Mice and antibodies

Wild-type C57Bl/6J (C57; CD45.2$^+$), B6.SJL-PtrcaPep3b/BoyJ (BoyJ; CD45.1$^+$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and (CD45.1×CD45.2) F_1 progeny of C57 and BoyJ mice obtained from a breeding colony at the Indiana University School of Medicine (Indianapolis, IN). Prior to this study, X-CGD mice, which carry a null allele for gp91*phox* [1], were backcrossed into C57 for more than 16 generations. X-CGD mice were crossed to BoyJ mice to generate CD45.1$^+$ X-CGD mice, which were bred with C57 X-CGD mice to produce (CD45.1$^+$ CD45.2$^+$) F_1 progeny. Mice were housed under pathogen-free conditions and fed autoclaved food and acidified water. Animal protocols were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine.

Antibodies and other reagents.

Antibodies against CD3-phycoerythrin (PE), Ter119-PE, B220-PE, Gr-1-PE, Mac-1-PE, sca-1-PECy7, CD34-fluorescein isothiocyanate (FITC), CD45.1-PE or allophycocyanin (APC) and CD45.2-FITC were purchased from BD Bioscience (San Jose, CA). Anti-CD150-PerCP-Cy5.5 and anti-CD16/32-APC-Cy7 were purchased from Biolegend (San Diego, CA). Purified anti-CD135, anti-rat-IgG-FITC and anti-IL7Ralpha-eFluor450 were purchased from eBioscience (San Diego, CA). Anti-KIT (3C11) conjugated to biotin and streptavidin conjugated to APC were purchased from Invitrogen (Carlsbad, CA). Dihydrorhodamine 123 (DHR 123) was obtained from Molecular Probes Inc (Eugene, OR).

Preparation of ACK2 and treatment of recipient mice

The ACK2 hybridoma cell line was a generous gift from S. Nishikawa (Kyoto University, Kyoto, Japan) [2]. Hybridoma cells were adapted to serum-free media and a high titer-producing clone was isolated by limiting dilution. Purified ACK2 antibody was prepared at the University of

Nebraska Medical Center Monoclonal Antibody Facility. Based on dosing used by Ogawa et al[2] and our preliminary studies showing a seven-fold reduction in marrow CFUS-12 content at seven days following intraperitoneal administration of 2 mg ACK2, 2 mg of ACK2 in 0.5 cc saline or 0.5 cc saline was administered by intraperitoneal injection into 8- to 12-week-old mice. On Day 4 after ACK2 administration, some of the mice were irradiated with 300 cGy. On Days 7, 10 and 14, mice were sacrificed and marrow harvested from femurs. Because of the marked reduction in marrow cellularity in mice treated with the combination of ACK2 and 300 cGy, mice in this cohort at Day 14 after ACK2 admnistration were transplanted with 2×10^5 CD45.2+ donor splenocytes on day 7 after ACK2. After erythrocyte lysis, the remaining cells were stained with antibodies prior to analysis by flow cytometry on a BD FACS-Aria. HSC frequency was determined by gating on $lin^-Sca\text{-}1^+KIT^+$ $CD135^-CD150^+$ cells and common myeloid progenitor cells were gated as $lin^-Sca\text{-}1^-KIT^+$ $CD34^{low}Fc\gamma R^{low}$. For the Day14 ACK2 and 300 cGy treated cohort, host HSCs and HPCs were determined with CD45.1 and CD45.2 staining in addition to regular HSC and HPC markers, showing that more than 95% of residual HSC and HPC in the marrow were host-derived. In some mice treated with ACK2, a colony forming unit (CFU) assay was also done to enumerate bone marrow granulocyte/macrophage progenitor cells on day 7. 2×10^4 bone marrow cells were plated in 35 mm culture dish with methylcellulose-based semisolid media (StemCell Technologies Inc., Vancouver, Canada) with 50U/ml mIL-3 (Peprotech, Rocky Hill, NJ) and cultured for 14 days at 37° C. Hematopoietic colonies were identified and counted by inverted microscope. Peripheral blood (30 µl) was collected from tail vein into BD Microtainer tubes with EDTA and analyzed with a *H*emavet Mascot hematology analyzer from Drew Scientific Group *(*Dallas*,* TX).

To estimate the concentration of ACK2 after administration, sera and purified ACK2 were diluted serially and incubated with primary mouse bone marrow-derived mast cells, which

express high levels of KIT. Goat-anti-rat IgG APC was added to each serial dilution; then the serum concentration of ACK2 was estimated using a relative standard curve derived from the mean fluorescence intensity of mast cell staining by the antibody. The correlation between mean channel fluorescence (MCF) and antibody concentration was analyzed by linear regression analysis (Microsoft Excel) similar to a previously described approach.[3]

For transplantation, recipient mice received ACK2 injections or saline on Day 1. On Day 4, some mice received 300 cGy (^{137}cesium source). On Day 7, recipients were transplanted with unfractionated fresh BM or BM cultured *ex vivo* for lentiviral-mediated gene transfer, as below.

