# Supporting Information

## Willinger et al. 10.1073/pnas.1019682108

### SI Materials and Methods

**Generation of hIL-3/GM-CSF KI Mice.** A targeting construct for replacing the mouse with the human *IL3* gene and the mouse with the human *CSF2* gene in a single targeting step was constructed using VELOCIGENE technology (1) using gap-repair cloning. Mouse sequences were obtained from BAC RPCI-23, clone 5E15, and human sequences from Caltech D library, BAC clone 2333J5. A gap-repair donor vector containing a p15 origin of replication was constructed by cloning a 5′ mouse homology arm immediately upstream of the m*Il3* ATG, a human 5′ *IL3* homology arm extending from the h*IL3* ATG to about 274 nts into the h*IL3* gene, a poly linker, a 3′ h*CSF2* beginning about 2.9-kb downstream of the polyA sequence of the h*CSF2* gene (about 233 bases), and a loxed drug selection cassette followed by a mouse 3′ homology arm having sequence downstream (about 2.9-kb downstream) of the m*Csf2* polyA sequence (Fig. S1). The gap-repair vector was linearized and inserted into *Escherichia coli* strain DH10B containing the human BAC clone 2333J5 and a recombination enzyme vector (1). Cells were grown in drug-selection medium. Individual clones were grown, gap-repair donor vector DNA was extracted, and portions of the vector were sequenced for proper mouse-human junctions. Pulsed field-gel electrophoresis was used to establish insert size and expected restriction fragment length. Captured donor containing mouse upstream and downstream homology boxes flanking the h*IL3* gene, the h*CSF2* gene, and the loxed drug selection cassette was obtained from repair donor vector, the captured donor was linearized, and linearized captured donor was introduced into *E. coli* DH10B containing RPCI23 clone 5E15 and pABG vector. Cells were grown in drug-selection medium. Individual clones containing captured donor DNA in RPCI23 clone 5E15 DNA (to form the targeting vector) were isolated, targeting vector DNA was extracted, and portions of the vector were sequenced for proper mouse-human junctions. Pulsed field-gel electrophoresis was used to establish insert size and expected restriction fragment length. The targeting vector was linearized and used to electroporate mouse ES cells as described (1). Electroporated mouse ES cells containing the targeting vector were further electroporated with a transient Cre-expressing vector to remove the loxed drug-selection cassette. The targeting vector was electroporated into *Rag2*$^{+/-}$ *Il2rg*Y/- ES cells. The parental ES cell line in which the *Rag2 Il2rg* targeting was done was a commercially available V17 ES cell line (BALB/c × 129 heterozygote). ES cells targeted with the h*IL3* and h*CSF2* genes were introduced into an eight-cell stage mouse embryo by the VELOCIMOUSE method (2). VELOCIMICE (F0 mice fully derived from the donor ES cell) bearing the humanized *IL3* and *CSF2* constructs were identified by genotyping for loss of mouse allele and gain of human allele using a modification of allele assay (1). These chimeric mice were first bred with Balb/cAnNCR and then mice heterozygous for *Rag2* and *IL2rg* plus the human IL-3/GM-CSF KI were bred with *Rag2*$^{-/-}$*IL2rg*$^{-/-}$ mice for engraftment studies.

**ELISA.** Mouse and human IL-3 and GM-CSF protein were detected with species-specific ELISA kits from R&D Systems according to the manufacturer's instructions. Splenocytes were activated with 5 μg/mL Con A and 100 U/mL IL-2 and supernatants harvested for ELISA after 24 and 48 h of stimulation.

