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SURVIVAL EFFICACY OF THE PEGYLATED G-CSFS MAXY-G34 AND NEULASTA IN A MOUSE MODEL OF LETHAL H-ARS, AND RESIDUAL BONE MARROW DAMAGE IN TREATED SURVIVORS

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Abstract

In an effort to expand the worldwide pool of available medical countermeasures (MCM) against radiation, the PEGylated G-CSF (PEG-G-CSF) molecules Neulasta and Maxy-G34, a novel PEG-G-CSF designed for increased half-life and enhanced activity compared to Neulasta, were examined in a murine model of the Hematopoietic Syndrome of the Acute Radiation Syndrome (H-ARS), along with the lead MCM for licensure and stockpiling, G-CSF. Both PEG-G-CSFs were shown to retain significant survival efficacy when administered as a single dose 24hr post-exposure, compared to the 16 daily doses of G-CSF required for survival efficacy. Furthermore, 0.1 mg kg⁻¹ of either PEG-G-CSF effected survival of lethally-irradiated mice that was similar to a 10-fold higher dose. The one dose/low dose administration schedules are attractive attributes of radiation MCM given the logistical challenges of medical care in a mass casualty event. Maxy-G34-treated mice that survived H-ARS were examined for residual bone marrow damage (RBMD) up to 9mo post-exposure. Despite differences in Sca-1 expression and cell cycle position in some hematopoietic progenitor phenotypes, Maxy-G34-treated mice exhibited the same degree of hematopoietic stem cell (HSC) insufficiency as vehicle treated H-ARS survivors in competitive transplantation assays of 150 purified Sca-1+cKit+lin-CD150+ cells. These data suggest that Maxy-G34, at the dose, schedule, and time frame examined, did not mitigate RBMD, but significantly increased survival from H-ARS at one-tenth the dose previously tested, providing strong support for advanced development of Maxy-G34, as well as Neulasta, as MCM against radiation.

Keywords

Blood; bone marrow; mice; radiation damage

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INTRODUCTION

In response to the growing threat of unintended radiation exposure from weapon or energy sources, efforts have been underway worldwide to develop and ultimately license medical countermeasures (MCM) against radiation for eventual stockpiling in case of a catastrophic nuclear event. While low dose exposure of 1–2 Gy or less is generally non-life threatening, exposures in the range of 2–10 Gy affect primarily the hematopoietic system, resulting in the Hematopoietic Syndrome of the Acute Radiation Syndrome (H-ARS). H-ARS is characterized by life-threatening neutropenia and thrombocytopenia, and possible death due to infection and or bleeding. As there are currently no drugs approved to treat severely irradiated personnel, physicians would likely rely on medications used to treat chemo- and radiotherapy-induced myelosuppression, such as hematopoietic growth factors (HGF), antibiotics, and fluids.

Likely HGF to be used after a radiation event include the granulopoietic cytokines granulocyte-colony stimulating factor (G-CSF, Neupogen) and PEGylated-G-CSF (Neulasta). Both molecules stimulate production of disease-fighting neutrophils, which help prevent mortality from opportunistic infections after irradiation. G-CSF has been shown to increase survival of lethally-irradiated animals when administered shortly after exposure (Schuening et al. 1989, Schuening et al. 1993, MacVittie et al. 2005), but must be administered in repeat daily doses for maximum efficacy. A single 1 or 2 mg kg⁻¹ dose of G-CSF 24h after 7–9.5 Gy exposure of mice was not able to improve survival (Neta and Oppenheim 1988, Neta et al. 1988, Uckun et al. 1990). The requirement for repeat dosing limits G-CSF's attractiveness in a radiation accident scenario where casualties will be numerous and medical facilities inundated.

Modification of G-CSF with polyethylene glycol (PEG) produces the molecule PEG-G-CSF, or Neulasta, which possesses a reduced renal clearance and prolonged circulating half-life compared to Neupogen (Abuchowski et al. 1984, Knauf et al. 1988, Molineux et al. 1999, Bailon et al. 2001). PEGylated growth factors, such as Neulasta, have a treatment advantage over non-PEGylated molecules due to their abbreviated dosing schedule, which would be especially advantageous after a nuclear event. Neulasta has demonstrated efficacy in the treatment of drug- and radiation-induced neutropenia in animals (Farese et al. 2012, Farese et al. 2013) and humans (Gabrilove et al. 1988, MacVittie et al. 1990, Crawford et al. 1991, Demetri 1992, Patchen et al. 1992, Bishop et al. 2000), following just one or two administrations.

Maxy-G34 (Maxygen, Inc) is a novel PEGylated G-CSF designed to possess enhanced efficacy compared to Neulasta via unique amino acid substitutions and additional PEG moieties, resulting in reduced receptor binding and increased circulation time. Reduced receptor binding results in reduced neutrophil-mediated elimination of Maxy-G34 compared to G-CSFs with greater receptor affinity, resulting in increased half-life. In normal volunteers, compared to Neulasta, Maxy-G34 was found to possess a 1.32.3-fold increased half-life, 3.7-fold increase in bioavailability, and a 1.6-fold increase in the neutrophil response using 50–70% less dose than Neulasta. In a clinical trial of breast cancer patients with chemotherapy-induced neutropenia, Maxy-G34 showed a 2-fold increased half-life, higher CD34+ cell counts post-nadir, and a lower incidence of febrile neutropenia compared to Neulasta over a range of doses, *some as low as 10% of Neulasta's labeled dose* (Schwartzberg et al. 2009). Half-life and neutrophil response were also superior in rat (Scholz et al. 2009) and non-human primate models (Farese et al. 2008) compared to Neulasta. The increased potency, efficacy, and fewer required doses make Maxy-G34 an attractive candidate MCM for use in the aftermath of a radiation event.

Exposure to radiation and or chemotherapy is known to result in a latent condition termed Residual Bone Marrow Damage (RBMD), whereby radiation-damaged hematopoietic stem cells (HSC) are unable to provide normal hematopoiesis under times of stress. RBMD is believed to be due to decreased self-renewal potential of HSC and induction of HSC senescence (Hellman and Botnick 1977, Botnick et al. 1979, Mauch et al. 1988, Meng et al. 2003, Wang et al. 2006). Many RBMD studies show restoration of peripheral blood cell counts, but a deficit of HSC and HPC function. The authors have recently examined RBMD in mice surviving H-ARS and have documented severely deficient HSC potential in competitive transplantation assays for up to 16 months post-exposure with severe skewing toward the myeloid lineage (Chua et al. 2012). All classes of peripheral blood cells as well as BM cellularity were significantly decreased, especially as H-ARS survivors aged. Given the current environment and threat of terrorist use of radiation, the long term effects and consequences of RBMD in survivors of H-ARS are of increasing concern. As survivors age, the incidence of malignancy and other delayed effects of acute radiation exposure (DEARE) increase, leaving H-ARS survivors particularly vulnerable to treatments resulting in hematopoietic stress, such as chemo- or radio-therapy, in addition to aging itself. Thus, mitigation strategies for not only H-ARS, but also for RBMD, are urgently needed.