Long-Term Repopulating Activity of Conditioned Marrow

Marrow HSC function in C57 mice treated with ACK2, LD-IR, or ACK2 + LD- IR was assessed using a competitive repopulation assay adapted from Harrison [4]. Bone marrow was harvested from femurs of 4–12 mice in each cohort and pooled; a mixture containing 1-1.33 femur equivalent of BM pooled from a cohort of treated mice ("test cells") and fresh untreated BoyJ marrow cells ("competitor cells") was transplanted into each of four lethally irradiated C57 recipients. In the cohorts treated with either ACK2 or LD-IR alone, the number of competitor cells was 1×10^6 while for the ACK2 + LD-IR cohort, either 0.2×10^6 or 0.5×10^6 competitors were used. For the untreated control group, a mixture containing 1×10^6 test (control) cells and 1×10^6 competitor cells was transplanted into each of 4 recipients. Test (CD45.2) and competitor (CD45.1) chimerism was assessed by flow cytometry 4–5 months after transplantation. The relative long-term repopulating ability of test cells compared to that of CD45.1 BoyJ competitor bone marrow cells was calculated for each mouse, and the mean was determined, as previously described [4-6]. Briefly, first, repopulating units (RU) were calculated as follows: RU = % test cell donor chimerism x C/(1 - % test cell donor chimerism), where C = competitor cell number/10^5. How well the test cells repopulated the recipient marrow per 10^5 test cells

used (referred to as long term repopulating ability) was calculated as equal to RU/actual number of test cells used/10^5 competitor cells.

Engraftment analysis

Transplantation of fresh unfractionated whole bone marrow into 8- to 12-week-old congenic recipients conditioned as above was performed as described. Donor engraftment was assessed at various time points post-transplantation as follows. Peripheral blood (PB) leukocytes were stained with anti-CD45.2-fluorescein isothiocyanate (FITC) or CD45.1-PE antibodies (BD Pharmingen; San Diego, CA), and the fluorescence was measured by flow cytometry using a BD FACSCalibur. The data collected was analyzed using Cellquest software (BD Biosciences; San Jose, CA); then donor chimerism was determined by the percentage of $CD45.2^+$ cells in PB leukocytes. T cells, B cells and granulocytes were also stained with CD3, CD19 and Gr1 to detect donor chimerism in different lineages.

Lentiviral Transduction of Bone Marrow

Lineage-negative cells were separated from the bone marrow of congenic $CD45.2^+$ X-CGD mice with MACS beads *(*Miltenyi Biotec*,* Bergisch Gladbach, Germany*)* and transduced with VSVG-pseudotyped CL20-gp91-OPT lentiviral vector [7], which is similar to CL20i4-EF1alpha-hgamma(c)OPT[8] but contains a codon-optomized gp91phox cDNA. The transduction was performed at an MOI of 10:1 overnight *in vitro* similar to as previously described [9]. Briefly, lin- bone marrow cells prepared from 6–12-week-old male X-CGD mice were plated in IMDM containing 20% fetal calf serum, 200 U/ml recombinant interleukin-6 and 100 ng/ml recombinant murine stem cell factor (Peprotech, Rocky Hill, NJ) with 8 μg/ml polybrene and concentrated lentiviral vector at MOI of 10. After overnight transduction, the gene-modified cells were counted and 5×10^5 cells injected on Day 7 into the tail vein of each 8- to10-week-old F1 ($CD45.1^+CD45.2^+$) X-CGD recipient, who were conditioned with either 300cGy or ACK2 +

300cGy according to the schedule in Figure 1A. Donor cell chimerism post-transplant was monitored by staining PB leukocytes for CD45.1 and CD45.2 expression as above. Expression of vector-transferred gp91^{phox} following transplantation was determined by monitoring NADPH oxidase activity in PB neutrophils of X-CGD recipients by flow cytometry using dihydrorhodamine 123 (DHR 123) fluorescence. [6]

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Table S1. Effect of ACK2 and/or LD-IR treatment on peripheral blood counts

