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Paul H Miller

Gabrielle Rabu


Margarita MacAldaz

David J H F Knapp

Alice M S Cheung

See next page for additional authors

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Authors

Paul H Miller, Gabrielle Rabu, Margarita MacAldaz, David J H F Knapp, Alice M S Cheung, Kiran Dhillon, Naoto Nakamichi, Philip A Beer, Leonard D. Shultz, R Keith Humphries, and Connie J Eaves



Analysis of parameters that affect human hematopoietic cell outputs in mutant c-kit-immunodeficient mice

Paul H. Miller^{a,b}, Gabrielle Rabu^a, Margarita MacAldaz^a, David J.H.F. Knapp^{a,b}, Alice M.S. Cheung^a, Kiran Dhillon^a, Naoto Nakamichi^a, Philip A. Beer^a, Leonard D. Shultz^c, R. Keith Humphries^{a,b}, and Connie J. Eaves^{a,b,d}

^aTerry Fox Laboratory, British Columbia Cancer Agency, Vancouver, BC, Canada; ^bDepartment of Medicine, University of British Columbia, Vancouver, BC, Canada; ^cJackson Laboratory, Bar Harbor, ME, USA; ^dDepartment of Medical Genetics, University of British Columbia, Vancouver, BC, Canada

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Xenograft models are transforming our understanding of the output capabilities of primitive human hematopoietic cells *in vivo*. However, many variables that affect posttransplantation reconstitution dynamics remain poorly understood. Here, we show that an equivalent level of human chimerism can be regenerated from human CD34⁺ cord blood cells transplanted intravenously either with or without additional radiation-inactivated cells into 2- to 6-month-old NOD-Rag1^{-/-}-IL2Rγc^{-/-} (NRG) mice given a more radioprotective conditioning regimen than is possible in conventionally used, repair-deficient NOD-Prkdc^{scid/scid}-IL2Rγc^{-/-} (NSG) hosts. Comparison of sublethally irradiated and non-irradiated NRG mice and W⁴¹/W⁴¹ derivatives showed superior chimerism in the W⁴¹-deficient recipients, with some differential effects on different lineage outputs. Consistently superior outputs were observed in female recipients regardless of their genotype, age, or pretransplantation conditioning, with greater differences apparent later after transplantation. These results define key parameters for optimizing the sensitivity and minimizing the intraexperimental variability of human hematopoietic xenografts generated in increasingly supportive immunodeficient host mice. Copyright © 2017 ISEH - International Society for Experimental Hematology. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

The discovery more than two decades ago that primitive normal human hematopoietic cells can engraft and regenerate hematopoiesis in CB17-*scid* mice proved to be a turning point in the analysis of the earliest stages of human hematopoiesis [1]. Subsequently, both empirically characterized and directly introduced genetic modifications have produced dramatic improvements in the diversity, level, and duration of human hematopoietic cell chimerism that can be obtained in xenografted mouse models [2–4]. These improvements have led to the identification of subsets of human CD34⁺ cells that recapitulate the concept, now well advanced in the mouse system, of multiple biologically distinct hematopoietic cell populations that produce different types of mature progeny over time in transplanted recipients [5–8].

Early studies of nongenetic variables that affect chimerism levels attained in nonobese diabetic–severe combined immunodeficient (NOD.CB17-*Prkdc*^{scid}/J, NS) mice included evidence of strong effects of the age of the mouse transplanted [9], whether other cells were coinjected [10], and the conditioning regimen used [11]. Most hematopoietic xenotransplantation studies now make use of NS mice into which a null mutation of the gamma chain of the interleukin 2 (IL2) receptor has been introduced (referred to as NOD-*Prkdc*^{scid/scid}-*IL2Rγc*^{-/-} [NSG] mice). This modification has the advantage of eliminating the generation of the fatal thymic lymphomas that appear universally in NS mice at between 3 and 6 months of age. NSG mice thus have a normal lifespan and the dynamics of transplanted human hematopoietic cells can be followed reproducibly for up to a year. NSG mice also lack natural killer (NK) cells, in contrast to NS mice, in which the levels of NK cells produced are still sufficient to suppress selectively the ability of most CD34⁺CD38⁺ human hematopoietic cells to

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Offprint requests to: Connie J. Eaves, PhD, Terry Fox Laboratory, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada; E-mail: ceaves@bccrc.ca

engraft the bone marrow (BM) and thus fail to display their innate short-term repopulating activity [6,12]. Evidence that the sex of the recipient can affect the level of chimerism obtained from either syngeneic (mouse) [13,14] or xenogeneic (human) transplantations [15] has also been reported.

