3, CD20, CD56) and HSPC markers (CD34, CD38). HLA-typing was determined by staining the $CD34^-$ fraction with HLA antibodies. Both $CD34^+$ and $CD34^-$ cells were washed and resuspended in freezing media (90% FBS, 10% DMSO) and stored at -80°C for later use.

Preparation of SCID-hu Thy/Liv mice. Co-implantation of thymus and liver fragments under the kidney capsule to create SCID-hu Thy/Liv mice was carried out as described (S3, S4). Male C.B-17 SCID (model #CB17SC-M, homozygous, C.B-*Igh-1*^{b}/IcrTac-*Prkdc*^{scid}) mice were obtained at 6–8 weeks of age from Taconic (Germantown, NY), and cohorts of 50–60 SCID-hu Thy/Liv mice were implanted with tissues from a single HLA-$A2^-$ donor. Implants were injected with HPSCs 24 weeks after surgical exposure of the implanted kidney of anesthetized mice.

Engraftment of SCID-hu Thy/Liv animals with HSPC. After approximately 22-26 weeks, SCID-hu Thy/Liv cohorts containing HLA-$A2^-$ Thymus/Liver grafts were irradiated with 175 cGy (from a cesium-137 source) immediately prior to transferring

$CD34^+$ cells. Frozen $CD34^+$ HSPCs were thawed during the irradiation time and washed and resuspended in sterile RPMI (no supplements) at a concentration of $10_{10}6$/mL. The thymic implant was then exposed by making a single incision in the peritoneum of each mouse and 50μL of each sample (fetal liver HSPC, fetal bone marrow HSPC, or adult bone marrow HSPC) was injected directly into individual thymic implants (5 mice/group). An additional group of mice received RPMI with no cells (mock/vehicle only). The incision was repaired by approximating the peritoneal lining with one stitch and using one to two staples to close the skin. Mice were returned to the specific pathogen-free barrier facility and monitored regularly for 7-8 weeks. At this time the thymic implants were harvested and processed to obtain thymocyte suspensions. These cells were then analyzed by flow cytometry, used for MLR proliferation assays, or sorted for RNA isolation and microarray analysis. Donor cells were identified by HLA-A2 expression. No HLA-$A2^+$ cells were ever seen in mock-injected implants.

Preparation of RNA for microarray. Sorted cells were checked for high purity (>98%) and immediately resuspended in RNA lysis buffer (Absolutely RNA microprep kit, Stratagene, La Jolla, CA) and stored at -80°C until enough samples were collected to isolate RNA for all samples simultaneously. Once all samples had been acquired, RNA was isolated according the manufacturer's protocol and yield was determined on a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Samples that had an appropriate yield (typically >50 nanograms total RNA) were subsequently analyzed for RNA integrity using an Agilent Bioanalyzer (Agilent 2100, Agilent Technologies, Santa Clara, CA). RNA that was determined to be of high quality was then converted to cDNA

and amplified (NuGEN WT-Ovation, NuGEN, San Carlos, CA), purified to remove residual RNA (QIAquick PCR purification kit, Qiagen, Valencia, CA), fragmented (NuGEN WT-Ovation Kit), and labeled (FL-Ovation cDNA biotin module, NuGEN) for subsequent microarray analysis. For thymocyte samples from SCID-hu Thy/Liv studies the RNA yield was too low to use the NuGEN Ovation kit for all samples so the NuGEN WT Ovation Pico kit (NuGEN) was used for these samples. Fragmented and labeled cDNA was hybridized to microarray chips (U133 plus 2.0 human gene expression chips, Affymetrix, Santa Clara, CA). Hybridization of samples and data acquisition was performed by the Gladstone Institutes Genomics Core (UCSF, San Francisco, CA).