**Flow Cytometry.** Cell suspensions were prepared from bronchoalveolar lavage, bone marrow, and blood of mice 10 to 14 wk posttransplantation. Lysis of RBC was performed using ACK lysis buffer (Lonza). Samples were then stained with fluorochrome-labeled mAbs against mouse and human cell surface antigens. The following mAbs were used: (*i*) Anti-human: CD3 (UCHT1), CD4 (RPA-T4), CD8 (HIT8a), CD11c (B-ly6), CD14 (MoP9), CD19 (HIB19), CD33 (WM53), CD34 (AC136), CD45 (HI30 and 2D1), CD56 (NCAM 16.2), CD66 (B1.1), CD116 (4H1), CD123 (9F5). (*ii*) Anti-mouse: CD45 (30-F11), F4/80 (BM8). CD34 mAb was from Miltenyi Biotec; CD116, CD45 (30-F11), and F4/80 mAbs were from eBioscience. All other mAbs were from BD Biosciences. Samples were analyzed on a FACSCalibur or LSRII flow cytometer (BD Biosciences).

**Methylcellulose CFU Assay.** Human CD34$^{+}$ bone marrow cells from engrafted mice were purified by cell sorting. Sorted cells (1-1.5 × 10$^{5}$) were cultured in Iscove's modified Dulbecco's medium (GIBCO) based methylcellulose medium (Methocult H4100; StemCell Technologies) that was supplemented with 20% FBS, 1% BSA, 2 mM L-glutamine, 55 μM 2-mercaptoethanol and the following human cytokines: stem cell factor (10 ng/mL), FLT3 ligand(10 ng/mL), thrombopoietin (50 ng/mL), IL-3 (20 ng/mL), IL-6 (10 ng/mL), IL-11 (10 ng/mL), GM-CSF (50 ng/mL), and erythropoietin (4 U/mL) (all R & D Systems). Cells were incubated in 60-mm Petri dishes at 37 °C/5% $CO_2$. The number of colonies was determined by microscopy after 12 to 14 d.

**Inflammatory Response to LPS.** Mice received two intraperitoneal injections of Ultrapure LPS *E. coli* 0111:B4 (Invivogen) 48 h apart (35 and 17.5 μg). Sera were harvested 2 to 3 h after each injection. Serum concentrations of human IL-6 were determined by a human-specific ELISA (R&D Systems). Mice were killed 72 h after the first LPS injection; blood was collected by cardiac puncture and used for flow cytometry as described above.

**Viral Burden After Influenza A Infection.** After intranasal infection with 2 × 10$^{2}$ or 2 × 10$^{4}$ PFU of influenza A /PR8 (H1N1) virus, lungs were harvested on day 3 or day 7 postinfection. Total RNA was extracted from lung homogenates and cDNA prepared using random hexamer primers. Quantitative RT-PCR for the matrix (M) gene was performed as described (3). Expression values were calculated using the comparative threshold cycle method and normalized to mouse *Hprt*.


1. Valenzuela DM, et al. (2003) High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. *Nat Biotechnol* 21:652–659.
2. Poueymirou WT, et al. (2007) F0 generation mice fully derived from gene-targeted embryonic stem cells allowing immediate phenotypic analyses. *Nat Biotechnol* 25: 91–99.
3. van Elden LJ, Nijhuis M, Schipper P, Schuurman R, van Loon AM (2001) Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. *J Clin Microbiol* 39:196–200.