Efficacy studies for screening candidate MCM, including Maxy-G34, require the use of relevant, applicable, and practical animal models adhering to the Food and Drug Administration's (FDA) Animal Rule. Efforts in the authors' laboratory to develop a murine model in C57BL/6 mice of H-ARS have recently been published (Plett et al. 2012). This model has been extensively validated through its use to screen multiple candidate radiomitigators, and can be used for Good Laboratory Practices (GLP) experiments, as recommended by the FDA's Animal Rule. The current study uses this murine model to assess the ability of Maxy-G34 to mitigate the acute effects of H-ARS in comparison to a known radiomitigator, Neulasta. In addition, mice surviving H-ARS after treatment with Maxy-G34 were assessed at various times up to 9mo post-exposure to determine whether treatment with Maxy-G34 for H-ARS in youth has any beneficial effect to mitigate the deleterious effects of RBMD in aged survivors of H-ARS.

MATERIALS AND METHODS

Mice

For H-ARS studies, specific pathogen free C57BL/6 mice (50/50 male/female; Jackson Laboratory, Bar Harbor, Maine) were received at 10 weeks of age, an age analogous to a "young adult" human. Weights of mice in the survival studies ranged from 15.0–22.5 g (females) and 20.0–28.0 g (males). Mice were identified by tattoo or ear punch, and or tail marks. Husbandry and health status monitoring were carried out as previously described (Plett et al. 2012). Briefly, mice were housed in microisolator cages and provided with autoclaved acidified water in sipper tubes and wet feed between d4–30 post-irradiation. Irradiated mice were observed twice daily and scored a scale of zero to three for signs meeting the criteria for early euthanasia based on the severity of hunched posture, squinted/closed eyes, and activity (Plett et al. 2012). Mice with scores of eight or nine underwent euthanasia by CO₂ inhalation followed by cervical dislocation (Plett et al. 2012). Overall, 11–67% of dead mice in these studies underwent humane euthanasia, as opposed to being found dead by animal caretakers.

For RBMD studies, mice were fed with regular chow and acidified water. Recipient mice and donors of competitor cells used in competitive transplantation assays were of the congenic P^{trc}aPep3^b/BoyJ (B6.BoyJ; CD45.1+) or the F1 hybrid of C57Bl/6 and B6.BoyJ mice (CD45.2+CD45.1+) strains. These two strains were used interchangeably as transplant recipients and competitor cell donors, and their leukocytes were distinguished from C57Bl/6

cells in transplant recipients by expression of CD45.1. B6.BoyJ and F1 hybrid mice were bred in-house and used between 8–12 wk of age. All studies were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Irradiation and dosimetry

Irradiation and dosimetry were performed as previously described (Plett et al. 2012). Mice for H-ARS studies were exposed in single chambers of a Plexiglas irradiation apparatus to single uniform total body irradiation (TBI) doses ranging from 7.76 to 9.04 Gy gamma radiation from a ^{137}Cs radiation source at (GammaCell 40; Nordion International, Kanata, Ontario, Canada; $0.60\text{--}0.66\text{Gy min}^{-1}$). Annual calibration of the radiation dose-lethality relationship in the authors' laboratory resulted in slightly different LDXX/30 doses over the time period that the studies were conducted, as shown in Table 1.

Complete blood counts (CBC)

Mice were tail-bled and blood was analyzed for complete blood count (CBC) values using a validated HEMAVET® 950FS Hematology System (Drew Scientific, Waterbury, CT) at least 10min after collection but within 24h.

Maxy-G34 manufacture

Maxy-G34 was engineered to contain 5 amino acid substitutions of the native human G-CSF protein and 3 polyethylene glycol (PEG) conjugations at unique sites. Maxy-G34 was selected by screening approximately 100 candidates to minimize G-CSF receptor-mediated clearance and renal excretion. The novel protein sequence and PEGylation pattern of Maxy-G34 resulted in reduced binding affinity for the G-CSF receptor and receptor-mediated internalization (unpublished data).

Neupogen, Neulasta and Maxy-G34 administration

The radiomitigators were administered subcutaneously post-TBI as described below. Control groups were similarly injected but with vehicle specified below.

Neupogen—One administration schedule was tested, 125 ug kg^{-1} , one dose administered at $24\pm 4\text{ h}$ post-TBI, then daily from d2 to d16 for a total of 16 doses. Vehicle: 0.9% saline (w/v) + 0.1% bovine serum albumin (BSA) solution, or 5% (D)-Glucose solution.

Neulasta—Two different administration schedules were tested, 1) 0.1 mg kg^{-1} , 0.3 mg kg^{-1} , or 1.0 mg kg^{-1} , one dose of each concentration administered to separate groups at $24\pm 4\text{ h}$ post-TBI, or 2) 1.0 mg kg^{-1} , two doses administered at $24\pm 4\text{ h}$ and d7 post-TBI. Vehicle: 0.9% saline (w/v) + 0.1% BSA solution. The 1 mg kg^{-1} dose of Neulasta used in this study was chosen based on doses used in chemotherapy myelosuppression models (Molineux et al. 1999, Lord et al. 2001).

Maxy-G34—Three different administration schedules were tested, 1) 0.1 mg kg^{-1} , 0.3 mg kg^{-1} , or 1.0 mg kg^{-1} , one dose of each concentration administered to separate groups at $24\pm 4\text{ h}$ post-TBI, or 2) 1.0 mg kg^{-1} , two doses administered at $24\pm 4\text{ h}$ and d7–8 post-TBI, or 3) 1.0 mg kg^{-1} , three doses administered at $24\pm 4\text{ h}$ and d7–8 and d14–15 post-TBI. Vehicle: Sodium acetate (10 mM), mannitol (45 mg mL^{-1}), polysorbate 20 (0.05 mg mL^{-1}), pH 4.0, solution. The doses of Maxy-G34 used in this study were chosen to be the same as doses of Neulasta.

For RBMD studies: two different Maxy-G34 administration schedules were used, 1) 1.0 mg kg^{-1} , one dose at $24\pm 4\text{ h}$ post-TBI, or 2) 1.0 mg kg^{-1} , two doses at $24\pm 4\text{ h}$ and d8 post-TBI.

Vehicle: Sodium acetate (10 mM), mannitol (45 mg mL⁻¹), polysorbate 20 (0.05 mg mL⁻¹), pH 4.0, solution.