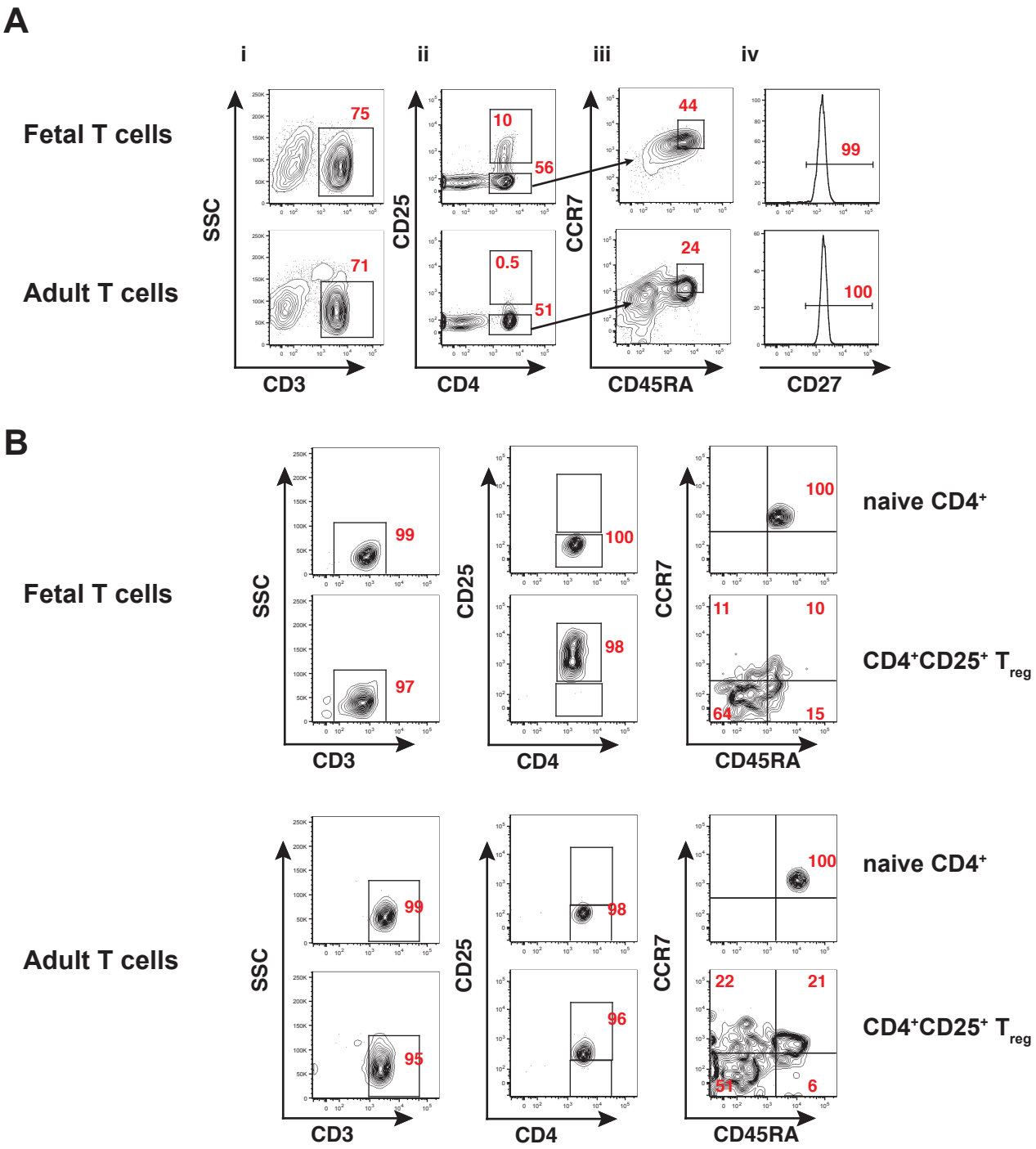
Microarray data analysis. Assessment of hybridization quality was performed using Bioconductor package *affyPLM*. Preprocessing of Affymetrix dataset followed these procedures: background correction, normalization using the quantile method (S5), summarization of probe set values using the RMA (Robust Multi-array Average) method (S6), which fits a specified robust linear model to the probe level data. Based on all quality control analysis performed by the UCSF bioinformatics core (part of the clinical translational sciences initiative (CTSI), UCSF) all arrays were found to be of good quality.

Statistical analysis.

Hierarchical clustering of gene expression samples was performed using a Euclidean distance metric and a complete linkage agglomeration method. Differentially expressed probesets were identified using Significance Analysis of Microarrays with false discovery

