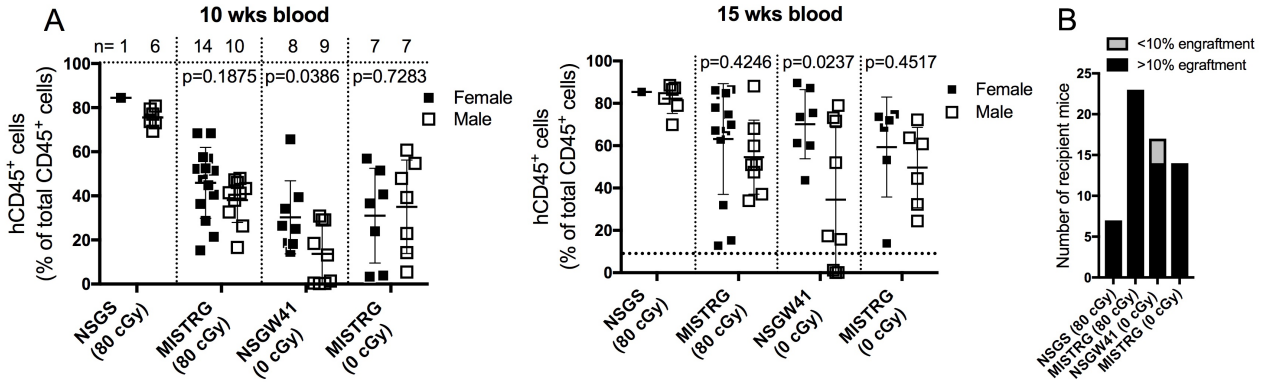
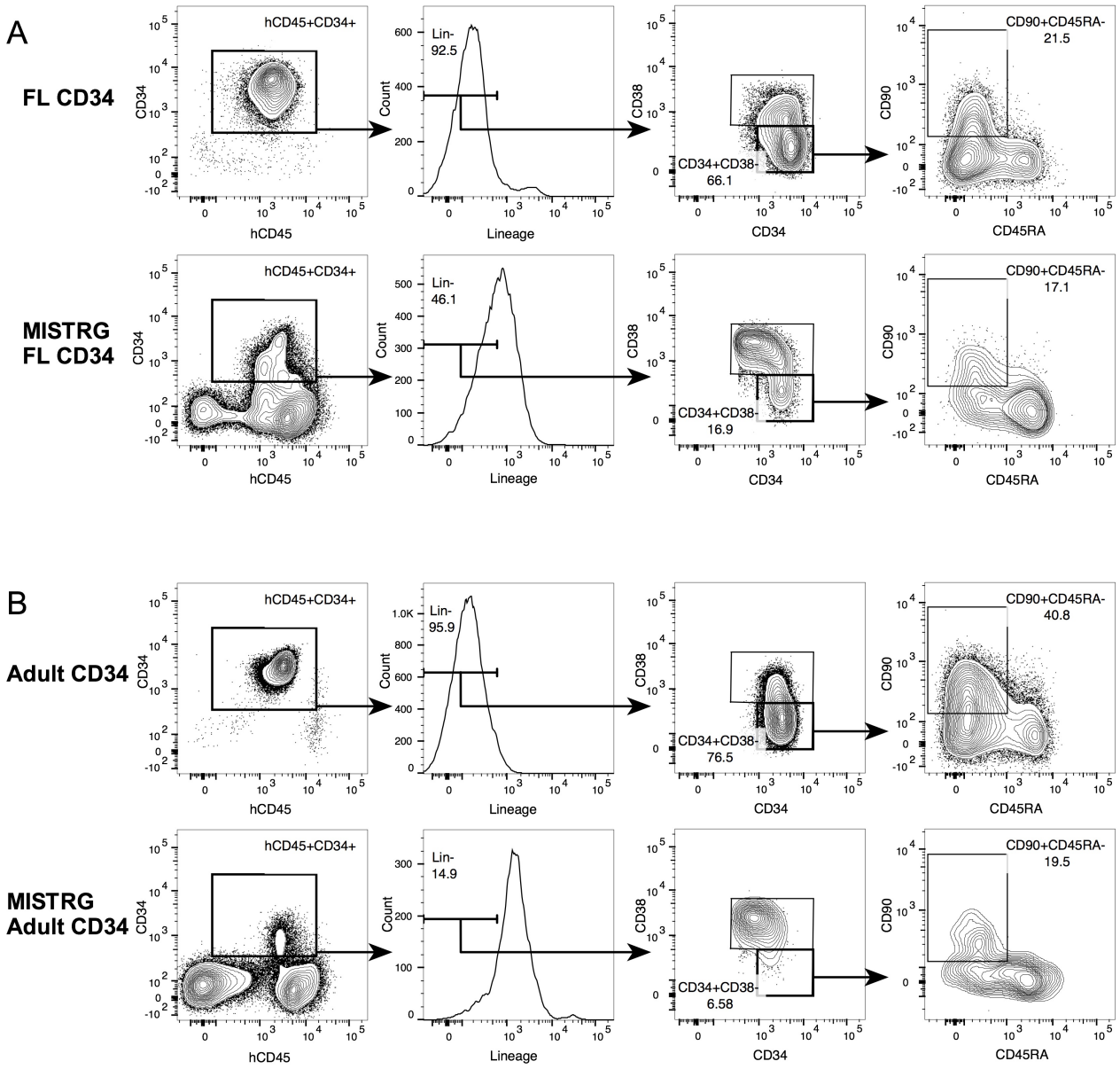