Residual bone marrow damage studies

Mice treated with one or two doses of 1.0 mg kg⁻¹ Maxy-G34 or vehicle in experiment 11.215, 8.29 Gy (Table 1), and which survived H-ARS, were assessed at 4 and 6 months (2 doses of Maxy-G34), and 7 and 9 months post-TBI (1 dose of Maxy-G34), along with non-TBI age-matched controls. Survival of mice at 30 days post-TBI (Table 1) was the same as that at 4 months post-TBI, the first time point analyzed in the RBMD studies. At each time point, mice were assessed for body weight, CBC, bone marrow (BM) cellularity, BM hematopoietic progenitor number, lymphoid progenitor number, primitive hematopoietic progenitor phenotypes, cell cycle analyses, and HSC multilineage reconstitution potential in competitive transplantation assays, all as previously described (Chua et al. 2012). Flow cytometry analyses of primitive hematopoietic progenitor populations analyzed Sca-1-bright cells, in addition to total Sca-1+ cells analyzed in previous studies Chua et al. 2012). Sca-1-bright cells represent a more primitive hematopoietic population than total Sca-1 cells. Data generated from either analysis did not differ in trend, so only Sca-1-bright analyses are presented in Results.

Study design, sample size, & statistical analyses

Survival studies—Each cage was randomized to a radiation exposure dose and individual mice were randomized to treatment groups by a study statistician. Studies testing MCM as mitigators were powered to detect a 30% reduction in mortality (i.e. 70% to 40%, 90% to 60%) with 80% power using a two-tailed 5% significance level. The primary outcome, 30-day survival, was examined using logistic regression, which also included sex, radiation dose and interactions of treatment with dose and gender to examine differential effects. Since the randomization was by mouse, “cage effects”, if present, did not bias the results but the model was adjusted using a Generalized Estimating Equations (GEE) method. Cage effects (differences between cages on the same treatment) can occur as a result of infection, or differences in environment due to cage position. Secondary analysis included time to death (overall survival), and was examined using a Cox proportional hazards regression model, which was analogous to the logistic regression and included treatment, gender and radiation dose. Mean survival Time (MST) of decedent mice was performed using analysis of variance including radiation dose as a factor.

Individual survival studies were conducted using 1–3 radiation doses and results from each were analyzed within each study for survival. For ease of presentation, results are presented within study in Table 1, but combined across radiation doses in the figures. Studies were powered to test for the main effect across all irradiation dose groups in the statistical model adjusted for dose. The statistical analysis included tests for interactions between the radiation dose and treatment for survival. If a significant interaction (indicating a differential treatment effect for the two doses) was detected, the dose groups were analyzed separately. The interactions between radiation dose and treatment for survival were not observed in any these studies and therefore the results for overall tests are presented.

RBMD studies—The Student’s unpaired t-test was used to compare the following parameters among TBI-cont, TBI-G34 and non-TBI: weight, CBC parameters, BM cellularity, percent and number per mouse of BM primitive hematopoietic cells, progenitor content per mouse, donor chimerism and %G0G1 of lin-, KSL and KSLCD150+ cells.

RESULTS

Survival of irradiated mice after administration of G-CSF, Neulasta, or Maxy-G34

The authors have previously published significantly increased survival of lethally-irradiated mice treated with 16 daily doses of 125 ug kg⁻¹ Neupogen in their murine model of H-ARS (Plett et al. 2012). They now show pooled data from 9 similar studies where a total of 212 lethally-irradiated mice were injected with 16 daily doses of 125 ug kg⁻¹ Neupogen and monitored for 30d survival. Neupogen treated mice showed significantly increased 30d survival (80.2%) compared to vehicle-treated mice (51.6%, $p=0.02$, Fig. 1a), further validating the use of this murine model of H-ARS to test candidate MCM and the strong survival efficacy of Neupogen in H-ARS.

The use of PEGylated HGF has favorable logistical advantages in the aftermath of a nuclear event since fewer injections are required for efficacy. To investigate efficacy of PEGylated granulopoietic HGF in our murine H-ARS model, groups of 20–30 C57BL/6 mice were exposed to lethal TBI with gamma radiation as previously described (Plett et al. 2012) and treated with either Maxy-G34 or Neulasta. One dose of 1.0 mg kg⁻¹ (24 ± 4 hr post-TBI) of Neulasta was found to be equally effective at increasing 30d survival as two doses of the same concentration, given at 24 ± 4 hr and on d7 post-TBI ($p=0.003$ and $p=0.004$, respectively, Figure 1b), suggesting the possibility that one administration might be sufficient for enhancing survival after a radiation event.

Likewise, different administration schedules of 1.0 mg kg⁻¹ Maxy-G34 were examined for survival efficacy: one dose at 24 ± 4 hr post-TBI, two doses at 24 ± 4 hr and d7 or 8 post-TBI, and three doses at 24 ± 4 hr, d7 or 8, and d14 or 15 post-TBI. Each administration schedule was examined in three to five separate studies for a total of eleven studies. Similar to Neulasta, each administration schedule of Maxy-G34 significantly increased 30d survival of treated mice compared to vehicle treated mice (Figure 1c), suggesting that this novel PEG-G-CSF may also provide sufficient survival efficacy in a one dose administration schedule.

Evident in these results was the variability in control group survival. The high survival in the control groups was an unintended consequence of the steep radiation dose response curve observed in inbred strains of mice (such as C57BL/6 used in these studies) and discussed in the authors' previous publication (Plett et al. 2012). The very steep curves mean that confidence intervals around the calculated LDXX/30 radiation doses span a larger fraction of the curve, thus pinpointing the LDXX/30 in subsequent experiments becomes difficult. Table 1 shows the intended and actual LDXX/30 in all experiments in Figures 1 and 2.

Mean survival time (MST) of decedent mice was significantly decreased in Neupogen-treated mice, compared to vehicle controls (14.3 and 17.6 days, respectively, $p<0.001$, Table 2). The shortened MST in the Neupogen-treated mice reflects the finding that onset of death in each group occurs at approximately the same time (day 9 in this study), but deaths in vehicle-treated mice continued to day 29, while the last death in the Neupogen group was on day 17. Since MST is calculated only from decedent mice, MST of groups where mice continue to die until late time points will be increased.

While the MST in the Neulasta group was slightly decreased compared to controls, data could not be analyzed statistically due to the fact that only 1 mouse died in each of the Neulasta groups (Table 2).

Similar to Neupogen, a significantly decreased MST was observed in the Maxy-G-34-treated mice compared to vehicle controls (13.3 and 19.9 days, respectively, combining all

Maxy-G34-treated and all vehicle-treated mice, $p=0.001$, Table 2). In these studies, as with Neupogen, lethality in each group commenced on the same day (day 8), but ended earlier in the Maxy-G34-treated mice (day 17) than in controls (day 28).