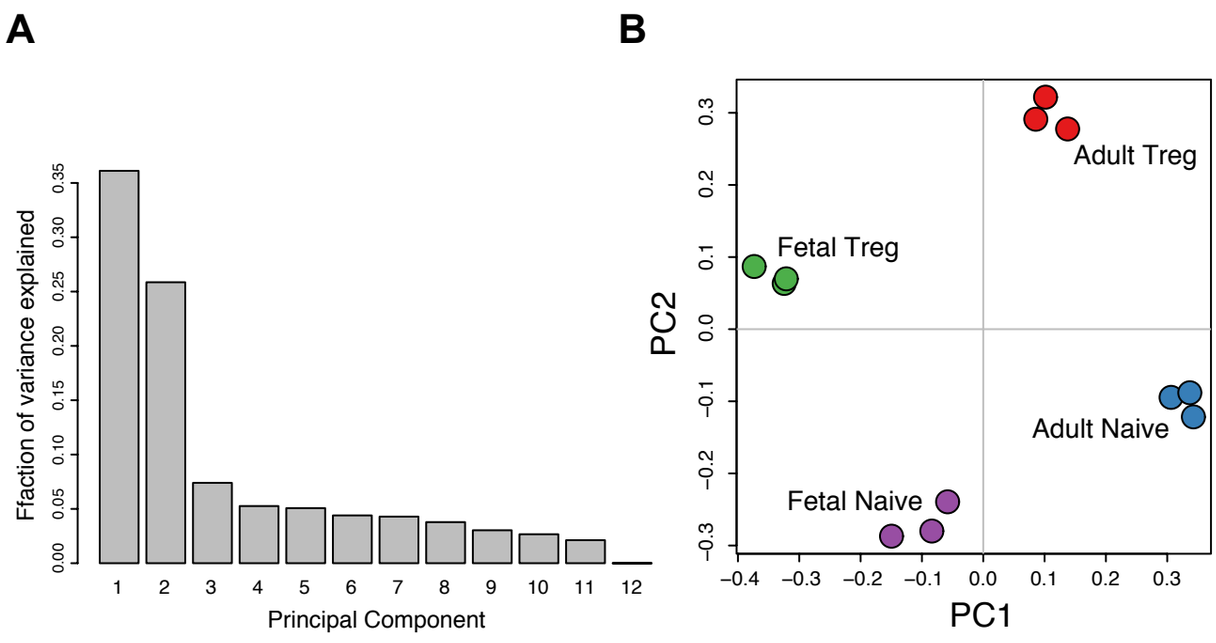
rate (FDR = 5%) and fold-change (greater than 2) thresholds (S7). Probesets were mapped to genes using AILUN (S8). Overlaps between sets of differentially expressed genes were assessed for independence using a chi-squared contingency test. Principal component analysis was performed after normalizing gene expression on a per-gene level such that the average expression of any gene across all samples was zero. This step controls for variation in mean expression level across genes, and results in the PCA being focused on variation in gene expression across samples. For the scatterplots in Fig. 4D and Supplementary Fig. 2, the component loadings were scaled by the square roots of the corresponding eigenvalues. All analysis was performed using R (cran.r-project.org) and Bioconductor (www.bioconductor.org).

Supplementary Figure 1



Supplementary Figure 1. Gating for naive and T_{reg} sorting from fetal and adult donors. (A) Sequential gating for fetal (top) and adult (bottom) $CD3^+$ T cell populations. The first panel (i) shows CD3 gating followed by (ii) CD4 gating for $CD4^+CD25^+$ T_{regs} (top gate) or $CD4^+CD25^-$ cells (bottom gate). $CD4^+CD25^-$ cells were subsequently serially gated on $CD45RA^+CCR7^+$ (iii) and $CD27^+$ (iv) to isolate naive T cells. (B) Representative sort purities for fetal (top) and adult (bottom) naive and T_{reg} populations.

Supplementary Figure 2



Supplementary Figure 2. Principal component analysis on fetal and adult peripheral T cell populations reveals segregation based on developmental stage (PC1) and T cell subtype (PC2). (A) Fraction of variance for different principle components. (B) Graph depicting the first two PC.