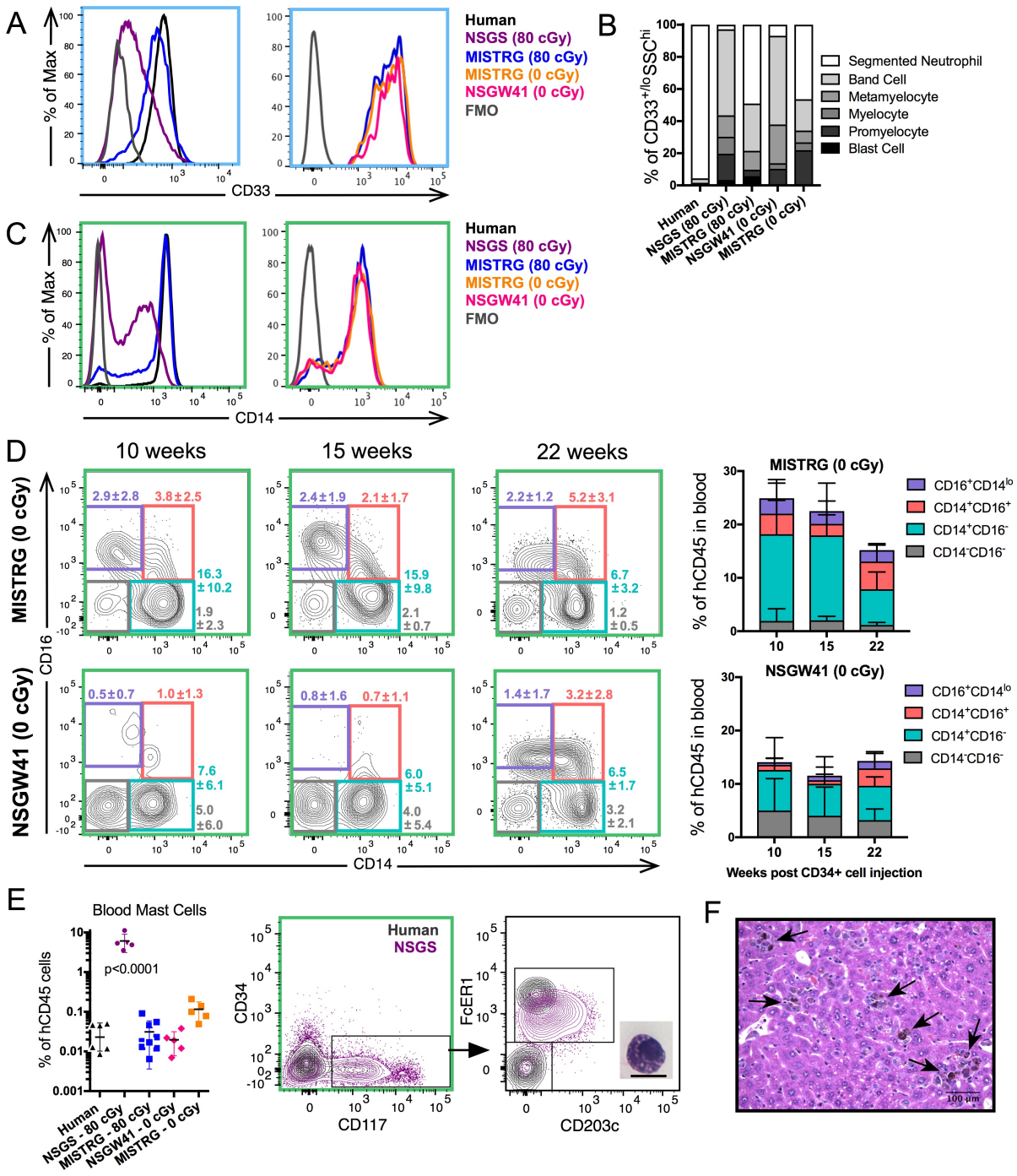
SUPPLEMENTAL FIGURE 1