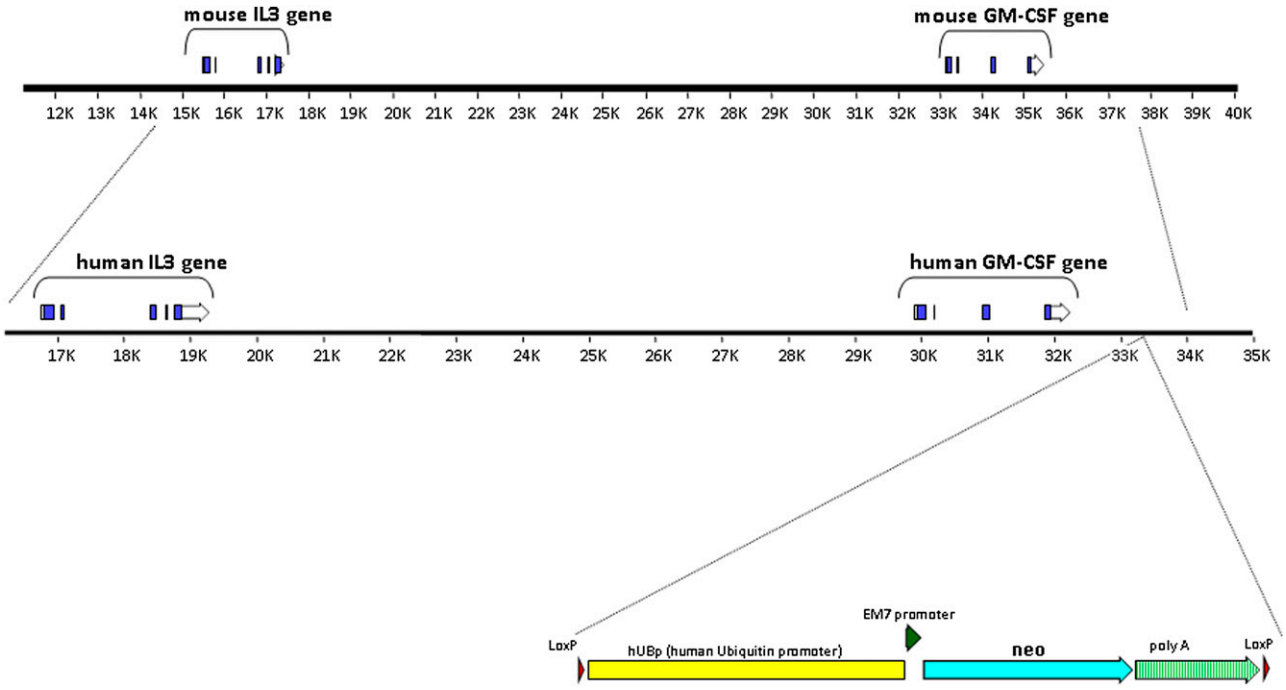


**Fig. S1.** Strategy to generate hIL-3/GM-CSF KI mice. Genomic organization of mouse (*Upper*) and human (*Lower*) *IL3* and *CSF2* loci are shown on chromosomes 11 and 5, respectively. Mouse loci were replaced with human loci as described in *Materials and Methods*.