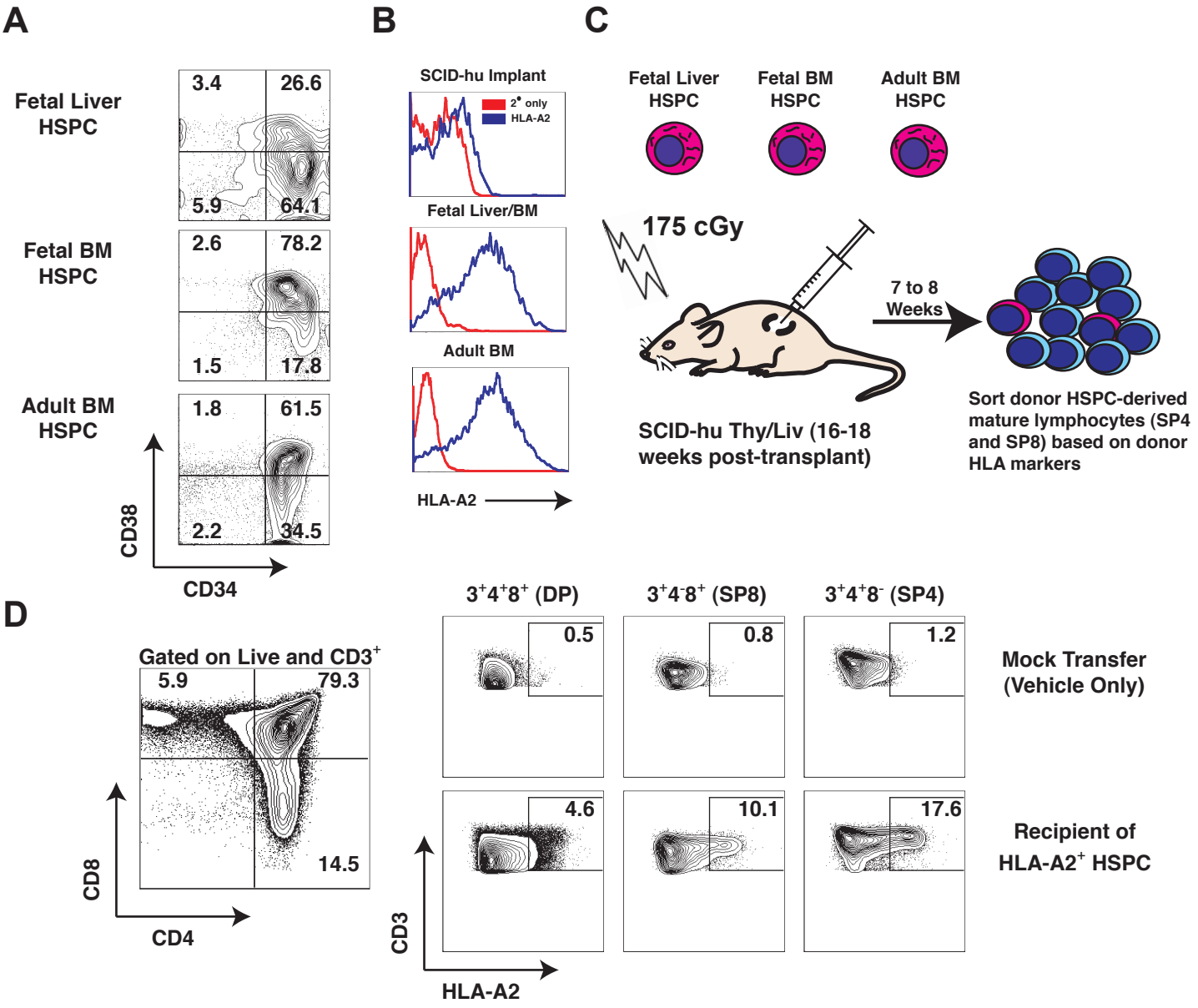
Supplementary Figure 3

	Gene Name	Alternative Name/Function
High in Fetal T cells	AUTS2	(KIAA0442) Autisim susceptibility gene unknown function
	GZMA	(Granzyme A) Serine protease with cytotoxic activity typically expressed by activated T cells or Treg cells
	GBP1	(Guanylate Binding Protein 1 and 4) Interferon induced genes that bind to GMP, GDP, and GTP and have GTPase activity
	GBP4	
	IGF2BP3	(Insulin-Like Growth Factor 2 mRNA-Binding Protein 3) Found to bind to 5'-UTR of IGF2 mRNA and may be involved in IGF2 secretion
	IL12RB1	(IL-12 Receptor Beta 1) Receptor for IL-12 involved in inflammatory responses
	LDB2	(LIM Domain Binding 2) Binds to diverse array of transcription factors
	LRRN3	(Leucine Rich Repeat Neuronal 3) Unknown function
	OAS1	(Oligoadenylate Synthetase 1) Interferon induced protein that catalyzes synthesis of 2'-5' oligomers of adenosine
	RGS1	(Regulator of G Protein Signaling 1) Involved in lymphocyte chemotaxis/migration
	TNFSF13B	(B Cell Activating-Factor, BAFF) A TNF family mamber that promotes B cell proliferation and survival and may be promote T cell apoptosis
High in Adult T cells	CDC14B	(Cell Division Cycle 14) Involved in G2 damage checkpoint
	NAP1L2	(Nucleosome Assembly Protein Like-1) Chromatin interacting protein
	NR3C2	(Mineralocorticoid Receptor) Steroid hormone receptor binds to aldosterone
	SERPIN B6	(Serine Peptidase Inhibitor Clade B, Member 6) Cathepsin B inhibitor thought to be involved in protecting immune cells during inflammation
	TRPC1	(Transient Receptor Potential Cation Channel, Member 1) Calcium channel known to be involved in immune cell signaling