Dose reduction of Maxy-G34 and Neulasta with retention of survival efficacy

To investigate whether doses lower than 1.0 mg kg^{-1} of the PEGylated-G-CSF molecules Maxy-G34 and Neulasta retain survival efficacy in lethally irradiated animals and therefore might be safer if administered to presumably healthy individuals in the aftermath of a nuclear event, 1.0, 0.3, and 0.1 mg kg^{-1} of each MCM were evaluated in the murine H-ARS model for survival efficacy. Remarkably, the lowest dose assayed of both Maxy-G34 and Neulasta provided 30d survival efficacy that was similar to a 10-fold higher dose of 1.0 mg kg^{-1} , and the 3-fold higher dose of 0.3 mg kg^{-1} (Figure 2, $p<0.001$ for all groups versus control). MST, unlike that in the previous Maxy-G34 studies discussed in Fig 1, were not statistically different between Maxy-G34- or Neulasta-treated mice compared to vehicle controls (Table 3).

These data suggest that doses lower than those currently proposed for use in lethally-irradiated personnel may provide sufficient survival efficacy, thereby reducing the potential for harmful side effects in people not at risk for neutropenia, as well as treatment costs.

Residual bone marrow damage in survivors of H-ARS treated with Maxy-G34

Exposure to radiation and or chemotherapy is known to result in a latent condition termed Residual Bone Marrow Damage (RBMD), whereby radiation-damaged HSC are unable to provide normal hematopoiesis under times of stress. The authors have examined RBMD for up to 2 years post-TBI in mice surviving H-ARS and have documented severe deficiencies in all classes of hematopoietic cells, especially as mice aged (Chua et al. 2012). Given the current environment and threat of terrorist use of radiation, the long term effects and consequences of RBMD in survivors of H-ARS are of increasing concern. In this regard, it is important to investigate whether mitigators that successfully increase survival from H-ARS, also mitigate some of the deleterious effects of RBMD in aged survivors.

To this end, mice from experiment 11.215, 8.29 Gy in Table 1 that were lethally irradiated, treated with one dose (on d1) or two doses (on d1 and 8 post-TBI) of 1.0 mg kg^{-1} Maxy-G34, and survived H-ARS, were assessed for RBMD as previously described (Chua et al. 2012). Such mice are referred to hereafter as “TBI-G34”. Survivors among vehicle treated control mice (referred to as “TBI-cont”), as well as non-irradiated age-matched controls (referred to as “non-TBI” mice) were also assessed for RBMD. Mice treated with two doses of Maxy-G34 were assessed at 4 and 6mo post-TBI, while mice treated with one dose of Maxy-G34 were assessed at 7 and 9mo post-TBI.

Body weights

Body weights of both groups of TBI mice at 4, 6, 7, and 9 mo post-TBI, as a whole, were significantly less than non-TBI mice ($p<0.01$, data not shown), similar to the authors' previously reported findings for TBI-cont mice (Chua et al. 2012). Fur color of both groups of TBI mice was also lighter than that of non-TBI mice, as previously reported (Chua et al. 2012).

CBC

Of interest, neutrophil counts in TBI-G34 mice were significantly higher than those in TBI-cont mice at 4mo post-exposure (Fig. 3b line graph and inset bar graph). Similarly, percentage of neutrophils was significantly higher in TBI-G34 mice at 4mo compared to non-TBI mice ($31.1 \pm 4.2\%$ versus $16.3 \pm 2.9\%$, respectively, $p=0.029$). These data illustrate

the persistence of the enhancing effect of Maxy-G34 on neutrophil recovery to as long as 4mo post-exposure. Neutrophil counts in all three groups of mice were similar at time points beyond 4mo (months 6, 7, and 9 post-TBI, Fig. 3b), although overall percentages of neutrophils were significantly increased in both TBI-cont and TBI-G34 compared to non-TBI mice (overall means $\pm$ SEMs: $34.4 \pm 4.4\%$, $32.2 \pm 2.8\%$, and $24.0 \pm 1.8\%$, respectively, $p=0.011$ for each comparison), similar to previously reported findings for TBI-cont mice (Chua et al. 2012).

The percentage of lymphocytes was significantly less in TBI-cont and TBI-G34 mice over time as compared to non-TBI mice (overall means $\pm$ SEMs: $61.5 \pm 4.5\%$, $63.8 \pm 2.6\%$, and $71.7 \pm 1.7\%$, respectively, $p=0.011$ for each comparison), illustrating the significant impact that radiation exposure poses on the immune system.

Differences in CBC parameters other than neutrophils at 4mo among the two TBI groups and non-TBI mice were not consistent or apparent (Fig. 3). These data are not surprising given the authors' previous findings that CBC parameters of TBI mice appeared similar to those of non-TBI mice up to approximately 9–10mo post-exposure, but tended to decrease at time points beyond 10mo as the mice aged (Chua et al. 2012).

BM cellularity and primitive hematopoietic phenotypes

Overall BM cellularity in both groups of TBI mice was significantly less than that in non-TBI mice by 30–70% of non-TBI values ($p<0.05$), with no apparent effects of Maxy-G34 treatment (Fig. 4a). Similar to the authors' previous report (Chua et al. 2012), the percentage of lineage-negative (lin-) cells was generally higher in both TBI groups compared to non-TBI mice, but because BM cellularity was lower in TBI mice, absolute numbers of lin- cells were similar between all three groups (data not shown).

The authors' previous data demonstrated significantly decreased percentage and number of primitive BM KSL cells in TBI mice compared to non-TBI (Chua et al. 2012), consistent with data reported herein (Fig. 4d and 4e). Of interest in the current study is that administration of Maxy-G34 as a radiomitigator for H-ARS appears to have effected a further decrease in the percentage and absolute numbers of primitive BM HPC populations. The overall percentage of Sca-1-bright+ cells, Sca-1-bright+ cKit+ cells, Sca-1-bright+ cKit+ lin-, and absolute numbers of Sca-1-bright+ cKit+ lin-, were all significantly decreased in TBI-G34 mice compared to TBI-cont and or non-TBI mice (Fig. 4b, 4c, 4d, and 4e, respectively). Expression of cKit on lin- cells did not differ in any of the three groups (data not shown).

Similar to the authors' and others' data (Simonet et al. 2009), expression of CD150 on KSL cells from both groups of TBI mice was significantly increased compared to non-TBI mice (Fig. 4g, $p=0.001$), while absolute numbers of KSLCD150+ cells were similar (due to lower numbers of KSL in TBI mice, Fig. 4h).