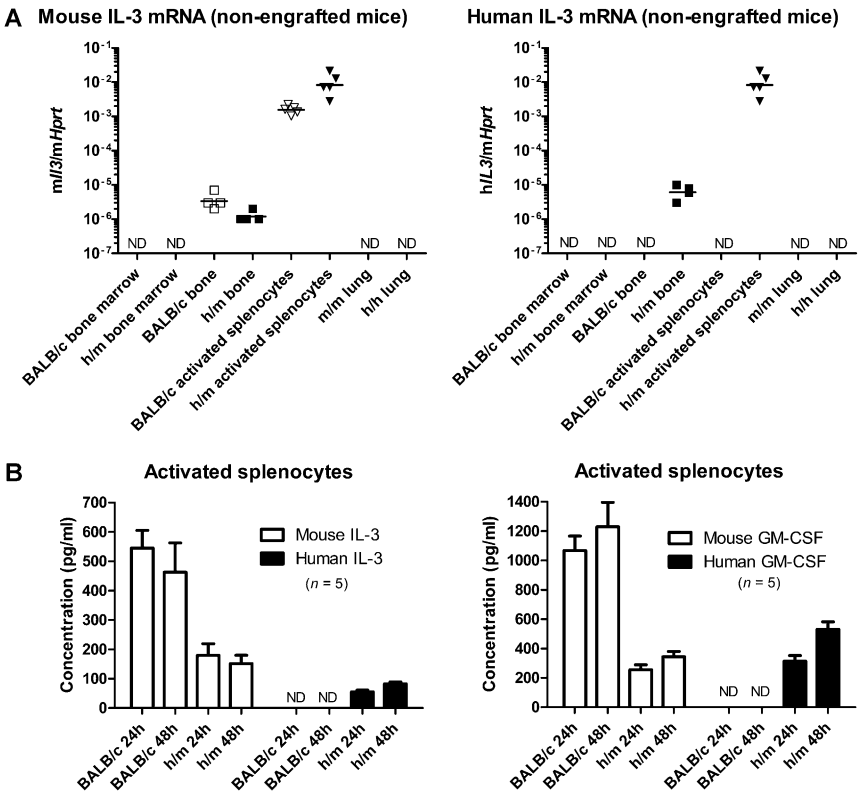


**Fig. S2.** Validation of human IL-3 expression in nonengrafted hIL-3/GM-CSF KI mice. (*A*) Quantitative RT-PCR analysis of mouse and human IL-3 mRNA expression in various tissues from BALB/c, *Rag2*$^{+/-}$ *Il2rg*$^{+/-}$ human/mouse (h/m) KI mice, or *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mouse/mouse (m/m), and human/human (h/h) KI mice (each $n$ = 3–5). Expression was normalized to mouse *Hprt*. ND, not detectable. (*B*) ELISA of mouse and human IL-3 and GM-CSF production by activated splenocytes. Splenocytes from either BALB/c or *Rag2*$^{+/-}$ *Il2rg*$^{+/-}$ h/m KI mice were stimulated with ConA and IL-2 and supernatants harvested after 24 h and 48 h (each $n$ = 5). Human IL-3 and GM-CSF were not detectable in m/m mice.