	Gene Name	Alternative Name/Function
High in Both Fetal and Adult T_{reg} cells	ANXA2	(Annexin 2) Involved in apical plasma membrane formation and lumen function
	CCR6	(Chemokine Receptor, CC Motif, Receptor 6) Inflammatory chemokine receptor expressed by Th17 and T_{reg} cells
	CTLA4	(Cytotoxic Lymphocyte Associated Protein 4) Provides a negative signal to inhibit T cell proliferation and activation
	DUSP4	(Dual Specific Phosphatase 4) Negatively regulates members of the mitogen-activated protein kinase superfamily
	FCRL3	(Fc-Receptor Like 3) Surface receptor in family of proteins related to Fc-Gamma Receptor with unknown function
	FOXP3	(Forkhead Box Protein 3) Transcription factor that directs and maintains the T_{reg} lineage
	IL2RA	(IL-2 Receptor Alpha (CD25)) High affinity receptor for IL-2 expressed by Treg cells
	IKZF2	(IKAROS Family Zinc Finger Protein 2 (Helios)) Transcription factor found to be involved in T_{reg} differentiation and function
	IKZF4	(IKAROS Family Zinc Finger Protein 4 (Eos)) Transcription factor found to be involved in T_{reg} differentiation and function
	MIRHG2	(MicroRNA host gene 2 (MIR155/BIC)) Suppresses MAF function and blocks Th2 differentiation of T cells
	PRDM1	(PR Domain-Containing Protein 1 (BLIMP1)) Transcription factor implicated in plasma cell differentiation and T cell activation and homeostasis
	RGS1	(Regulator of G Protein Signaling 1) Involved in lymphocyte chemotaxis/migration
	SOCS2	(Suppressor of Cytokine Signaling 2) Blocks activation of cytokines and thus downregulates inflammatory responses

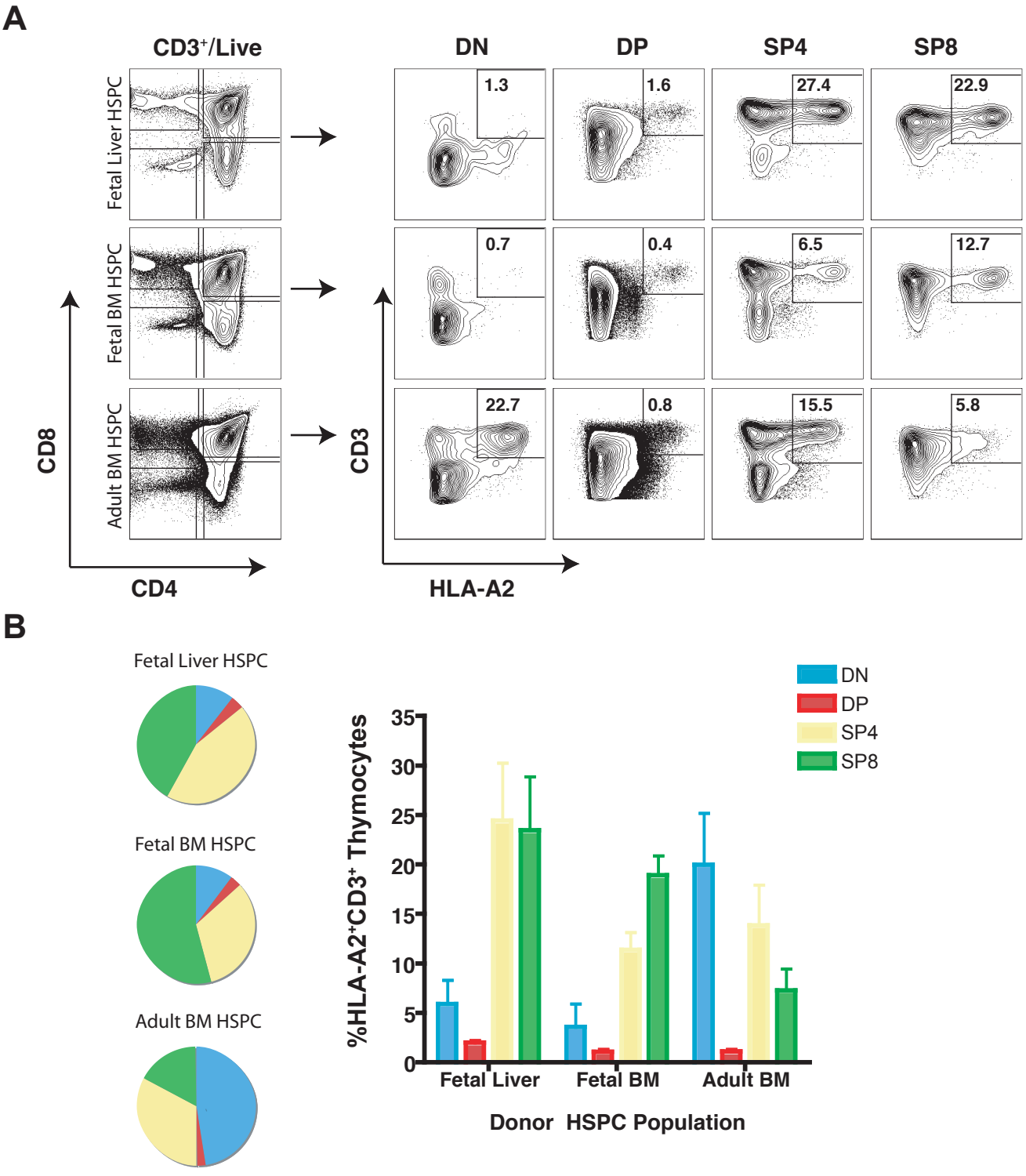
Supplementary Figure 3. List of selected genes found to be overexpressed by fetal, adult, or Treg cells in microarrays on peripheral T cell populations