BM hematopoietic progenitor cells (HPC) and pre-B lymphoid colonies

Absolute numbers of the BM hematopoietic progenitors CFU-GM, BFU-E, CFU-GEMM, and pre-B cell progenitors were all found to be significantly decreased in both groups of TBI mice compared to non-TBI mice (Figs. 5a–d), similar to the authors previous findings (Chua et al. 2012). There were no significant differences in progenitors from TBI-G34 versus TBI-cont mice.

Competitive transplantation and lineage reconstitution

The long term engraftment potential of 150 purified, sorted KSLCD150+ donor cells derived from Maxy-G34 or vehicle treated TBI mice was determined at 4, 6, 7, or 9 mo post-TBI in a competitive repopulation assay along with similar cells from non-TBI mice. Fig. 6 shows donor-derived chimerism at 6 mo post-transplant for a total of 38 recipients of TBI-cont cells, 30 recipients of TBI-G34 cells, and 36 recipients of non-TBI cells. Evident from the figure is that chimerism for both groups of TBI mice was severely depressed compared to non-TBI mice at every time point analyzed post-TBI, similar to the authors' previous findings with TBI-cont KSLCD150+ cells (Chua et al. 2012).

Recipients with at least 0.05% donor chimerism at 6mo post-transplant were further analyzed for multilineage reconstitution of peripheral blood CD8+ T cells, CD4+ T cells, B220+ B cells, and Gr-1+ granulocytes by flow cytometry. As previously described for TBI-cont mice (Chua et al. 2012), recipients of KSLCD150+ cells isolated from both groups of TBI mice were deficient in B-cell reconstitution with skewing toward the myeloid lineage at every time point analyzed (Fig. 7a and 7b) compared to recipients of similar cells from non-TBI mice (Fig. 7c), or competitor cells co-transplanted with any of the test groups of cells (Fig. 7d, 7e, and 7f). Reconstitution of CD8+ T-cells did not differ among groups, while reconstitution of CD4+ cells was significantly less in TBI-G34 mice compared to non-TBI (Fig. 7b and 7c).

Cell cycle analysis

One of the hallmarks of HSC is their high degree of mitotic quiescence, which is believed to be essential for life long hematopoiesis, self-renewal, differentiation, and efficient homing to their BM microenvironment following transplantation (Verfaillie and Miller 1995, Peters et al. 1996, Traycoff et al. 1996, Ladd et al. 1997, Nilsson et al. 1997, Gothot et al. 1998, Habibian et al. 1998). Data in Figs. 6 and 7 show deficient long term repopulating ability of candidate HSC isolated from aged mice that survived the lethal effects of H-ARS after treatment with Maxy-G34, as well as few mice that survived without treatment. Data in Fig. 8a–c show significantly increased cell cycling of primitive lin-, KSL and KSLCD150+ from TBI-cont mice compared to similar cells from non-TBI mice, consistent with the authors' previous findings (Chua et al. 2012). Despite a few time points where the %G0/G1 of these phenotypes from TBI-G34 mice were similar to those from non-TBI mice, in general there was no consistent effect of Maxy-G34 administration months prior on cell cycling during the observed 4–9 months post-TBI time frame.

DISCUSSION

This paper investigates survival efficacy, effective dose levels, and administration schedules of three granulopoietic growth factors of potential value in a radiation/nuclear event for treatment of personnel suffering from H-ARS. G-CSF (Neupogen) and the PEG-G-CSF molecules Neulasta and Maxy-G34, all significantly increased 30d survival from lethal radiation in a mouse model of H-ARS. Of these, G-CSF is currently stockpiled for use in case of a radiation event. G-CSF has proven survival efficacy in several animal models of H-ARS including canine (Schuening et al. 1989, Schuening et al. 1993, MacVittie et al. 2005), mini-pig (Moroni et al. 2013), non-human primate (Farese et al. 1996, Drouet et al. 2008, Farese et al. 2013), in addition to mouse (Patchen et al. 1990, Patchen et al. 1992, Patchen et al. 1993, Patchen and MacVittie 1994, Patchen 1995), and is the lead candidate MCM for licensure under the FDA's Animal Rule. The authors have shown that survival efficacy of G-CSF in murine H-ARS is strongly linked to the secondary parameter of neutrophil recovery (Plett et al. 2012), providing a plausible mechanism of action for this MCM.

The relatively short half-life of G-CSF requires that it be administered as repeat daily injections for maximum survival efficacy (Neta and Oppenheim 1988, Neta et al. 1988, Uckun et al. 1990). To circumvent this apparent disadvantage for a mass causality event, efforts have been underway in the authors' and others laboratories (Dainiak et al. 2003, Herodin et al. 2007, Drouet et al. 2008) to investigate survival efficacy of longer acting granulopoietic growth factors. The authors have shown enhanced neutrophil recovery in a radiation-induced myelosuppression model in rhesus macaques using the PEGylated form of G-CSF, Neulasta (Farese et al. 2012). In the current study, Neulasta was shown to provide enhanced survival in a *lethal* radiation model of H-ARS in mice. Of particular importance, one dose of Neulasta given 24h post-exposure was shown to be as efficacious as 2 doses given on days 1 and 7, a dosing schedule with obvious advantages in the aftermath of a radiation event.

In efforts to expand the pool of MCM worldwide to avoid shortage in the event of widespread need, other pegylated G-CSF are being evaluated for survival efficacy in H-ARS. Of these, Maxy-G34 (Maxygen, Inc) is a unique PEG-G-CSF that has been specifically designed to provide prolonged activity. Maxy-G34 has been shown in both normal volunteers and cancer patients to possess a longer half-life and more potent hematopoietic effects than Neulasta, at lower doses than those recommended for Neulasta (Schwartzberg et al. 2009). Similar results have been found in animal models (Farese et al. 2008, Scholz et al. 2009). In the current study, 1 dose of Maxy-G34 was found to be as effective as 2 or 3 doses to significantly increase 30d survival in a mouse model of H-ARS, similar to Neulasta. Given its favorable dosing schedule, improved leukocyte response (Schwartzberg et al. 2009), and longer half-life compared to Neulasta, Maxy-G34 is an extremely attractive candidate for licensure and stockpiling in preparation for a possible radiation event.