More recently, NSG mice with various mutant *c-kit* alleles have been created and shown to be even more permissive than NSG mice in terms of their support of human hematopoietic cells obtained from transplantations of human cord blood (CB) cells [16–18]. Of particular interest are *c-kit*-deficient NSG mice expressing a homozygous W^{41} allele because these remain fertile. In C57BL/6(B6)- W^{41}/W^{41} mice, maintenance of the hematopoietic stem cell (HSC) and downstream erythroid progenitor compartments is sufficiently compromised to enable their repopulation by transplanted B6-+/+ HSCs without being subjected to a lethally myeloablative treatment [19–21]. Key findings from assessments of previously described “NSG-W41” mice transplanted with human hematopoietic cells include an increase in the levels of human chimerism that they support compared with NSG mice, including the production of human erythroid precursors and platelets detectable even in the absence of any prior host conditioning [16–18].

However, for many future studies, particularly those with the objective of assessing treatments of engrafted human malignant cells, the *scid* mutation (that encodes a defective DNA repair protein [22]) is undesirable because it sensitizes all of the host tissues markedly to many radiomimetic drugs that would be likely candidates for inclusion in test treatment protocols. Therefore, we initiated an examination of a radio-resistant alternative to NSG-W41 mice and evaluated variables that might affect the level and duration of human hematopoietic chimerism that would be supported.

Here, we report the relevance of a number of variables in mice genetically identical to NSG mice but with a homozygous *Rag1*^{null} genotype and wild-type *Prkdc* genotype to retain the same level of immunodeficiency but a normal DNA repair capacity. We then introduced the W^{41}/W^{41} genotype into NOD-*Rag1*^{-/-}-*IL2Rγc*^{-/-} (NRG) mice to generate a derivative strain. We refer to the latter as “SRG-W41” mice because they have been selected in the F2 generation to be homozygous for the *Sirpa* [23], *Rag1*-null, and *IL2rγc*-null alleles of the NRG mouse, as well as the introduced W^{41} allele on an otherwise mixed NOD-B6 genetic background.

Materials and methods

Mice

Breeding colonies of NOD.Cg-*Rag1*^{tm1Mom}*Il2rg*^{tm1Wjl}/SzJ (NRG) and B6-*Kit*^{W⁴¹/W⁴¹} (B6- W^{41}/W^{41}) mice originally obtained from The Jackson Laboratory have been established for many years in the Animal Resource Centre of the British Columbia Cancer Agency. Mice referred to here as “SRG-W41” were F2 mice

generated by intercrossing the F1 progeny of NRG with B6- W^{41}/W^{41} matings and then selecting the progeny that were homozygous for the *Sirpa* allele, the null *Rag1* and *Il2rg* alleles of the NRG mouse, and the W^{41} mutant *Kit* allele of the B6- W^{41}/W^{41} mouse to obtain all of these alleles on an otherwise mixed segregating NODxB6 (STOCK) background. Genotyping of ear notch samples was performed by polymerase chain reaction (PCR) gel electrophoresis, except for determining the presence of two, one, or no copies of the single nucleotide mutation that encodes the W^{41} allele, which were detected by Sanger sequencing of amplified PCR products. All animal breeding and experiments were performed according to regulations approved by the Animal Care Committee of the University of British Columbia.

Human cells

CD34⁺-enriched suspensions of human low-density CB cells (>80% purities) were obtained using an EasySep kit (STEMCELL Technologies, Vancouver, Canada) from cryopreserved cells originally acquired and processed as described previously [5]. Human BM cells were obtained as discarded anonymized autologous transplantation harvests from deceased patients. All cells were obtained and used according to procedures approved by the University of British Columbia Research Ethics Board.

Transplantation

Cells were injected intravenously into 7- to 12- or 21- to 25-week-old male and female mice that had either not been irradiated at all or had been irradiated with ¹³⁷Cs γ-rays or 250 kVp X-rays, as indicated. All acute exposures involved irradiating mice at a dose rate of 100–150 cGy/minute (for both types of ionizing radiation). All NRG mice used as transplantation recipients were irradiated with 900 cGy of ¹³⁷Cs γ-rays given over a 3 hour period (5 cGy/min) to allow significant repair of DNA damage in nonhematopoietic cell types that have such capacity (e.g., intestinal epithelial cells [24]). In mice given transplantations accompanied by coinjected accessory cells, the latter consisted of 10⁶ irradiated (15 Gy) human BM cells. Mice were maintained under specific pathogen-free conditions using protocols approved by the Animal Care Committee of the University of British Columbia.

Flow cytometry

Absolute numbers of human CD34⁺ cells were determined using appropriate antibodies and AccuCheck counting beads (Life Technologies, Carlsbad, CA) as described previously [25]. Human CD45⁺, glycophorin A (GPA) erythroid, CD45⁺CD33/15⁺ granulocyte-macrophage (GM), CD45⁺CD19⁺ B-cells, and FSC^{low}CD41⁺CD61⁺ platelets were also quantified using previously described antibodies and their absolute numbers determined in recipient peripheral blood (PB) samples using AccuCheck counting beads (Life Technologies) [5,25].

Statistical analysis

Data analysis and graph generation made use of R version 3.2.3 [26]. Data shown are geometric means ± SEM, with reconstitution data log transformed to improve fit to assumptions of normal distribution and equal variance. Kaplan–Meier curves were produced using the R “survival” package. Pairwise differences were examined using the Student *t* test. Multiple group comparisons were examined using one-way ANOVA with post hoc Tukey’s honest significant difference analysis.