Blood Parameters	Control	ACK2	IR	ACK2+IR
RBCs (m/μl)	11.6 ± 0.6	9.2 ± 0.1**	9.1 ± 0.4**	7.2 ± 0.8**
Hemoglobin (g/dl)	17.0 ± 0.5	13.1 ± 0.4**	13.2 ± 0.6**	9.8 ± 1.2**
Hematocrit (%)	48.5 ± 2.3	39.0 ± 0.9**	39.2 ± 1.7**	29.2 ± 3.5**
WBC (k/μl)	22.1 ± 3.6	16.9 ± 1.4*	8.8 ± 1.3**	1.9 ± 0.9**
NE (k/μl)	5.6 ± 0.5	1.8 ± 0.2**	5.5 ± 0.8	0.5 ± 0.2**
LY (k/μl)	15.0 ± 2.6	14.0 ± 1.3	2.4 ± 0.4**	1.2 ± 0.5**
MO (k/μl)	0.8 ± 0.1	0.6 ± 0.1*	0.3 ± 0.1**	0.1 ± 0.1**
PLT (k/μl)	1307.0 ± 60.1	526.0 ± 70.7**	1313.3 ± 235.3	419.7 ± 66.1**

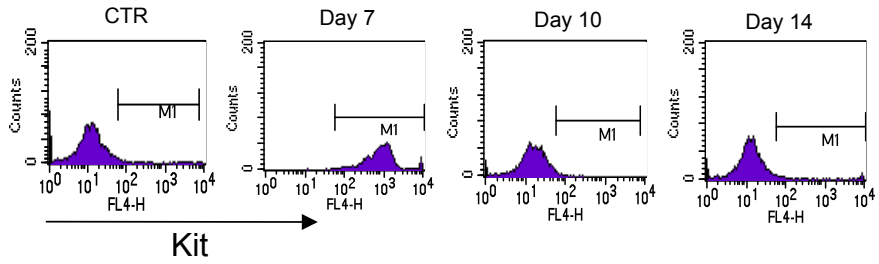
Peripheral blood was collected from control mice and mice that had been treated with ACK2 and/or IR, with administration of ACK2 10 days prior and LD-IR 7 days prior (n = 3 mice in each group). Several hematological parameters were assessed. **: $P<0.01$ and *: $P<0.05$ compared with control group.

Figure S1. ACK2 clearance post administration. (A) After administration of 2 mg ACK2, sera were collected on Days 7, 10 and 14, and incubated with murine marrow-derived mast cells expressing high levels of KIT. ACK2 was still detected in sera on Day 7 and but not on Days 10 and 14 after administration. **(B)** Flow cytometry was used to determine the relative staining intensity of marrow-derived mast cells, stained using serum obtained at Day 7 following ACK2 or with known concentrations of ACK2.

Figure S2. Multilineage donor chimerism in peripheral blood and in marrow HSC following transplantation. (A) Multilineage analysis of donor CD45.2 cells in representative transplanted mice. Recipient mice received ACK2 +/- LD-IR, followed by transplantation of 1×10^6 or 20×10^6 marrow cells from donor mice as described in Figure 2B. The percentage of donor CD45.2 lymphoid (CD3 and CD19) and myeloid (Gr-1) cells in peripheral blood is shown based on the FACS profile at 12 weeks post-transplantation (n = 4 in each group). (B) Chimerism in peripheral blood leukocytes and marrow HSC ($KIT^+lineage^-Sca\text{-}1^+CD135^-CD150^+$) of primary recipients at 32 weeks post-transplantation (shown also Fig. 2A). Gray bar shows the 300cGy cohort and dark solid bar shows ACK2 + LD-IR cohort (n = 4 in each cohort). ** indicates $P < 0.01$.

