Supplementary Methods

$CD34^+$ cell isolation. $CD34^+$ cells were obtained from fetal liver tissue incubated at 37˚C in complete RPMI [RPMI 1640 medium (Sigma Chemical Co.), 10% fetal bovine serum (FBS) (Mediatech Inc.), 50 U/mL penicillin (Sigma), 50 mg/mL streptomycin (Sigma), 2 mM glutamine (Sigma), 1 mM sodium pyruvate (Sigma)] (cRPMI) supplemented with 1 mg/mL collagenase/dispase (Roche, Mannheim), and 0.5 U/mL DNase I (Roche). The tissue was gently disrupted by cutting into small pieces and pipetting the supernatant up and down every15 minutes for 1 hour. The cell suspension was filtered through a 70-µm mesh, and mononuclear cells were isolated by Ficoll gradient separation. $CD34^+$ cells were isolated using immunomagnetic beads (Miltenyi Biotech) as previously described.[1,2] Isolated $CD34^+$ cells were then subsequently stained with the mouse anti-human monoclonal antibodies to CD34 (clone 581, Pharmingen) and CD3 (clone HIT3a, Pharmingen) or similarly labeled isotype controls and analyzed by flow cytometry on a FACSCalibur using CellQuest Pro (version 4.0.2) (Becton Dickinson (BD)) for CD34 expression and residual $CD3^+$ T cells. This protocol yielded greater than 90-95% pure $CD34^+$ cells and less than 0.6% $CD3^+$ T cells. Cells were immediately frozen (-80˚ C) in 90% FBS and 10% dimethyl sulfoxide (Fisher) following isolation and stored in liquid nitrogen until transplanted.

Tissue mononuclear cell isolation. Bone marrow and spleen mononuclear cells were isolated as previously described.[3] Thymocytes were isolated by excising the thymic organoid from the kidney. The tissue was finely minced into small fragments and disrupted in 5 ml cRPMI using a 3 ml syringe plunger and the supernatant filtered through a 70 µm cell strainer. The pellet was washed in solution B [1 liter Phosphate Buffered Saline (PBS) (Sigma), 5 grams BSA (Sigma), 50 U/ml Pennicillin (Sigma), 50mg/ml Streptomycin (Sigma), 1% Citrate Phosphate Detrose (Sigma)] and resuspended in 15 mls Red Blood Cell (RBC) lysis buffer (500 ml dH_2O, 4.15 g NH_4Cl (Sigma), 0.5 g $KHCO_3$ (Sigma) and 0.019 g EDTA (Sigma)) for 10 minutes. The cells were centrifuged at 1500 rpm at 4˚C for 10 minutes and washed with solution B. To harvest liver mononuclear

cells, mice were anestitized and the peritoneum opened to expose the liver. The anterior vena cava was tied off with suture and the portal vein cut to allow for peripheral blood drainage. The mouse liver was perfused through the right atrium with PBS or RPMI (37˚C) at 3 ml/minute (10 ml syringe with a 21 gauge needle). Following perfusion, the liver was removed and diced into small pieces, and the tissue disrupted using a 3 ml plunger in a 100 mm Petri dish. The cell suspension was filtered through a 40 µm nylon filter and centrifuged at 1500 rpm at 4˚C for 10 minutes. The cells were then resuspended in 5 ml 40% Percoll (Sigma) and cRPMI in a 15 ml conical tube, underlayed with an equal volume of 70% Percoll and cRPMI and spun at 2400 rpm at 20˚C for 20 minutes. Lymphocytes were isolated from the 40% -70% interface and washed in cRPMI. Red blood cells were lysed with RBC lysis buffer and washed with solution B. Intact lungs were removed and perfused through the trachea with PBS to remove peripheral blood mononuclear cells. Lungs were minced into small pieces with scissors and treated with collagenase enzyme cocktail (5mls RPMI, 12 mg Collagenase type 1, 100 µg DNAse) and incubated for 30 minutes at 37˚ C. The tissue was further disrupted by passing the cell suspension through a 16 gauge needle and filtering through a 70 µm cell strainer. Cells were resuspended in 35ml cRPMI then underlayed with 12 ml of 70% Percoll/cRPMI and centrifuged at 2000 rpm for 20 minutes. Cells were collected from the interface and washed with cRPMI. Red blood cells were lysed as previously described and MNCs washed with solution B. Isolated mononuclear cells were resuspended in 5 mls RPMI and filtered again through a 70 µm cell strainer. All isolated tissue mononuclear cells were analyzed for human reconstitution and immune subsets by flow cytometry as described below.

Human reconstitution analysis by flow cytometry. Mononuclear cells (MNCs) from BLT mice or control mice were isolated from the bone marrow, spleen, lymph nodes, lung, liver and thymic organoid tissues. The percentage of human leukocytes ($CD45^{+}$) and hematopoietic lineages were determined by 4 color flow cytometry using antibodies to human hematopoietic markers as previously described.[1-3] For further characterization, subsets of myeloid and lymphoid cells were stained with appropriate antibodies and analyzed by flow cytometry on a FACSCalibur (BD) collecting a minimum of 10,000 live