Optimal use of PEGylated HGF in the aftermath of a nuclear event would be targeted to those whose level of exposure places them at risk for life-threatening neutropenia and in need of HGF treatment. At least two challenges exist to such optimal delivery of treatment. First, safe treatment with such potent MCM relies on timely and accurate biodosimetry to identify those victims who face the greatest risk. Current biodosimetric methods are time-consuming and imprecise, and may not provide accurate biological dose-estimations within a logistically- or medically-feasible time frame. Second, the so-called "worried-well" may want medical treatment that may not be warranted or in their best interest. Treatment of healthy individuals with PEGylated HGF exposes them to the known side effects of the drug and is difficult to "undo" due to the long half-life imparted by the PEG moiety. A potential solution to this dilemma would be to administer lower doses of PEGylated MCM that retain survival efficacy in lethally-irradiated personnel, but would be less dangerous if given to healthy, non-irradiated people, since side effects would be presumably less severe and subside more rapidly. The data presented herein show equal survival efficacy of Maxy-G34 and Neulasta at *one-tenth* the dose previously tested in murine models of H-ARS. These data suggest that lower doses of PEG-G-CSF than those previously thought to be required for survival efficacy may be used, which would likely limit the harmful effects if administered to non-irradiated personnel.

The dose of Neulasta used in this study was selected based on previous studies modeling bone marrow myelosuppression in mice treated with 150 mg kg⁻¹ 5-FU (Molineux et al. 1999), or 200 mg kg⁻¹ cyclophosphamide plus 90 mg kg⁻¹ temozolomide (Lord et al. 2001). Molineux et. al. (Molineux et al. 1999) showed that 1 mg kg⁻¹ Neulasta was more effective than 0.5 mg kg⁻¹ Neulasta or 5 daily doses of 300 ug kg⁻¹ Neupogen at reducing both the depth and duration of neutropenia in the myelosuppressed mice. Lord et. al. showed that 1 mg kg⁻¹ Neulasta was as effective as 4 daily doses of 125 ug kg⁻¹ Neupogen at inducing

granulopoiesis (Lord et al. 2001). Doses of Neulasta used in the Molineux study (Molineux et al. 1999) were chosen by the authors to provide the equivalent amount of G-CSF protein only; the molecular weight of the PEG moiety was factored into the calculations.

Radiation-induced neutropenia results in loss of the “neutrophil sink” as a clearance mechanism of G-CSF, which may contribute to the observed enhanced efficacy of lower doses of these two PEG-G-CSF molecules through increased PK in irradiated mice. At lower radiation doses than those used herein, or where a portion of the mouse is shielded during exposure, some neutrophils may remain or at least be regenerated more quickly. These neutrophils will absorb the PEG-G-CSF molecules, remove them from circulation, resulting in shortened half-life compared to the longer half-life observed during absolute neutropenia. In the current study, such a situation may have a larger impact on the half-life of Neulasta than Maxy-G34, since Maxy-G34 is designed to have low receptor affinity, thus its half-life would not be influenced by circulating neutrophils to the same degree as the half-life of Neulasta. It is tempting to speculate that in such a scenario, efficacy of low doses of Maxy-G34 may be greater than low doses of Neulasta, since half-life of Maxy-G34 would be longer. Future studies are warranted to investigate this possibility as current modeling studies predict that a considerable percentage of the population would experience partial marrow shielding during a radiation event, allowing survival of neutrophils that would contribute to clearance of G-CSF MCM.

G-CSF treatment has been associated with secondary malignancy in diseases such as ALL (Relling et al. 2003), breast cancer (Smith et al. 2003), and severe congenital neutropenia (Rosenberg et al. 2006, Germeshausen et al. 2007, Touw and Bontenbal 2007), raising concern that use of granulopoietic cytokines in irradiated personnel may also result in increased incidence of secondary malignancy later in life. An additional attractive feature of using lower doses of PEG-G-CSF MCM during a mass casualty event is that lower doses may reduce the possibility of treatment-associated secondary malignancy. To date, the incidence of malignancy in the current studies does not appear to be increased in mice treated with Maxy-G34 compared to vehicle treated mice (data not shown), but long term monitoring of H-ARS survivors and larger group sizes are necessary to adequately investigate risk of malignancy. Such studies were beyond the scope of this investigation. Additional studies are required to examine whether a further dose reduction is possible, while maintaining survival efficacy.

The authors have previously described residual bone marrow damage (RBMD) in murine survivors of H-ARS up to 20mo post-TBI [(Chua et al. 2012) and unpublished observations]. In those studies, CBC of TBI survivors were comparable to those of non-TBI age-matched controls up to approximately 10mo of age, but became significantly decreased at later time points. HSC and HPC were significantly decreased in TBI mice at every time point analyzed up to 20mo post-TBI [(Chua et al. 2012) and unpublished observations]. Clearly, survival from H-ARS will be met with a myriad of chronic afflictions later in life, including RBMD, and mitigation strategies against the delayed effects of acute radiation exposure (DEARE) must be actively pursued. To this end, the authors compared RBMD in mice surviving H-ARS after treatment with 1.0 mg kg⁻¹ Maxy-G34 (TBI-G34), vehicle (TBI-cont), and non-TBI controls to examine whether Maxy-G34 treatment for H-ARS also mitigated of the some of the deleterious effects of RBMD.

Treatment with Maxy-G34 for H-ARS resulted in significantly increased neutrophils at 4mo post-TBI. The authors have previously shown that significantly increased survival from H-ARS is linked to enhanced recovery of neutrophils in G-CSF-treated mice (Plett et al. 2012). The current data suggest that significant increases in neutrophil levels of mice treated with granulopoietic cytokines may persist to 4mo post-TBI, or alternatively, that the longer half-

life of Maxy-G34 exerts neutrophilic effects beyond the 30d time frame of H-ARS that may not be seen with shorter-acting G-CSF. Studies comparing neutrophil recovery beyond 30d in H-ARS after treatment with either G-CSF or Maxy-G34 are needed to investigate any potential differences between the two MCM in long-term neutrophil reconstitution.

Of particular interest in the current study is the overall significant decrease in lineage-negative BM cells expressing Sca-1 in mice treated with Maxy-G34. The consequence, if any, of the observed decrease in Sca-1 expression for hematopoiesis and or RBMD in Maxy-G34-treated mice is unknown. In fact, the function of Sca-1 in hematopoiesis is largely unknown, and the existence of a ligand for the Sca-1 receptor remains an enigma. Sca-1 is expressed on all classes of murine HSC and HPC, and mechanisms for its up- and down-regulation are unclear (Kondo et al. 1997, Akashi et al. 2000). In mice and normal humans, G-CSF administration has been shown to increase plasma levels of TNF-alpha and TGF-beta, which in turn resulted in decreased Sca-1 expression on myoblasts and T cells (Long et al., Xu et al. 1996, Hirayama et al. 2002). Decreased Sca-1 expression in these studies was, however, reversible and only apparent during the time frame that G-CSF was present, and thus cannot directly explain our findings of decreased Sca-1 expression 9 mo after a single injection of Maxy-G34.