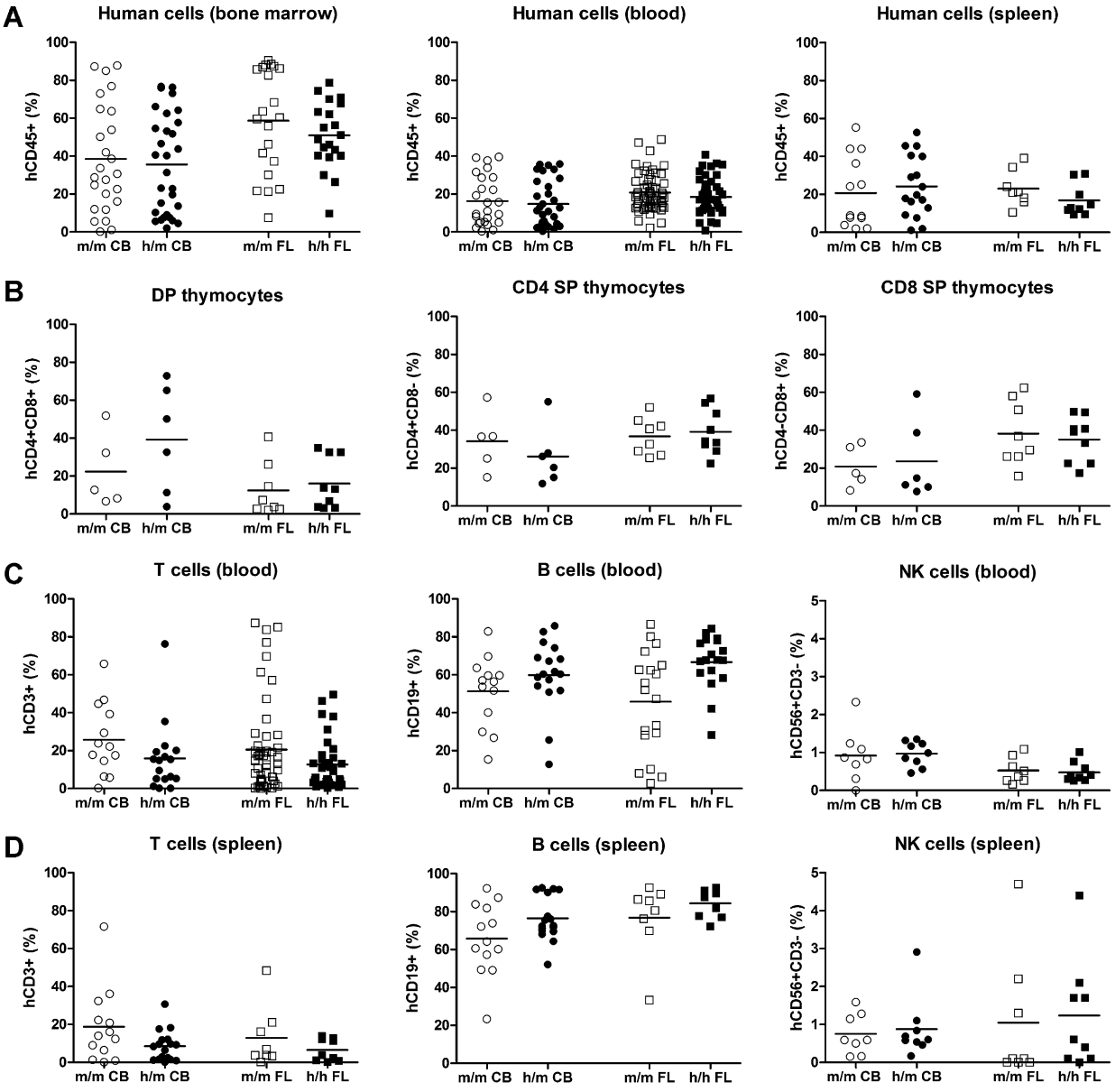


**Fig. S3.** Human hematopoietic cell engraftment and lymphocyte development in engrafted hIL-3/GM-CSF KI mice. The frequency of human hematopoietic ($hCD45^+$) cells in bone marrow ($n$ = 20–30 per group), blood ($n$ = 25–61 per group), and spleen ($n$ = 8–17 per group) (*A*); human thymocytes ($n$ = 5–9 per group) (*B*); human T ($n$ = 13–51 per group), B ($n$ = 13–19 per group), and NK cells ($n$ = 8–9 per group) in blood (*C*) and spleen ($n$ = 8–17 per group) (*D*) was determined by flow cytometry in m/m, h/m, and h/h KI mice engrafted with either cord blood or fetal liver $CD34^+$ human cells, as indicated. Each dot represents one mouse. Horizontal bars indicate mean frequencies.