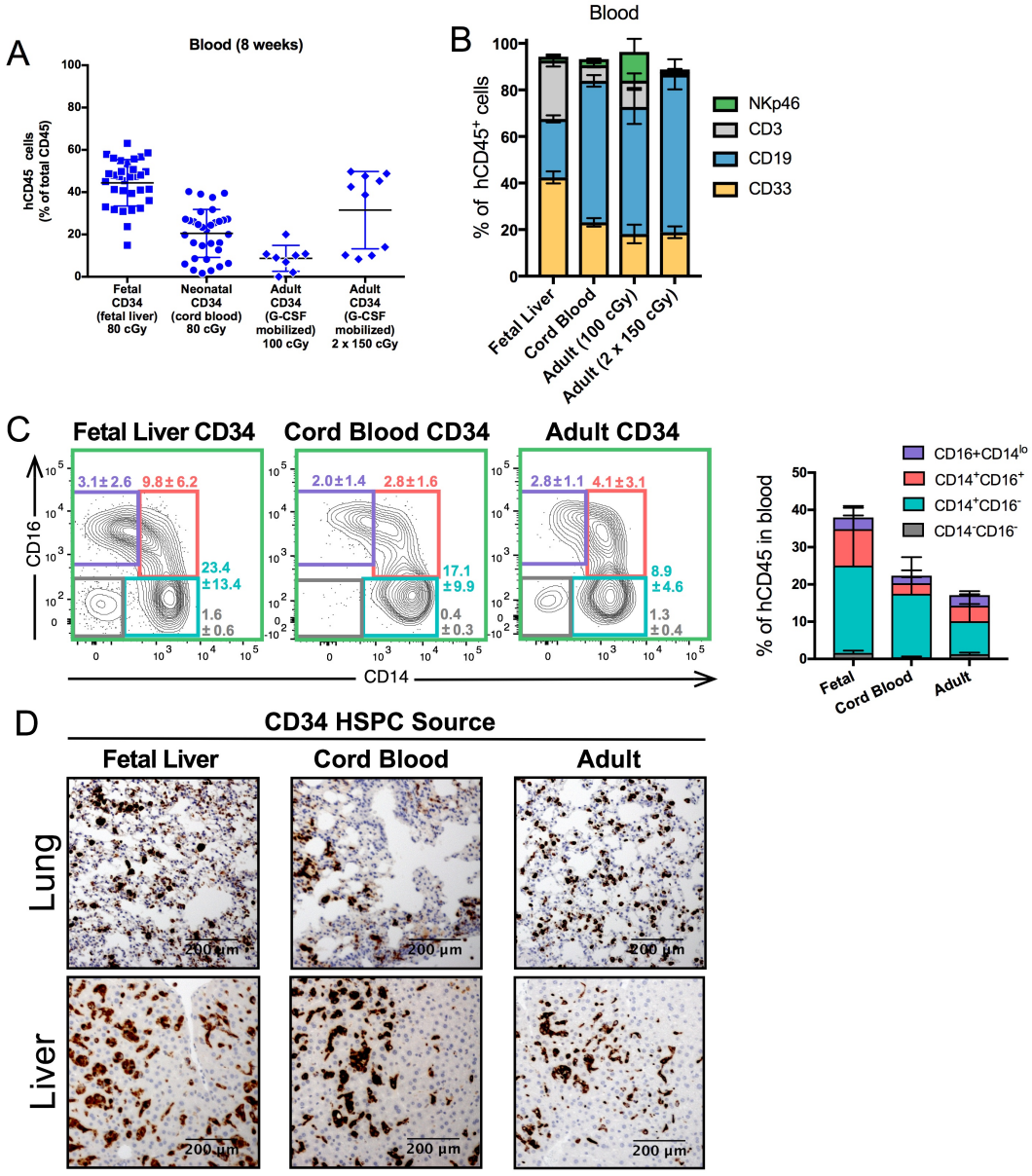
SUPPLEMENTAL FIGURE 2



SUPPLEMENTAL FIGURE 3



SUPPLEMENTAL FIGURE 4



Supplemental methods.

Mouse strains. NSGS (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ; strain 013062)[13,14] and NSGW41 (NOD.Cg-*Kit*$^{W-41J}$ *Prkdc*^{scid} *Il2rg*^{tm1Wjl}/WaskJ; strain 026497)[15] breeding pairs were purchased from the Jackson Laboratory and bred in-house. NSGS mice overexpress three human cytokines (SCF, GM-CSF and IL-3; or SGM3) under the control of a strong constitutive promoter[12] in NSG genetic background. NSGW41 harbor homozygous W41 inactivating mutation in the *Kit* gene, resulting in functional deficiency of mouse HSCs.[15] MISTRG mice (M-CSF$^{h/h}$ IL-3/GM-CSF$^{h/h}$ SIRP$\alpha^{h/m}$ TPO$^{h/h}$ RAG2$^{-/-}$ IL-2R$\gamma^{-/-}$) were previously reported.[20,21] In these mice, several genes (*Csf1*, *Il3/Csf2*, *Sirpa* and *Thpo*) are humanized by knockin replacement of the mouse allele by its human ortholog, from ATG to stop codon, in a *Rag2 Il2rg* double knockout 129xBALB/c (N2) background.[11,16-19] MISTRG mice are used under Material Transfer Agreements with Regeneron Pharmaceuticals and Yale University. They were re-derived by embryo transfer at Charles Rivers and bred in-house. All mice were housed in an enhanced barrier (with restricted access and enhanced personal protective equipment requirements) under specific pathogen free conditions, with continuous prophylactic enrofloxacin treatment (Baytril, 0.27 mg/ml in drinking water). Animal experiments were approved by Fred Hutch's Institutional Animal Care and Use Committee (protocol # 50941).

Human CD34^{+} cell isolation and engraftment. De-identified human fetal liver tissues (4 donors, 15-22 weeks of gestation), collected with informed consent from the donors, were obtained from Advanced Bioscience Resources, Inc. and their use was determined as non-human subject research by Fred Hutch's Institutional Review Board (6007-827 and 6007-985). Fetal livers were cut in small fragments, treated for 45 min at 37°C with collagenase D (Roche, 100 ng/ml), and a cell suspension was prepared. Hematopoietic cells were enriched by density gradient centrifugation (Lymphocyte Separation Medium, MP Biomedicals)

followed by positive immunomagnetic selection with anti-human CD34 microbeads (Miltenyi Biotec). Cells were frozen at -80°C in FBS containing 10% DMSO.