Results

NRG mice can support similar levels of human hematopoietic cell chimerism as NSG mice

Given the different radiation sensitivities of NRG and NSG mice [27], we first undertook experiments to develop a conditioning regimen for NRG mice that would exploit the selectively enhanced repair capacity of many of their nonhematopoietic tissues. As expected, acute exposure (150 cGy/min) to increasing doses of X-rays showed 750 cGy caused 100% mortality within 3 weeks, whereas 600 cGy was the maximum dose that allowed the full survival of all mice in that test group. However, an estimated equivalent split dose protocol (two acute exposures of 400 cGy separated by a 6-hour interval) also allowed all six mice tested to survive (Fig. 1). Subsequent studies showed that a similar result could be obtained with 900 cGy of ^{137}Cs γ -rays spread over 3 hours (5 cGy/minute). This latter protocol was then adopted for all subsequent experiments.

We then compared human CD34^+ CB transplantation outcomes in young (8- to 12-week-old) NRG mice with those obtained in sex- and age-matched groups of NSG mice conditioned with a radiobiologically similar, single acute exposure (~ 100 cGy/min) to 315 cGy of ^{137}Cs γ -rays (i.e., a near maximum sublethal dose that allows the full long-term survival of NSG mice [27]). The dynamics of human hematopoietic cell chimerism obtained from 2×10^4 human CD34^+ CB cells (\pm coinjected 10^6 irradiated BM cells according to the experiment) in the BM and PB of these recipients was then tracked for up to 30 weeks. The results showed that, for at least 10 weeks, both strains thus conditioned supported similar outputs of total human CD45^+ , GM, B-lymphoid, and erythroid cells, as well as platelets, with a slight but insignificant favoring of the NSG host thereafter (Fig. 1B).

Differential effects of recipient sex, age, and use of coinjected irradiated human BM cells on the levels of human chimerism obtained in NRG mice

We next examined the role of the sex and age (up to 6 months) of the NRG host or a coinjected dose of 10^6 irradiated (1500 cGy) human BM cells on the ability of 2×10^4 purified human CD34^+ CB cells to regenerate both rapid and sustained multilineage grafts in sublethally irradiated NRG mice. Consistent with earlier data [15], assessments of the chimerism seen in both the BM (Fig. 2A) and the PB (Fig. 2B) showed that female mice generally supported a greater output of all human lineages regardless of their age when transplanted or whether additional cells were injected. However, this greater supportive activity of the female hosts appeared slightly more pronounced on the production of human B-lymphoid cells than on the production of GM cells and the negative effect of using male recipients was more marked when these were older or were coinjected with irradiated cells.

Both irradiated and untreated SRG-W41 hosts support elevated human chimerism compared with NRG mice

We then compared the human multilineage hematopoietic cell outputs obtained from $2\text{--}5 \times 10^4$ human CB CD34^+ cells transplanted into both sublethally irradiated or untreated male and female SRG-W41 and NRG mice. Previous studies had shown 400 cGy of ^{137}Cs γ -rays (given at a high dose rate) to be an optimal sublethal conditioning regimen for preparing $\text{B6-W}^{41}/\text{W}^{41}$ mice as hosts of B6 cells [20]. However, NRG mice are considerably more radiosensitive than B6 mice, with an acute dose of 600 cGy X-rays on NRG mice (Fig. 1) being approximately equal to the effect of an acute dose of 900 ^{137}Cs γ -rays (~ 800 cGy X-rays) on B6 mice. We thus estimated (and subsequently found) that a single dose of 150 cGy of ^{137}Cs γ -rays given at the high dose rate would be a conservatively sublethal conditioning dose for SRG-W41 mice.

Analysis of sequentially harvested BM aspirate cells from the four different groups of sublethally irradiated recipients again showed generally higher levels of human chimerism in female versus male mice of the same genotype, particularly at later time points after transplantation (Fig. 3A and B). In the male hosts, the outputs of human cells in the SRG-W41 mice were consistently enhanced compared with the NRG mice. In the female hosts, the levels obtained in the NRG mice were initially already very high, except in the erythroid lineage, so, not surprisingly, there was little evidence of increased chimerism in the SRG-W41 hosts.

The BM content of human hematopoietic cells obtained from the same CD34^+ CB cells injected into male and female SRG-W41 and NRG mice that had received no prior treatment are shown in Figure 3C and D. Readily detectable multilineage human cell chimerism was evident in both the non-irradiated male and female SRG-W41 mice for the entire 30 weeks of follow-up. Unexpectedly, parallel analyses of the non-irradiated female NRG recipients also revealed detectable, albeit much lower, levels of human GM and B-lymphoid cells in the BM. Even in the non-irradiated male NRG recipients, low and transient levels of human B-lymphoid cells were seen during the first 20 weeks.