Supplementary Figure 4



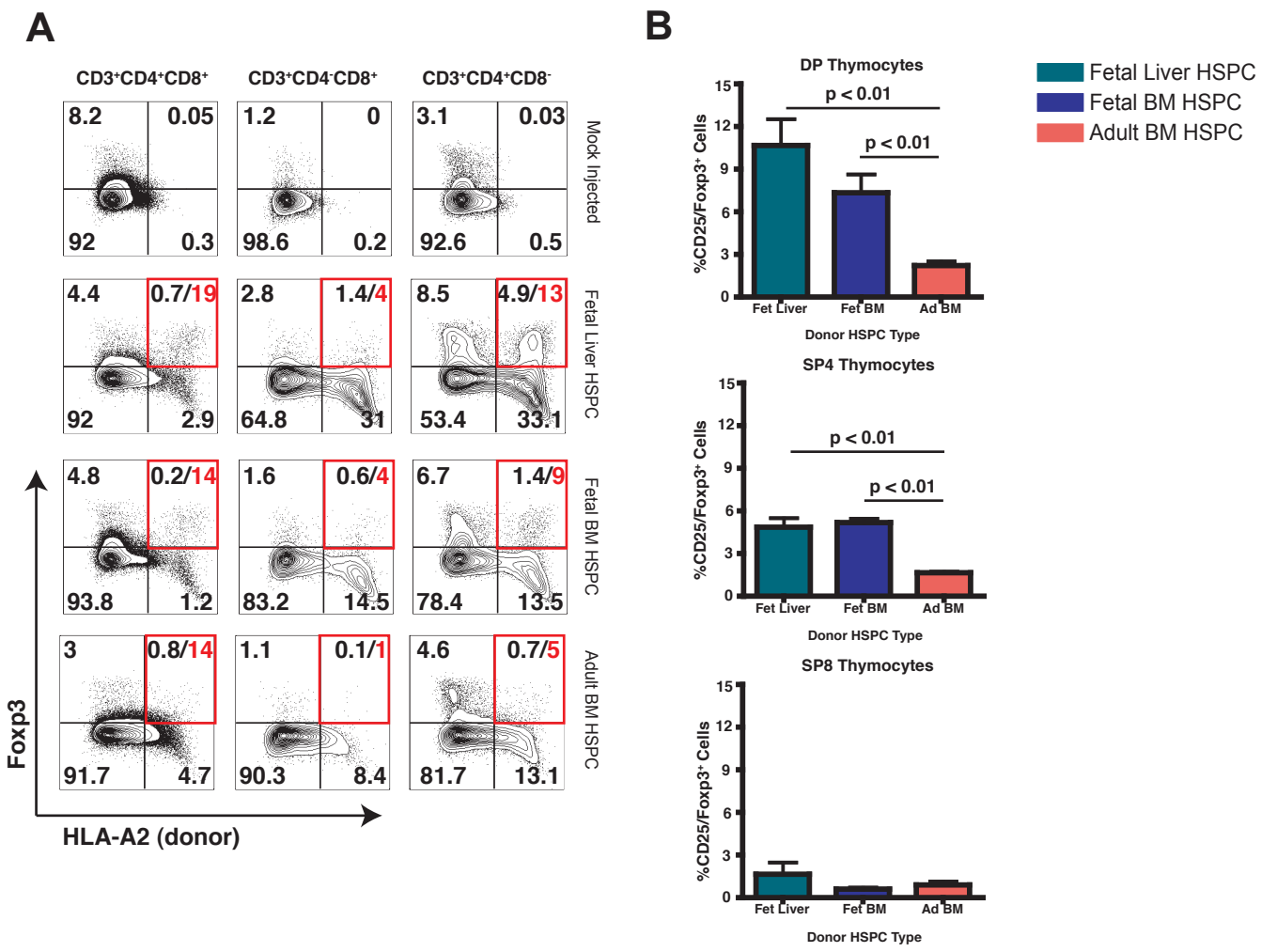
Supplementary Figure 4. Experimental strategy to derive mature thymocytes from fetal and adult hematopoietic stem/progenitor subsets in vivo. (A) CD34 and CD38 staining on fetal liver, bone marrow (BM), and adult BM HSPC populations. Cells were processed from fresh human fetal liver and BM (gestational age 18-22 g.w.) or from adult BM (age 19-43 y.o.). Total mononuclear cells were then incubated with anti-CD34 antibodies conjugated to magnetic beads and $CD34^+$ cells were positively selected by MACS separation. Purity was determined by staining for HSPC markers CD34/CD38 and lineage markers (CD3, CD20; not shown). (B) HLA-A2 expression on recipient thymic graft (negative) and on donor HSPC populations (positive). (C) Schematic for how HSPC transfer and thymocyte maturation was achieved. (D) Representative staining shown for mature donor-derived thymocyte populations 7-8 weeks following transfer of HSPC. Top panels show a thymic implant receiving a mock transfer (vehicle only) and bottom panels depict a thymic implant receiving HLA-$A2^+$ donor HSPC. The small percentage of DP thymocytes that express HLA-A2 is likely due to the low expression of HLA Class I antigens on the surface of the majority of the more “immature” DP thymocytes (S9).