De-identified cord blood (3 donors) was obtained from normal deliveries under Swedish Medical Center (3834S-03) and Fred Hutch's (5647) Institutional Review Board approval, after consent was obtained. Cord blood $CD34^+$ cells were isolated using the EasySep Human Cord Blood CD34 Positive Selection Kit II from StemCell Technologies.

Adult $CD34^+$ cell donors were enrolled, and mobilization and apharesis collection were performed following standard methods approved by Fred Hutch's Institutional Review Board (3942). Briefly, healthy donors underwent four days of G-CSF injections (7.5 μg/kg body weight) and apharesis on the 4th and 5th days. $CD34^+$ cells were purified using the Miltenyi immunomagnetic bead method of positive selection, using the Miltenyi CliniMacs device. Cells from five donors were used.

Newborn mice (day 1-3) were sublethally irradiated (80 cGy gamma rays in a Cesium-137 irradiator) or not, as indicated, and 12,000-50,000 fetal or newborn $CD34^+$ cells in 20 µl of PBS were injected into the liver with a 22-gauge needle (Hamilton Company), as previously described.[4,5] For transplantation of adult mobilized $CD34^+$ cells, mice were preconditioned with 100 cGy or 2x150 cGy and 130,000-265,000 cells were injected. Engraftment levels were measured as the percentage of human $CD45^+$ cells among total (mouse and human combined) $CD45^+$ cells in the blood. Primary recipient mice in which the engraftment level was inferior to 10%, 15 weeks post-transplantation, were excluded from the analyses. All experiments were repeated twice independently with cohorts of mice transplanted with $CD34^+$ cells from different donors. Results from the two experiments were combined for data analysis.

For comparison of fetal, newborn and adult $CD34^+$ cells, the engraftment levels were measured 8 weeks post-transplantation.

Flow cytometry analysis. Mouse blood was obtained by retro-orbital collection and bone marrow cells were flushed from the tibia and femur. Red blood cells were eliminated by ammonium-chloride-potassium (ACK) lysis and the cells were analyzed by flow cytometry, following standard procedures. The following antibody clones were used (all purchased from Biolegend):

Human lineage cocktail: CD2-biotinylated (RPA-2.10), CD3-biotinylated (OKT3), CD4-biotinylated (OKT4), CD8-biotinylated (RPA-T8), CD11b-biotinylated (M1/70), CD14-biotinylated (HCD14), CD15-biotinylated (HI98), CD16-biotinylated (3G8), CD19-biotinylated (HIB19), CD20-biotinylated (2H7), NKp46-biotinylated (9E2), Streptavidin-BV605.

Anti-human antibodies: CD3-AF700 (HIT3a), CD14-APC-Fire750 (M5E2), CD14-PE-Dazzle (M5E2), CD16-FITC (3G8), CD16-BV785 (3G8), CD19-PE-Cy7 (HIB19), CD33-APC (WM53), CD34-PE (581), CD38-FITC (HB-7), CD45-Pacific Blue (HI30), CD45-AF700 (HI30), CD66b-FITC (G10F5), CD117-APC-Fire750 (104D2), CD203c-BV605 (NP4D6), FcεR1-AF700 (AER-37 (CRA-1)), IL-6-PE (MQ2-13A5), NKp46-PE (9E2), Siglec 8-PE-Cy7 (7C9), TNF-α-APC (Mab11).

Anti-mouse antibodies: CD45-BV605 (30-F11), CD45-PerCP (30-F11), Ter119-PerCP (TER-119).

Human hematopoietic cells were gated based on expression of human CD45 and exclusion of mouse CD45 and Ter119 staining. Dead cells were excluded by staining with 7-Aminoactinomycin D (7-AAD) Biolegend). Data were acquired with FACSDiva on an LSRII flow cytometer (BD Biosciences) and analyzed with the FlowJo software.