The results of concurrently assessed PB samples obtained from the same irradiated and untreated mice tracked in these experiments were similar to those obtained for the BM. Absolute counts of circulating human hematopoietic cells in both female genotypes were higher than in the corresponding males independent of their prior conditioning; furthermore, the results obtained in the SRG-W41 mice were consistently higher than in the matched groups of NRG mice (Fig. 4). Importantly, these differences extended to the output of human platelets, which was uniquely sustained for 30 weeks in untreated as well as irradiated female SRG-W41 hosts.

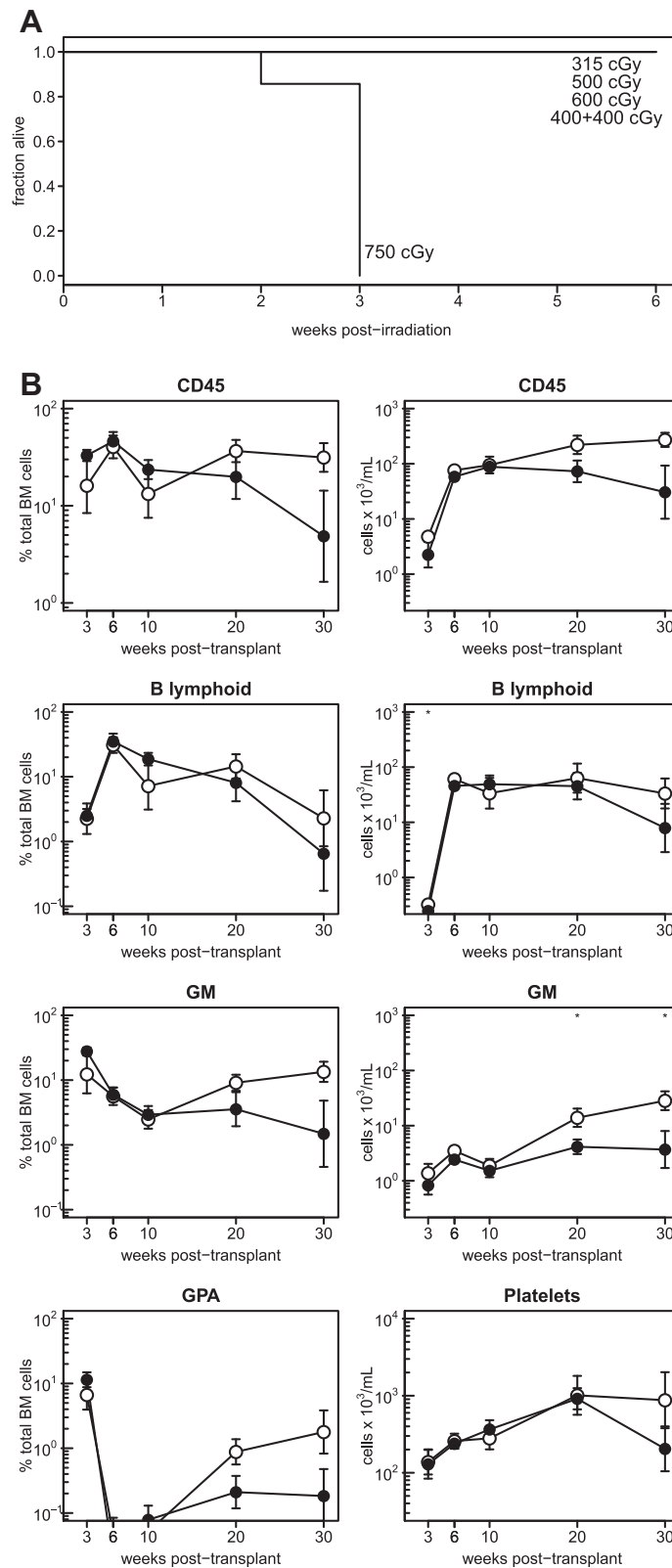


Figure 1. Similar human cell reconstitution of NSG and NRG mice. **(A)** Cohorts of 7- to 12-week-old NRG mice were X-irradiated at a high dose rate with 315 cGy (4 mice), 500 cGy (4 mice), 600 cGy (6 mice), 750 cGy (7 mice), or with two doses of 400 cGy separated by 6 hours (6 mice) and their survival was then tracked. **(B)** Kinetics of human CD45⁺, GM, B-lymphoid, and erythroid cell reconstitution of the BM (left column) and of numbers of human CD45⁺, GM, and B-lymphoid cells and platelets per milliliter of PB (right column) of NSG (open symbols) and NRG (filled symbols) mice after their transplantation with 2×10^4 human CD34⁺ CB cells plus 10^6 irradiated human BM cells. Data are pooled from three replicate experiments with a combined total of 11 mice per group. Asterisks indicate statistical significance ($*p \leq 0.05$).