Other studies suggest a role for Sca-1 in HSC self-renewal and lineage determination (Ito et al. 2003, Bradfute et al. 2005), two key characteristics of HSC. Data in the current paper show no difference in either parameter between TBI-G34 and TBI-cont mice. Given the authors' previous findings that hematopoietic deficiencies in H-ARS survivors were most pronounced beyond 10mo of age (Chua et al. 2012), it is possible that differences between TBI-G34 and TBI-cont mice may be more apparent at later time points, when the stress of RBMD is coupled with that of aging. Whether Sca-1 expression in other organ systems (bone, muscle, kidney, brain, etc.) (Holmes and Stanford 2007) is similarly affected by prior Maxy-G34 administration, is unknown, but of potential importance in the context of DEARE.

The decreased expression of Sca-1 resulted in significantly decreased percentage and absolute numbers of KSL cells in TBI-G34 mice compared to TBI-cont. However, due to the radiation-induced increased expression of CD150 on KSL (Simonnet et al. 2009, Chua et al. 2012), absolute numbers of KSLCD150+ cells were similar among all three groups, consistent with the authors' previous report (Chua et al. 2012).

Competitive multilineage repopulating potential, the only true test of HSC, of KSLCD150+ cells purified from both groups of TBI mice was significantly decreased at every time point analyzed compared to similar cells from non-TBI mice. Both groups of TBI cells provided predominantly myeloid reconstitution and deficient lymphoid reconstitution, similar to the authors' previous findings (Chua et al. 2012) for TBI-cont mice. These data illustrate the severity of damage in HSC and the hematopoietic system in survivors of H-ARS, and inability of Maxy-G34, albeit its successful mitigation of H-ARS, to enhance recovery from RBMD.

It is possible, as previously suggested for Neupogen in myelosuppressive protocols (van Os et al. 2000), that Maxy-G34 stimulated proliferation and differentiation of the few remaining HSC following exposure to lethal radiation at the expense of self renewal, resulting in the manifestations of RBMD. This is unlikely since the cell cycle status of some classes of HPC in TBI-G34 mice were not significantly different overall from non-TBI mice, while such cells from TBI-cont mice were more actively cycling. The less-damaged marrow and higher number of HPC/HSC remaining after the myelosuppressive protocol used in the van Os

study compared to the severe BM damage after TBI myeloablation, are key differences that may impact the effects of granulopoietic cytokines on primitive HPC function.

The similar cell cycle status of TBI-G34 and non-TBI primitive HPC was most apparent at 4 and 6mo post-TBI in lin-cells. It is curious that changes in Sca-1 expression in TBI-G34 mice were also most apparent in the lin- population. The biological significance, if any, of these findings is unknown. Given the strong association of mitotic quiescence with HSC function, further investigation of these findings may be warranted since the possibility exists that they may signify a potential mechanism to restore quiescence in HSC from H-ARS survivors.

It is possible that HSC in H-ARS survivors are different phenotypically and/or reside in a different location than those from steady state marrow, and were missed in the current analyses. Given the ability of granulopoietic cytokines to mobilize HSC from the marrow, coupled with a radiation-damaged HSC niche, the possibility of extramedullary hematopoiesis in TBI-G34 mice is worth investigation. The low levels of chimerism (<10%) from both groups of TBI mice may have meant that the assay was near the lower limit of detection, and that detecting differences in the negligible HSC potential between the two groups of H-ARS survivors would not have been possible. However, the significance of determining small differences in HSC potential, when levels are so low regardless, is questionable.

Transplantation of purified HSC phenotypes in this study, rather than unfractionated bone marrow, allowed differentiation between radiation-induced deficits in *quality* versus *quantity* of HSC. The authors' finding of lower chimerism from both groups of TBI KSLCD150+ cells indicates functional damage to HSC (i.e. reduced quality). Had unfractionated cells been transplanted instead of purified HSC phenotypes, it would have not been possible to distinguish between lower chimerism levels due to reduced numbers of HSC (quantity) or reduced function (quality), but would have allowed detection of HSC in the event that the phenotype had changed in H-ARS survivors.

CONCLUSIONS

Collectively, these data document significantly increased 30 day survival in a murine model of H-ARS of three candidate radiomitigators, G-CSF (Neupogen), PEG-G-CSF (Neulasta), and the novel PEG-G-CSF, Maxy-G34, which possesses increased half-life and enhanced hematopoietic activity compared to Neulasta. Both PEG-G-CSFs were shown to retain significant survival efficacy when administered as a single dose, and at one-tenth the dose previously used in survival studies, favorable characteristics for use in a mass casualty event. Mice surviving from H-ARS were examined for RBMD up to 9mo post-exposure. Despite differences in Sca-1 expression and cell cycle position in some hematopoietic phenotypes, TBI-G34 mice possessed the same degree of hematopoietic insufficiency as vehicle treated TBI-cont mice in competitive transplantation assays. These data suggest that Maxy-G34, at the dose, schedule, and time frame examined, did not mitigate RBMD, but significantly increased survival from H-ARS at one-tenth the dose previously tested, providing strong support for advanced development of Maxy-G34 as a MCM against radiation.

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Fig. 1.

Survival of lethally-irradiated mice after Neupogen, Neulasta, or Maxy-G34 administration. Fig 1a: mice were exposed to a single dose of radiation between 7.76–9.04 Gy and injected subcutaneously with one dose of 125 ug kg⁻¹ Neupogen (G-CSF) at 24 ± 4h, then daily to d16 (filled bars). Control mice were similarly injected but with vehicle (open bars). Figure 1a includes data previously published by the authors (Plett et al. 2012). Fig 1b: mice were exposed to a single dose of radiation (7.76 or 7.96 Gy) and injected subcutaneously with one dose of 1.0 mg kg⁻¹ Neulasta (PEG-G-CSF) at 24 ± 4h, or two doses at 24 ± 4h and d7 post irradiation (filled bars); control mice were similarly injected but with vehicle (open bars). Fig 1c: mice were exposed to a single dose of radiation between 7.76–8.29 Gy and injected

subcutaneously with one dose of 1.0 mg kg^{-1} Maxy-G34 (PEG-G-CSF) at $24 \pm 4\text{h}$, two doses at $24 \pm 4\text{h}$ and d7–8, or three doses at $24 \pm 4\text{h}$, and d7–8 and d14–15 post irradiation (filled bars); control mice were similarly injected but with vehicle (open bars). Thirty-day survival was significantly increased for all G-CSF- or PEG-G-CSF-treated mice compared to vehicle-treated controls. Bars represent mean $\pm$ SEM, * $p < 0.05$ comparing drug to vehicle treated mice, $n=212\text{--}221$ mice per group (fig 1a), $n=20$ mice per group (fig 1b), and $n=70\text{--}110$ mice per group (fig 1c).