Red blood cell (RBC) counts. Blood (20 µl) was collected in K_2EDTA coated microtainer tubes (BD Biosciences), and RBC counts were measured using an ADVIA 2120i Hematology System.

Colony-forming unit (CFU) and secondary transplantation assays. Total human $CD45^+$ cells were isolated from the BM of irradiated NSGS and MISTRG humanized mice by

immunomagnetic depletion of mCD45$^+$ and mTer119$^+$ cells using biotinylated antibodies (clones 30-F11 and Ter119, Biolegend) and streptavidin microbeads (Miltenyi Biotec). Human CD34$^+$ cells were isolated from the BM of NSGW41 and MISTRG mice with anti-human CD34 microbeads (Miltenyi Biotec).

For CFU assays, 5,000 hCD45$^+$ or 2,000 CD34$^+$ cells were seeded into 1 ml MethoCult H4435 (StemCell Technologies). Hematopoietic colonies were scored after 12-14 days. Arising colonies were identified as colony forming unit- (CFU-) granulocyte (CFU-G), macrophage (CFU-M), granulocyte-macrophage (CFU-GM) and burst forming unit-erythrocyte (BFU-E). Colonies consisting of erythroid and myeloid cells were scored as CFU-GEMM.

For serial transplantations, 1.8x10^6 human BM CD45$^+$ cells or 1-2.5x10^5 CD34$^+$ cells were injected intrahepatically into pre-conditioned (80 cGy) newborn MISTRG mice, as described above.

Cytospin. Human or humanized mouse blood was flow sorted using a FACS Aria II cell sorter (BD Biosciences). Lymphocytes and singlets were gated by forward and side scatter. Myeloid cells were identified within the hCD45$^+$ population as either CD33$^{+/lo}$ SSC^{hi} (granulocytes) or CD33^{hi} SSC^{lo} divided based on expression of the CD14 and CD16 markers (monocytes); mast cells were found in the CD33^{hi} SSC^{lo} population. Blood samples from 3 mice were pooled together and 100,000 cells in 100 µl were spun at 1,000 rpm for 5 minutes onto Superforst Plus Gold microscope slides (Fisher) using a Cytospin 3 centrifuge (Shandon), and stained with Diff-Quik using a HEMA-TEK 2000.

Ex vivo monocyte stimulation. Human or humanized mouse blood underwent ACK lysis to remove RBCs. Remaining WBCs were plated at 1x10^5 cells per well in 200 µL RPMI + 10% FBS + 1% Pen-Strep in a 96 well round bottom plate and stimulated with 100 ng/mL LPS or 10 µg/mL R848 in the presence of 1X Brefeldin A (Biolegend) for 24 hours. Unstimulated cells were

incubated with Brefeldin A alone. Cells were stained for intracellular TNF-α and IL-6 using a standard protocol (Biolegend). Percent positive cells were identified by flow cytometry within the $CD33^{+}SSC^{lo}$ population.

Immunohistochemistry. Tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich), and embedded in paraffin. Sections were stained with anti-human CD68 (clone PG-M1, Dako) followed by an HRP-conjugated anti-mouse secondary antibody (Leica) and revealed with the peroxidase substrate 3, 3′-diaminobenzidine (Leica).

Statistical analysis. All datasets were analyzed for similar variance using an F test prior to performing subsequent statistical analysis. For direct comparison between two groups, an unpaired t-test was used. For comparison between multiple groups, one-way ANOVA with Tukey's multiple comparison test was used. For analysis of groups over time, a repeated-measures 2-way ANOVA was used. Survival curves were analyzed using a log-rank Mantel-Cox test.

Supplemental Figure legends

Supplemental Figure 1. Rate of successful engraftment in female and male recipient mice.