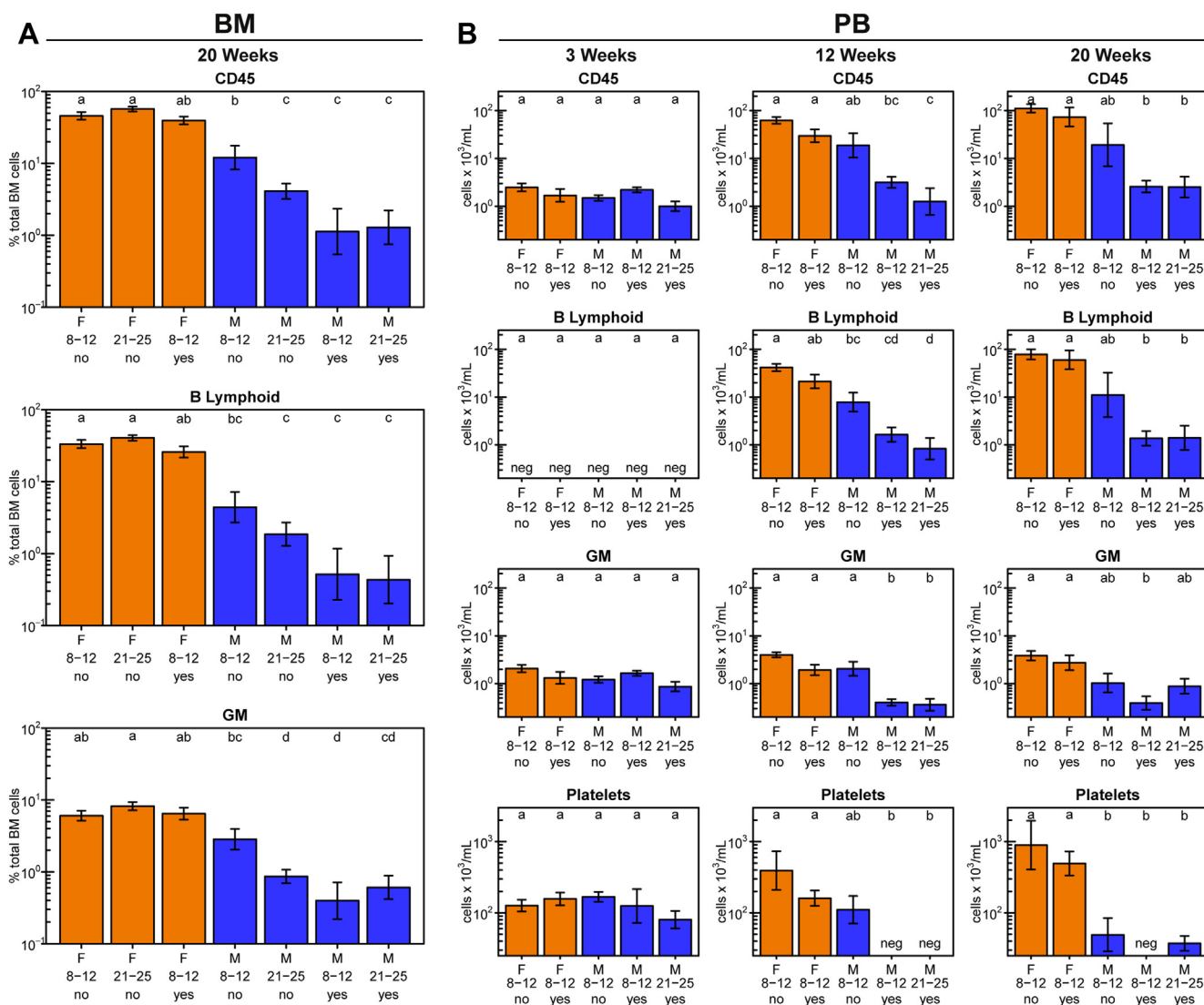


Figure 2. Female NRG mice support higher human cell outputs than male hosts. Female (orange bars) and male (blue bars) mice were each transplanted with 2×10^4 human CD34⁺ CB cells and assessed for percentage of viable human cells in the BM 20 weeks later (A), and numbers per milliliter of PB 3 (left column), 12 (middle column), and 20 (right column) weeks later (B). The X-axis labels indicate the recipient sex, recipient age at transplantation in weeks, and whether irradiated human BM cells were cojointed with the CB cells. Letters above the bars indicate groups that are not significantly different from each other. Total numbers of mice in each group (from two experiments) were as follows: female, 8-12 weeks and no accessory cells: 20 mice; female, 21-25 weeks and no accessory cells: 18 mice; female, 8-12 weeks and accessory cells: 5 mice; male, 8-12 weeks and no accessory cells: 24 mice; male, 21-25 weeks and no accessory cells: 20 mice; male, 8-12 weeks and accessory cells: 4 mice; male, 21-25 weeks and accessory cells: 4 mice.