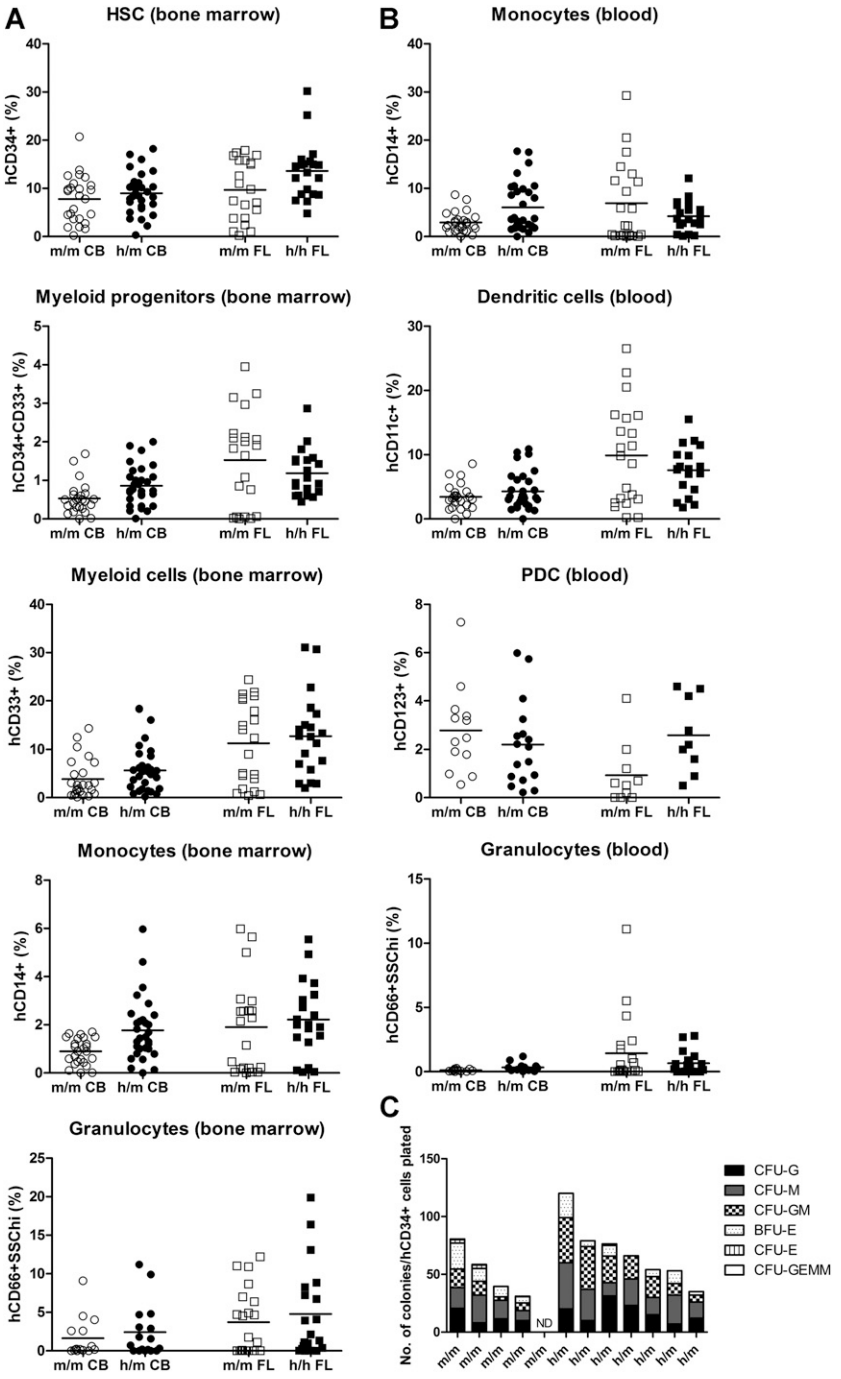


**Fig. S4.** Steady-state human myelopoiesis in engrafted hIL-3/GM-CSF KI mice. (*A*) The frequency of human hematopoietic stem cells ($n$ = 20–30 per group), myeloid progenitor cells ($n$ = 20–30 per group), total myeloid cells ($n$ = 20–30 per group), monocytes ($n$ = 20–30 per group), and granulocytes ($n$ = 15–21 per group) in bone marrow was determined by flow cytometry in m/m, h/m, and h/h KI mice engrafted with either cord blood or fetal liver CD34$^+$ human cells, as indicated. Each dot represents one mouse. Horizontal bars indicate mean frequencies. (*B*) The frequency of human monocytes ($n$ = 19–31 per group), dendritic cells (DC) ($n$ = 19–31 per group), plasmacytoid DCs (PDC) ($n$ = 9–17 per group), and granulocytes ($n$ = 12–21 per group) in blood was determined by flow cytometry as in *A*. (*C*) Colony formation assay of human bone marrow cells from engrafted m/m or h/m KI mice ($n$ = 5–7 per group). The number of CFU or burst-forming units (BFU) per human CD34$^+$ cells plated is shown. Each bar represents data from an individual mouse. ND, none detected. CFU-G, CFU-