(A) Engraftment levels, measured as the percentage of $hCD45^+$ cells among total blood $CD45^+$ cells, in the blood of female and male recipient mice, 10 and 15 weeks post-transplantation. The bars represent mean ± S.D. (n=1-14, unpaired t-test). The dashed line indicates the 10% threshold for successful engraftment at 15 weeks. **(B) Success rates** for engraftment in NSGS, NSGW41 and MISTRG recipients 15 weeks post-transplantation (n=7-23, combining males and females).

Supplemental Figure 2. Phenotype of $CD34^+$ cells isolated from humanized MISTRG BM and from human donors.

Long-term hematopoietic stem cell, with self-renewing and multilineage differentiation potential are contained within the lineage-negative $CD34^+$ $CD38^{lo}$ $CD90^+$ $CD45RA^-$ cell population. **(A)** Flow cytometry characterization of this cell population among purified human fetal $CD34^+$ cells, and human $CD34^+$ cells in the BM of MISTRG humanized with these cells. **(B)** Similar analysis of purified human adult G-CSF mobilized $CD34^+$ cells, and human $CD34^+$ cells in the BM of MISTRG humanized with these cells. This result shows that lineage-positive cells, within the CD34+ population, are expanded in humanized mice compared to humans; but Lin^- $CD34^+$ $CD38^{lo}$ have a similar phenotype.

Supplemental Figure 3. Phenotypic characterization of human myeloid cells.

(A) Histograph comparing CD33 expression levels on blood $CD33^{+/lo}$ SSC^{hi} granulocytic cells (blue gate in Figure 2C), in the indicated mice and human healthy donor blood. FMO, 'fluorescence minus one' control, gated on human donor SSC^{hi} blood cells. **(B) Neutrophil**

maturation stages of sorted CD33$^{+/lo}$ SSC^{hi} populations flow sorted from human or humanized mouse blood and stained by Diff-Quik, represented as frequency among n=40-50 cells from 2 mice/group. **(C) Histograph comparing CD14 expression levels** on blood CD33^{hi} SSC^{lo} CD117^{-} FcεR1^{-} CD203c^{-} monocytic cells (blue gate in Figure 2C) in the indicated mice and human healthy donor (black) blood. FMO control (grey), human blood. **(D) CD14^{+} CD16^{+} and CD14^{lo} CD16^{+} monocyte subsets** develop at late time points in NSGW41 recipient mice. **(E) Frequency of mast cells** (CD33^{hi} SSC^{lo} CD117^{+} FcεR1^{+} CD203c^{+}) in human and humanized mouse blood (left panel, n=4-9; one-way ANOVA with Tukey's multiple comparison test), and representative flow cytometry analysis (pre-gated on CD33^{hi} SSC^{lo} cells) of healthy human donor (black) and NSGS (purple) blood. The inset shows a representative image (scale bar: 20 μm) of human mast cells from NSGS mouse blood. **(F)** Human tissue macrophages phagocytose mouse red blood cells, which this is particularly visible (arrows) by H&E staining of humanized MISTRG livers.

Supplemental Figure 4. Transplantation of fetal, newborn and adult CD34^{+} cells in MISTRG mice.

Newborn mice were pre-conditioned with the indicated radiation dose and fetal liver (40,000-50,000 cells, 2 donors), cord blood (12,000-18,000 cells, 3 donors) or adult G-CSF mobilized (132,000-207,000 cells, 5 donors) CD34^{+} cells were transplanted by intrahepatic injection. **(A) Blood human CD45^{+} immune cell chimerism** measured 8-10 weeks post-transplantation. Error bars indicate mean ± S.D. (n=8-32). **(B) Composition of human white blood cells** (error bars indicate mean ± S.E.M.). **(C) Representative flow cytometry analysis and frequency of human monocyte subsets**, defined by CD14 and CD16 expression among CD33^{hi} SSC^{lo} cells. Error bars indicate mean ± S.D. (n=6-23). **(D) Human tissue macrophages** in the lungs and livers of recipient mice, as identified by immunohistochemistry for human CD68.