Discussion

Much interest is now focused on defining the hematopoietic potential and activity of different types of primitive human hematopoietic cells, how these properties are intrinsically regulated, how they are elicited, and how they might be manipulated. This interest inevitably relies on combining molecular and genetic analyses of different phenotypes with robust readouts of the number and types of maturing progeny that they can generate under defined in vitro and in vivo conditions. The use of sublethally irradiated highly immunodeficient mice as hosts of intravenously transplantable human hematopoietic cells has proven an important platform for gaining insights into such issues and revealing

the complexity of this system [2,3]. Despite many advances, however, species differences between humans and mice continue to pose challenges to optimizing the sensitivity and specificity of current assays that rely on transplantation readouts. For example, assessments of human repopulating cell activity that rely on measurements of total chimerism obtained in recipients of bulk transplantations are often used to infer HSC frequencies in the cell suspension being evaluated. Such surrogate approaches are valuable because they circumvent the cost and challenges of larger-scale limiting dilution transplantation assays. However, any alteration in the numbers or types of the cells produced, or their assessment under conditions in which inputs

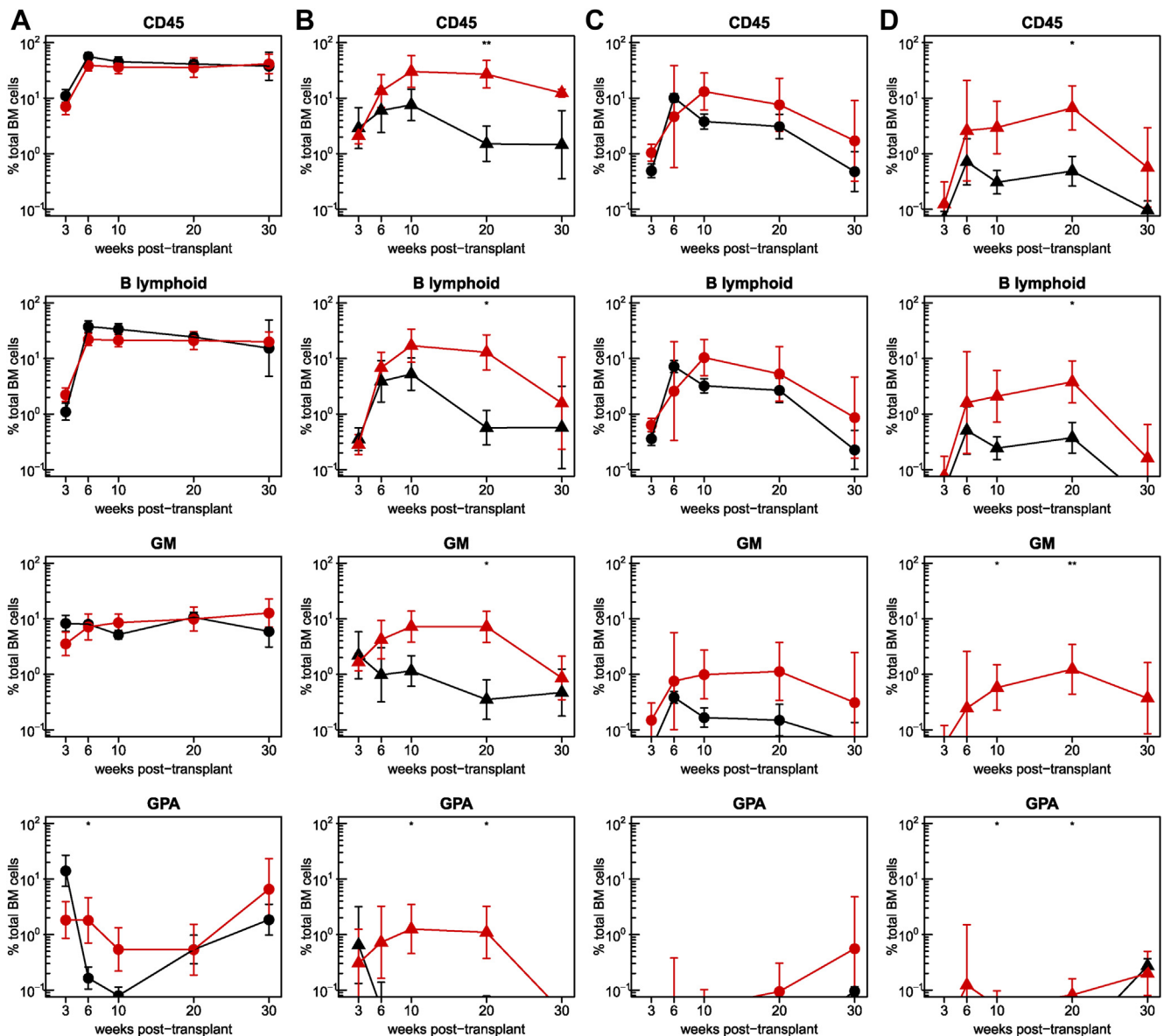


Figure 3. Enhanced human cell chimerism in the BM of SRG-W41 versus NRG mice. Shown are the kinetics of human cell reconstitution of the BM of irradiated female (A), irradiated male (B), non-irradiated female (C), and non-irradiated male (D) mice (all 7–12 weeks of age at the time of the transplantation). Results in SRG-W41 hosts are shown by the red symbols and for the parental NRG hosts in black. Mice were transplanted with $2\text{--}5 \times 10^4$ human CD34⁺ CB cells and the percentages of total human CD45⁺, GM, B-lymphoid, and erythroid cells in the BM were assessed in serial aspirates obtained 3, 6, 10, 20, and 30 weeks later. Circles and triangles indicate female and male mice, respectively. Results are pooled from three experiments with a combined total of six to 10 mice per group. Asterisks indicate statistical significance (* $p \leq 0.05$; ** $p \leq 0.01$).

and outputs are nonlinear, can compromise interpretation of the results and the validity of using this approach to infer input frequency values. The recent and anticipated continuing creation of increasingly permissive recipient mice for such studies of normal and perturbed human xenografts underscores the continuing need to address these issues.