cell gated events. Live cells were identified based on their characteristic side scatter versus forward scatter (data not shown). Subsequently, live human MNCs were identified with mouse anti-human $CD45^{+}$ (clone HI30, Pharmingen) to determine the percentage of human reconstitution. Myeloid cells were identified first by expression of human CD45 (CD45 gate), then for expression of CD33 (CD33 gate) (clone P67.6, BD) and analyzed for the monocyte and macrophage markers CD14 (clone M5E2, Pharmingen) and CD16 (clone 3G8 Pharmingen). Dendritic cells were identified using the BD DC Kit gating through mononuclear cells that were lineage negative, HLA DR bright (Lin^{-}, $HLA^{-}DR^{++}$ gate) and further characterized for CD11c (clone B-ly6, Pharmingen) and CD123 (clone 7G3, Pharmingen) expression. DC (Lin-$HLA^{-}DR^{++}$ and $CD123^{+}$ or $CD11c^{+}$ gate) were also characterized for expression of the activation markers CD40 (clone 5C3, BD), CD80 (clone BB1, Pharmingen), CD86 (clone FUN-1, BD) and the maturation marker CD83 (clone HB15e, Pharmingen). Lymphocytes were gated through human $CD45^{+}$ cells and CD19 (clone HIB19, Pharmingen) for B cells, $CD3^{-}$ $CD56^{+}$ (clone B159, Pharmingen) for natural killer cells and $CD3^{+}$ cells for T cell subsets. T cells ($CD45^{+}CD3^{+}$ gate) were further analyzed for CD4 (clone SK3, BD) and CD8 (clone SK1, BD) subsets, gd TCR (clone B1.1, BD) expression, CD25 (clone M-A251, Pharmingen) expression and for naïve versus memory T cell subsets using CD45RA (clone HI100, Pharmingen) and CD27 (clone MT-271, Pharmingen). The T cell Vb repertoire (Vb1 clone BL37.2, Vb2 clone MPB2D5, Vb3 clone CH92, Vb8.1 and Vb8.2 clone 56c5 clone, Vb14 clone CAS1.1.3, Vb17 clone E17.5F and Vb21.3 clone IG125, Immunotech) was monitored by gating through $CD45^{+}$ and $CD3^{+}$ cells. All flow cytometry data was collected and analyzed using CellQuest Pro.

Immunohistochemistry analysis. Tissues were collected and placed in fresh-phosphate buffered 4% paraformaldehyde for 4 to 6 hours, washed with 80% ethanol and stored in 80% ethanol until embedded in paraffin. Immunohistochemistry was performed using a biotin-free polymer approach (MACH-3™; Biocare Medical) to analyze all BLT and human control tissues. Immunohistochemistry was performed on 5 µm tissue sections mounted on glass slides, dewaxed and rehydrated with PBS (pH 7.4). Antigen retrieval was performed by heating sections in 1X EDTA Decloacker™ reagent (Biocare

Medical) in a 95°C water bath for 20 minutes followed by cooling to room temperature. Nonspecific Ig-binding sites were blocked with Blocking Reagent (Biocare Medical) for 1h at room temperature. Endogenous peroxidase was blocked with 3% (v/v) H_2O_2 in PBS (pH 7.4). Primary antibodies were diluted in 10% Blocking Reagent in 1,3,5-trinitrobenzene and incubated overnight at 4°C. The sections were then analyzed using either the mouse or rabbit MACH-3™ polymer systems (Biocare Medical) according to the manufacturer's instructions and developed with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA). Sections were counterstained with Harris Hematoxylin (Surgipath, Richmond), mounted in Permount (Fisher Scientific) and examined by light microscopy. Primary antibodies used for immunohistochemistry were mouse anti-human CD3 (clone F7.2.38, DakoCytomation), mouse anti-human CD4 (clone 1F6, Novocastra Laboratories, Newcastle-upon-Tyne), mouse anti-human CD8 (clone 295, Novocastra Laboratories) or rabbit monoclonal anti-human CD8 (SP16, NeoMarkers), mouse anti-human CD14 (clone 223, Novocastra Laboratories), mouse anti-human CD20 (clone L26, DakoCytomation), mouse anti-human CD45 LCA (clone 2B11 + PD7/26, DakoCytomation), mouse anti-human CD68 (clone KP1, DakoCytomation), mouse anti-human CD83 (clone 1H4b, Novocastra Laboratories), anti-human BLC/BCA-1/CXCL13 (clone AF801, R&D Systems) and anti-human 6Ckine/SLC/CCL21 (clone AF366, R&D Systems). Isotype matched negative control antibodies used were mouse IgG1 (clone X 0931, DakoCytomation), mouse IgG2a (clone X 0943, DakoCytomation), goat ChromPure IgG (Jackson Immuno-Research) and rabbit ChromPure IgG (Jackson Immuno-Research).

Vector stock preparation and $CD34^+$ cell transductions. Lentivirus vector used was produced and titered essentially as previously described.[2] Briefly, isolated human fetal liver $CD34^+$ cells were maintained in stem cell medium[2] and transduced with a vector expressing EGFP at 5 infectious units/cell at 0, 16 and 48 hours. Forty-eight hours after the last addition of vector, both transduced and mock tranduced (no vector) cells were analyzed for CD34 and EGFP expression by flow cytometry. Cells were frozen and stored at -80° C until transplant. NOD/SCID-hu thy/liv mice were transplanted with $2x10^5$ cells per mouse. Twelve weeks post transplant, mice were sacrificed and analyzed for

human reconstitution. Human CD45^{+} cells were further characterized for multilineage reconstitution and EGFP expression.

Sheep red blood cell administration. Sheep Red Blood Cells (SRBC) (Quadfive, Ryegate) were washed with pyrogen free PBS (B. Braun Medical Inc.) and resuspended at a concentration of 20% SRBCs in pyrogen free saline. Mice were anestitized and inoculated with 50 µl per rear footpad, 50 µl subcutaneous in the scruff of the neck, and 200 µl intraperitoneal.[4] Mice were sacrificed at 5 days and PB, BM, thymic organoid, spleen and lymph nodes (axillary, cervical, inguinal, and popliteal) were isolated and analyzed by flow cytometry for human reconstitution and immunohistochemistry for germinal center formation.

References

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