Supplementary Figure 5



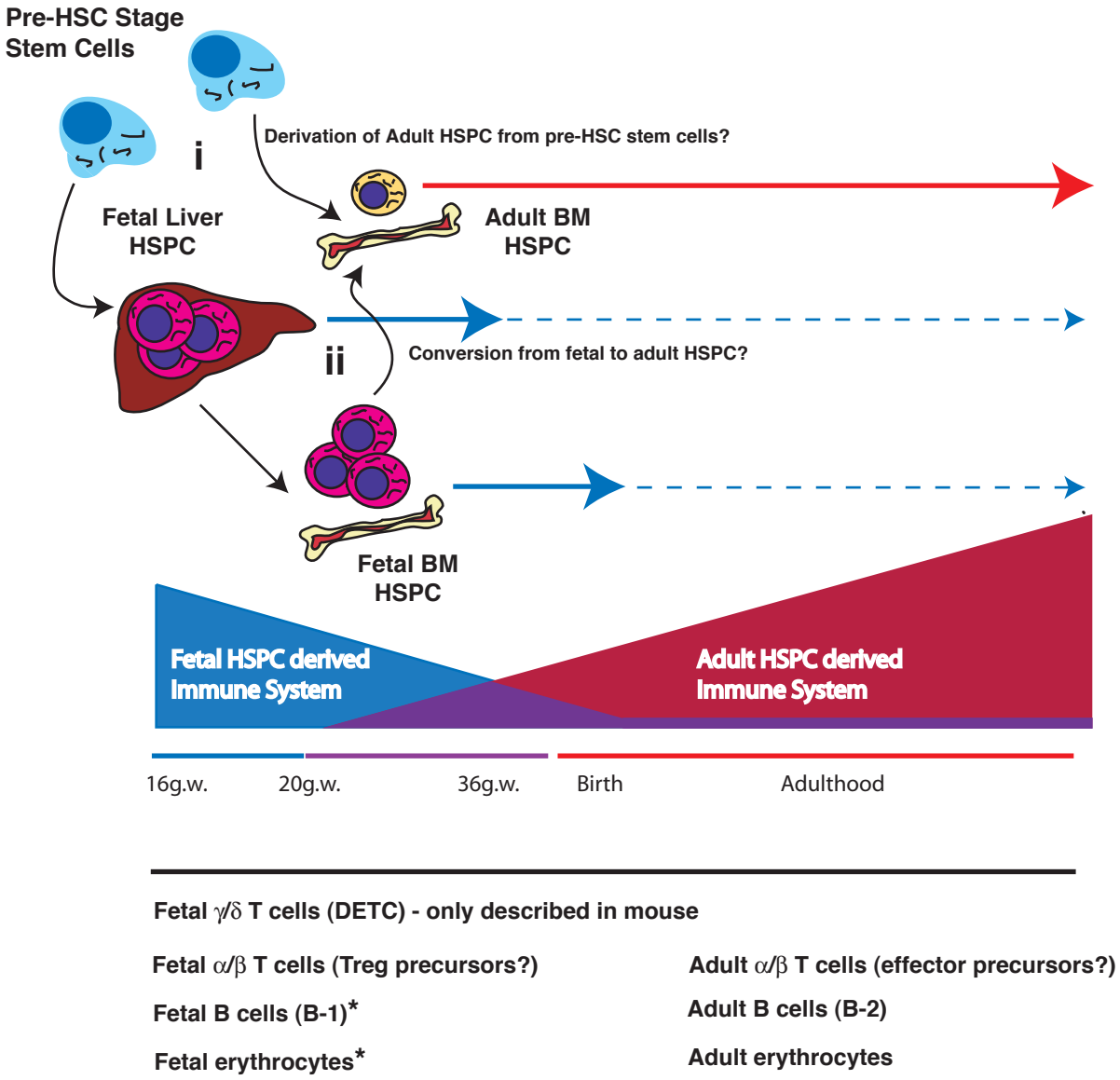
Supplementary Figure 5. Relative distribution of CD3+ thymocyte subsets derived from different HSPCs in SCID-hu thymic implants. (A) Frequency of $CD3^+CD4^-CD8^-$ (DN), $CD3^+CD4^+CD8^+$ (DP), $CD3^+CD4^-CD8^+$ (SP8), and $CD3^+CD4^+CD8^-$ (SP4) expressing donor HLA (HLA-A2) observed in implants injected with fetal liver, fetal BM, or adult BM HSPCs at 7-8 weeks post-injection. The low frequency of cells observed in the DP populations reflects the low expression of HLA Class I by most immature thymocytes present in this fraction (S9). (B) Relative frequencies (pie charts) and actual frequencies (bar graph) of $HLA\text{-}A2^+CD3^+$ thymocyte subsets in 4-5 difference mice injected with fetal liver, fetal BM, or adult BM HSPC. Error bars represent standard deviation.

Supplementary Figure 6



Supplementary Figure 6. Expression of Foxp3 by DP, SP8, and SP4 thymocytes from Thy/Liv implants receiving different HSPC populations. (A) Representative flow cytometry plots showing Foxp3 expression within the recipient (top left quadrant) and donor (top right quadrant) thymocyte subsets. Numbers in red indicate the frequency of Foxp3+ T_{regs} within the donor-derived population for each specific thymocyte subset (highlighted in red). (B) Summary of at least 4 separate animals from a single experiment showing statistically significant elevation of Foxp3+ cells within the DP and SP4 populations derived from fetal liver or fetal BM HSPCs compared to those derived from adult BM HSPCs. No differences were seen within the SP8 populations. Statistics calculated by unpaired Student's t-test.

Supplementary Figure 7



Supplementary Figure 7. Model for transitions between fetal and adult hematopoiesis. There are two predominant models that could account for the switch from fetal to adult hematopoiesis: (i) the generation of separate hematopoietic stem cells or progenitor cells from a single precursor population that exists upstream of the identified HSC populations; (ii) a transition directly from existing fetal HSC to adult HSC triggered by changes in the environment in which they reside. In each case the possibility exists that small populations of fetal HSC persis throughout life (dashed lines) which may contribute to low levels of "fetal" hematopoiesis. Several examples of fetal and adult lineages are listed in the bottom half of the model including T cells (γ/δ and α/β), B cells (B1 vs B2), and erythrocytes (F cells vs adult erythrocytes). * Both B-1 cells and fetal erythrocytes have been generated from HSC populations that are present during adult life under certain conditions (S10, S11).

SOM References

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