The *Sirpa* polymorphism and *scid* and *IL2rg* mutations of NSG mice contribute to enhancing their reconstitution by transplantations of human hematopoietic cells by restraining multiple components of the host mouse immune

system. Substitution of an inactivating mutation of the *Rag1* or *Rag2* gene for the *scid* mutation results in a recipient that is immunologically similar, but less radiosensitive. Replacement of the *c-kit* gene with a mutant (e.g., *W⁴¹*) allele enables even higher levels of human chimerism to be obtained by impairing the competitiveness of the primitive hematopoietic cells of the recipient. This latter genetic alteration also enhances the output of human erythroid precursors selectively due to the important role of KIT in sustaining erythropoiesis in both mice and humans [28,29]. The ability of an added KIT deficiency to improve the

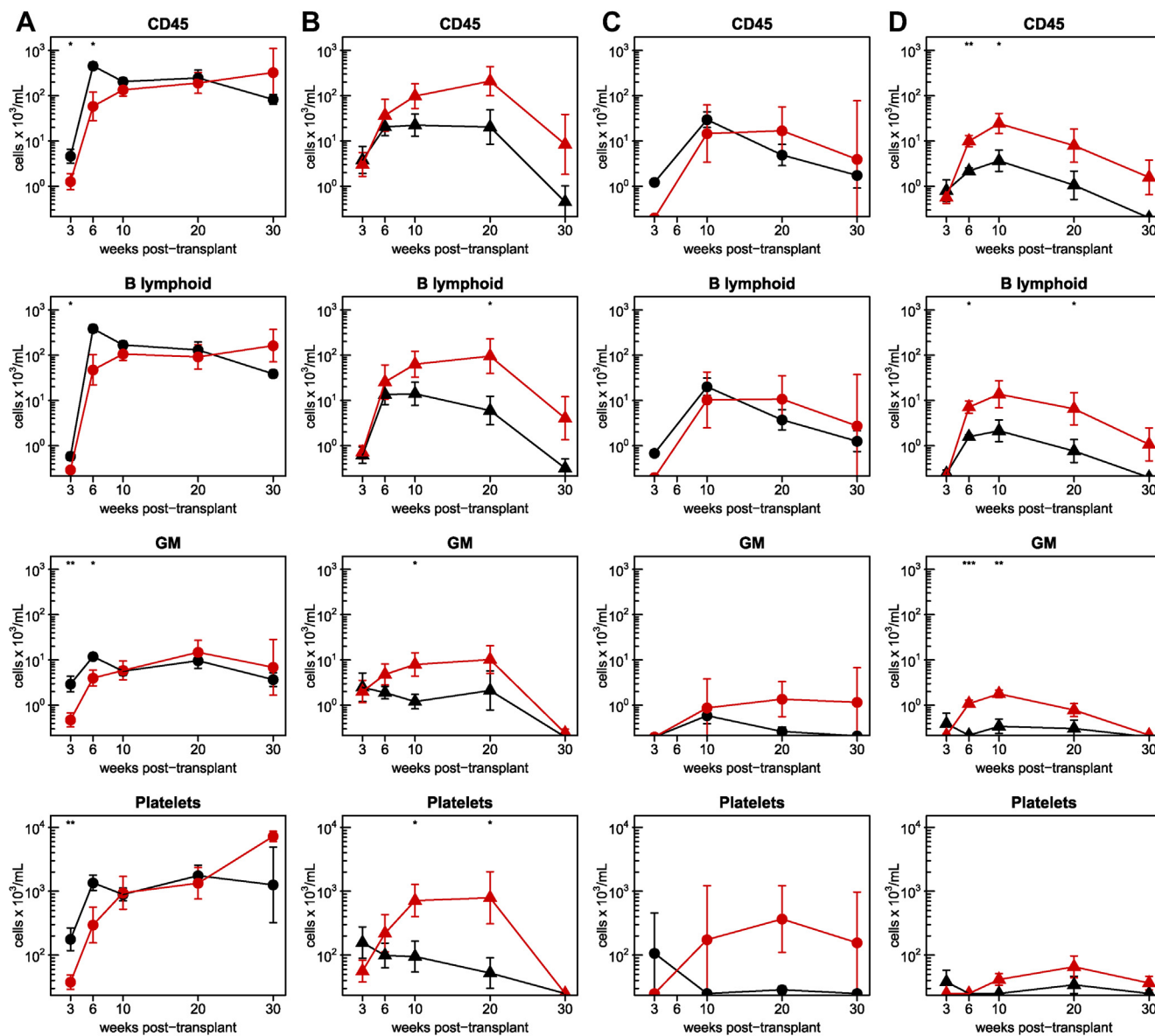


Figure 4. Enhanced human cell numbers in the PB of transplanted SRG-W41 versus NRG mice. Kinetics of human cell numbers in the PB of the irradiated female (A), irradiated male (B), non-irradiated female (C), and non-irradiated male (D) mice described in Figure 3. Results in SRG-W41 mice are again shown by the red symbols and for the NRG mice in black. Numbers of human CD45⁺, GM, and B-lymphoid cells and platelets per milliliter of recipient blood were determined 3, 6, 10, 20, and 30 weeks after transplantation. Circles and triangles indicate female and male mice, respectively. Asterisks indicate statistical significance (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

chimerism obtained in NOD, BALB/c, and mixed NOD-B6 genotypes has been reported recently by several groups [16–18]. Although a number of mutant *c-kit* alleles have been shown to confer this effect, the W^{41} mutation has the advantage of not compromising the fertility of the host strain. Here, we extend the demonstration of its utility when combined with a *Rag1*^{null} instead of a *scid* genotype, to enable less radiotoxic conditioning regimens to be used. This model should thus offer advantages in future studies designed to test the effects of cytotoxic therapies that might not be tolerated in *c-kit*-deficient NSG mice.

A systematic examination of variables historically reported to affect the level, durability, and lineage contributions to the human chimerism obtained in NS and NSG mice showed that concerns with the use of >12-week-old mice as recipients [9] and the need for coinjected human “carrier” cells for transplantations of small cell numbers [10] no longer pertain with NRG mice. In contrast, we found the previously reported superiority of female recipients [15] remains a critical influence in SRG-W41 and NRG recipients, regardless of whether they are irradiated before performing the transplantation, with the most

pronounced effects seen at later time points up to 30 weeks after transplantation.