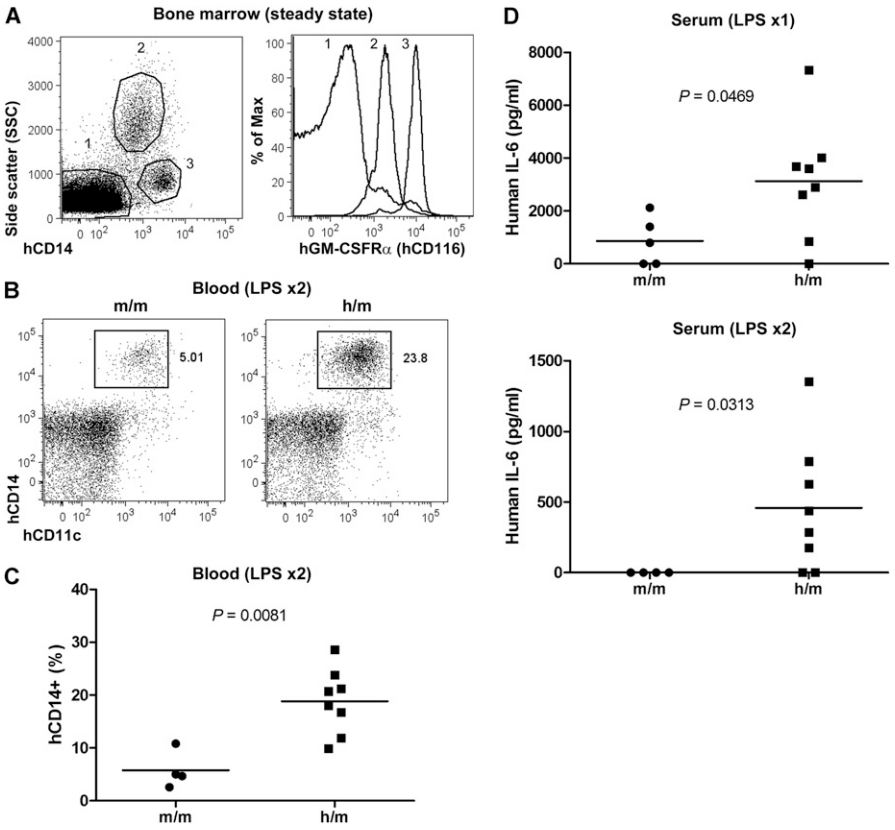


**Fig. S5.** Enhanced human inflammatory responses in engrafted hIL-3/GM-CSF KI mice. (*A*) Flow cytometry analysis of human bone marrow cells from engrafted hIL-3/GM-CSF h/m KI mice in steady state. Dot plot (*Left*) is gated on hCD45$^+$mCD45$^-$ cells. Histogram (*Right*) shows GM-CSF receptor α (CD116) expression on CD14$^-$ cells (population 1), CD14mid/SSChi granulocytes (population 2), and CD14hi monocytes (population 3). One representative example of a total of 12 mice analyzed is shown. (*B*) Representative flow cytometry analysis of human blood cells from cord blood-engrafted m/m or h/m KI mice 72 h after two intraperitoneal injections of LPS. Plots are gated on hCD45$^+$mCD45$^-$ cells. Numbers next to boxed areas indicate the percentages of human CD14$^+$ cells. (*C*) Frequency of human CD14$^+$ blood cells in engrafted m/m ($n$ = 4) or h/m KI mice ($n$ = 8) 72 h post-LPS injections. (*D*) ELISA of human IL-6 in sera from engrafted m/m ($n$ = 4–5) or h/m KI mice ($n$ = 8) 2–3h after first (*Upper*) and second (*Lower*) LPS injection. One m/m mouse died after the first LPS injection. Each dot represents one mouse. Horizontal bars indicate mean values. Results are representative of two independent experiments.