Figure S1

A


CTR
Day 7
Day 10
Day 14
Counts
FL4-H
M1
Kit

B

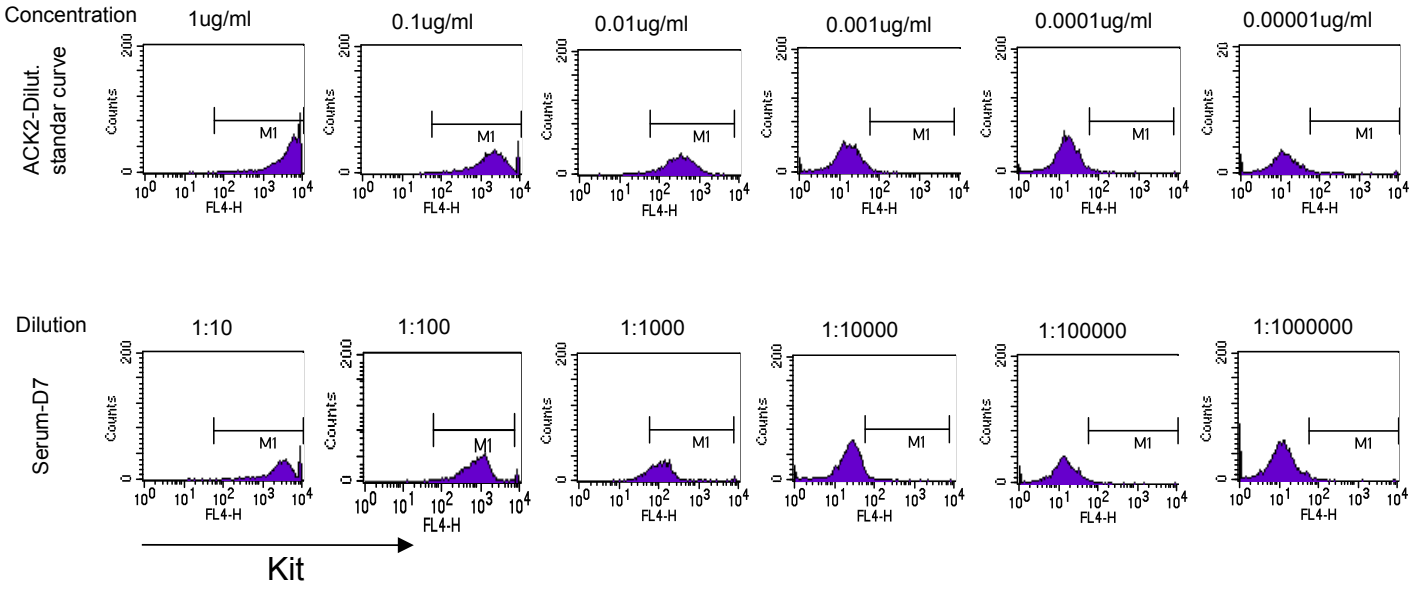
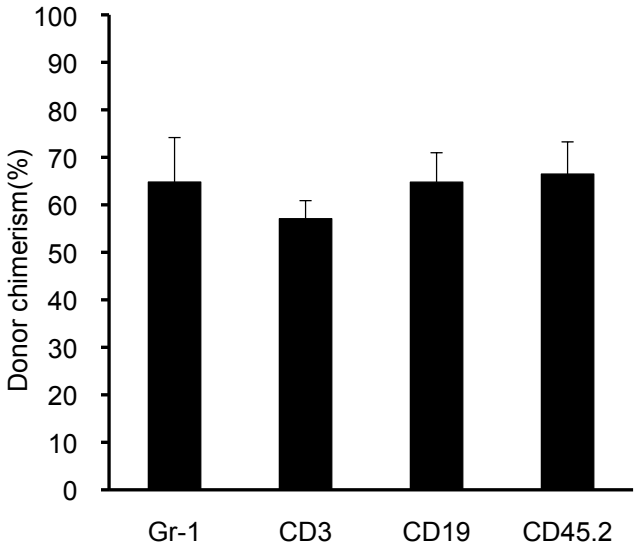
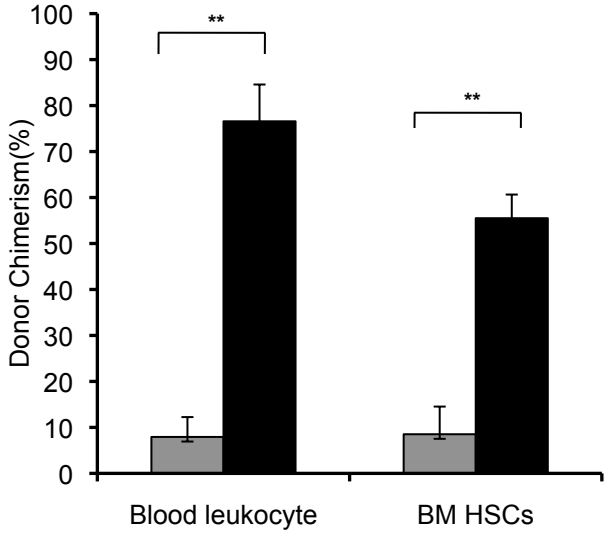

Concentration
ACK2-Dilut. standar curve
1ug/ml
0.1ug/ml
0.01ug/ml
0.001ug/ml
0.0001ug/ml
0.00001ug/ml
Dilution
Serum-D7
1:10
1:100
1:1000
1:10000
1:100000
1:1000000
Counts
FL4-H
M1
Kit

Fig. S2

A


100
90
80
70
60
50
40
30
20
10
0
Donor chimerism(%)
Gr-1
CD3
CD19
CD45.2

B


100
90
80
70
60
50
40
30
20
10
0
Donor Chimerism(%)
**
**
Blood leukocyte
BM HSCs