As predicted by previous studies with NSG-W41 mice [16–18], we found that SRG-W41 mice support greater human chimerism than parental NRG mice, although this difference was masked in the irradiated female recipients studied here by the high chimerism already achieved in the NRG mice by the number of human CD34⁺ CB cells injected. Of particular note was the marked effect obtained in female NRG and SRG-W41 mice in enhancing the number of circulating human platelets. This finding, together with the enhanced human erythropoiesis also seen, confirms the utility of immunodeficient mice with an additional *W⁴¹* mutant genotype for studies of manipulations that cause or might correct the delayed platelet recovery characteristic of many human transplantations. Importantly, all of the experiments reported here made use of expanded F2 progeny generated from NRG × B6-W41 F1 mice selected exclusively for homozygosity for the four allelic differences between these two strains anticipated to be relevant to their utility as improved hosts of human cells. The consistency of the xenograft results obtained using them reinforces previous evidence that the *Sirpa* allele unique to NOD mice accounts for most, if not all, of the permissivity that it contributes [23,30]. Continued backcrossing to obtain more broadly homogeneous genotypes on both NOD and B6 backgrounds is ongoing and will eventually allow this inference to be tested more rigorously.

The extent to which any of the observed recipient sex, genotype, or conditioning regimen-associated differences in chimerism are driven by effects on the initial level of repopulating cell engraftment or stimulation obtained, as opposed to subsequent downstream effects on their output of differentiating cells, remains unresolved. The high frequency of clones derived from injections of highly purified human HSCs [8] argues in favor of the latter, at least for cells with long-term *in vivo* repopulating activity. However, this latter observation does not rule out a contribution from an increased seeding efficiency or initial activation of human cells in an environment where their endogenous mouse counterparts have a reduced responsiveness to the KIT ligand.

In summary, we have analyzed multiple factors that affect the level of repopulation of immunodeficient mice by intravenously transplanted human CD34⁺ CB cells. The results demonstrate variable effects early after transplantation, with maximal and readily detectable levels of human B-lineage cells, GM cells, erythroid precursors, and circulating platelets evident for at least 30 weeks in sublethally irradiated female SRG-W41 mice. These findings should facilitate future investigation of the mechanisms of this enhanced chimerism in both irradiated and unconditioned recipients and show the utility of these mice for improving human cell and gene therapy and in studies of human leukemic cell growth and treatment response.

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P.H.M., G.R., M.M., P.A.B., and L.D.S. assisted in the derivation of the SRG-W41 strains. G.R., with P.H.M. and D.J.H.F.K., performed sex, age, and accessory cell experiments on NRG mice. P.H.M., G.R., P.A.B., D.J.H.F.K., and N.N. optimized transplant analysis protocols. P.H.M., A.M.S.C., and K.D. performed NSG and NRG comparison experiments. P.H.M., R.K.H., and C.J.E. designed the experiments. P.H.M. and C.J.E. wrote the manuscript, which was reviewed and approved by all co-authors.

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