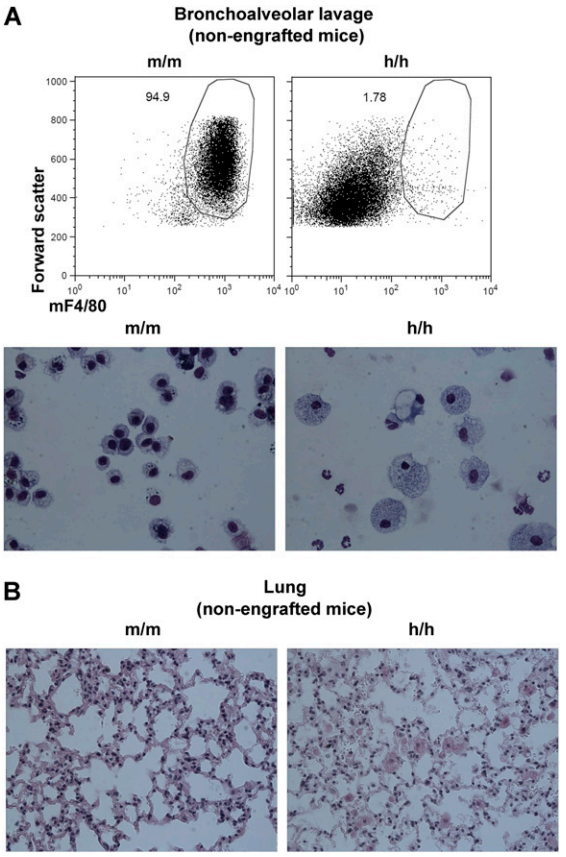


**Fig. S6.** Nonengrafted homozygous hIL-3/GM-CSF KI mice develop PAP. (*A*) Flow cytometry analysis (*Upper*) and DiffQuick staining (*Lower*) of bronchoalveolar lavage cells from nonengrafted m/m or h/h KI mice. Magnification: 600×. One representative example of a total of 10 to 11 mice analyzed per group in two independent experiments is shown. (*B*) Periodic acid-Schiff staining of lung tissue sections from nonengrafted m/m or h/h KI mice. Magnification: 400×. One representative example of a total of 12 mice analyzed per group is shown.

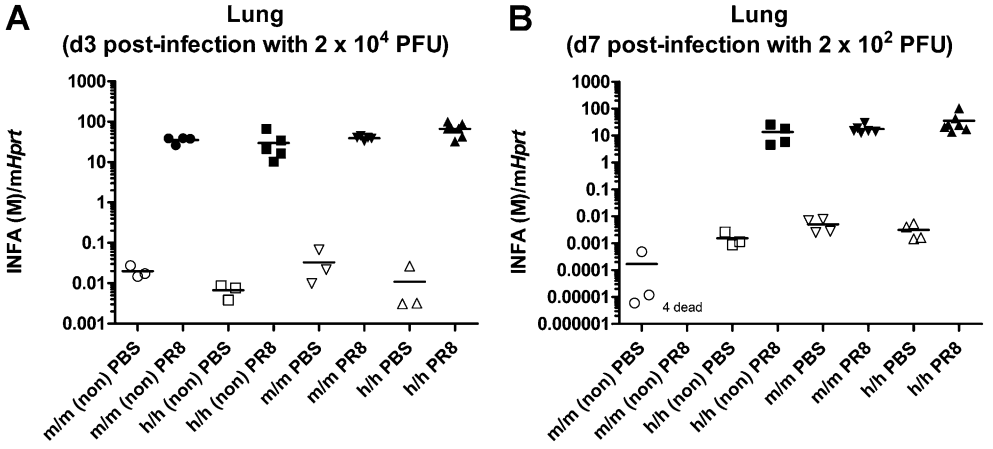


**Fig. S7.** Viral burden after infection with influenza A. Quantitative RT-PCR analysis of influenza A matrix [INFA (M)] RNA expression in lung tissue from nonengrafted (non) and engrafted m/m and h/h KI mice 3 (*A*) or 7 d (*B*) after intranasal infection with $2 \times 10^4$ (*A*) or $2 \times 10^2$ (*B*) PFU of influenza A (PR8) virus, respectively (each $n = 4–7$). Intranasal application of PBS was used as a control (PBS) (each $n = 3–4$). Expression was normalized to mouse *Hprt*.