Fig. 2. Survival of lethally-irradiated mice after administration of low doses of Maxy-G34 or Neulasta. Mice were exposed to 8.7 Gy and injected subcutaneously with one dose of 0.1, 0.3, or 1.0 mg kg⁻¹ Maxy-G34 (Fig. 2a, filled symbols) or Neulasta (Fig. 2b, filled symbols) at 24 ± 4h post irradiation. Control mice were similarly injected but with vehicle at 24 ± 4h post irradiation (open symbols, Fig. 2a and 2b). Thirty-day survival was significantly increased for all mice treated with Maxy-G34 or Neulasta. * p<0.001 comparing drug to vehicle treated mice, n=30 mice per group.

Fig. 3.

CBC profiles in TBI-cont, TBI-G34 and non-TBI mice. At various times between 4 and 9mo post-exposure, TBI-cont, TBI-G34 and non-TBI mice were assessed for peripheral white blood cells (WBC, panel a), neutrophils (NE, panel b), lymphocytes (LY, panel c), red blood cells (RBC, panel d) and platelets (PLT, panel e). Lines represent mean $\pm$ SEM. Data from all time points were pooled for statistical analyses: ‡ $p < 0.05$ comparing non-TBI to TBI-G34, † $p < 0.05$ comparing non-TBI to TBI-cont, * $p < 0.05$ comparing TBI-G34 to TBI-cont. $n = 2-3$ mice per group per time point.

Fig. 4.

Bone marrow (BM) cellularity and primitive hematopoietic phenotypes in TBI-cont, TBI-G34 and non-TBI mice. TBI-cont, TBI-G34 and non-TBI mice were sacrificed between 4 and 9mo post-exposure and LDBM isolated. LDBM was enumerated and absolute numbers of LDBM cells per mouse (BM cellularity) were calculated as previously described (Chua et al. 2012) (panel a). The percentage of different primitive hematopoietic cell phenotypes was determined by flow cytometry as described (Chua et al. 2012), and multiplied by BM cellularity in panel a to give the absolute number of the different phenotypes per mouse. The following data are shown: panel b, %Sca-1+ of lineage-negative (lin-) cells; panel c, the %Sca-1+ckit+ of lin- cells; panel d, %KSL cells; panel e, the absolute number of KSL cells per mouse; panel f, %KSLCD150+ cells; panel g, the %CD150+ cells within the KSL population; panel h, the absolute number of KSLCD150+ cells. Lines represent mean $\pm$ SEM. Data from all time points were pooled for statistical analyses: ‡ $p < 0.01$ comparing non-TBI to TBI-G34, † $p < 0.05$ comparing non-TBI to TBI-cont, * $p < 0.05$ comparing TBI-G34 to TBI-cont. $n = 3$ mice per group per time point.

Fig. 5.

Hematopoietic and pre-B lymphoid progenitors in TBI-cont, TBI-G34 and non-TBI mouse BM. 1.0×10^5 LDBM from TBI-cont, TBI-G34 and non-TBI mice isolated at 4 and 6mo post-exposure was suspended in duplicate in 1 mL of methylcellulose media containing muSCF, muIL-3, rhuIL-6 and rhuEPO (for hematopoietic progenitors), or in 1 mL of methylcellulose media containing rhuIL-7 (for pre-B lymphoid colonies). Cells were incubated in 100% humidified 5% CO₂ in air at 37°C and enumerated 13 days later for CFU-GM, BFU-E and CFU-GEMM colonies, or 7 days later for pre-B lymphoid colonies. The total number of CFU-GM, BFU-E, CFU-GEMM and pre-B lymphoid colonies per mouse are shown in panels a, b, c, and d, respectively. Bars represent mean $\pm$ SEM. Data from all time points were pooled for statistical analyses: † $p < 0.05$ comparing non-TBI to TBI-G34, ‡ $p < 0.01$ comparing non-TBI to TBI-cont. $n = 3$ mice per group per time point.

Fig. 6.

Long-term engraftment potential of TBI-cont, TBI-G34 and non-TBI HSC in competitive transplantation assays. Lethally irradiated congenic murine recipients were transplanted with 150 KSLCD150+ cells isolated from TBI-cont, TBI-G34 or non-TBI donors, along with 1.0×10^5 LDBM competitor cells of congenic origin. Peripheral blood from tail-snips was obtained from transplanted recipients at monthly intervals and was analyzed by flow cytometry to determine donor chimerism using antibodies against CD45.1 and CD45.2. Lines represent mean $\pm$ SEM donor chimerism at 6 months post-transplant. Data from all time points were pooled for statistical analyses: † $p < 0.001$ comparing non-TBI to TBI-G34, ‡ $p < 0.001$ comparing non-TBI to TBI-cont. $n = 5-12$ recipients per group per time point.

Fig. 7.

Lineage reconstitution of TBI-cont, TBI-G34 and non-TBI HSC in competitive transplantation assays. Mice transplanted with TBI-cont, TBI-G34 or non-TBI KSLCD150+ cells in Fig. 6 were assayed for donor-derived lineage reconstitution at 6 months post-transplantation. Peripheral blood from tail snips was stained with fluorescently tagged antibodies to CD45.1, CD45.2, CD4, CD8, B220 and Gr1, and analyzed by flow cytometry for donor- or competitor-derived CD4+ and CD8+ T cells, B cells and granulocytes. Panel a, b and c depict the lineage reconstitution of 150 KSLCD150+ cells from TBI-cont, TBI-G34 and non-TBI donors, respectively (expressed as a percentage of total TBI-cont, TBI-G34 or non-TBI donors, respectively). There are no data at the 4 month time point in panel a and only n=1 mouse in panel b at 4 months, since transplanted mice at these time points did not exhibit high enough donor chimerism (0.05% donor-derived chimerism) to allow lineage analysis. Panels d, e and f show the lineage reconstitution by the congenic competitor cells (expressed as a percentage of total competitor cells) that were co-transplanted with KSLCD150+ from TBI-cont, TBI-G34 and non-TBI donors, respectively, which acts as an

internal standard for normal lineage reconstitution. Bars represent mean + SEM. Data from all time points were pooled for statistical analyses: † $p < 0.05$ comparing non-TBI to TBI-G34, ‡ $p < 0.001$ comparing non-TBI to TBI-cont. $n = 1-12$ recipient mice per group per time point.

Fig. 8.

Cell cycle analysis of TBI-cont, TBI-G34 and non-TBI hematopoietic populations. TBI-cont, TBI-G34 and non-TBI mice were sacrificed at various times between 4 to 9mo post-exposure, and BM hematopoietic populations were analyzed by flow cytometry for cell cycle position using the DNA stain DAPI. Panels a, b and c show the cell cycle status of lineage-negative (lin-), KSL and KSLCD150+ cell populations, respectively. Bars represent mean $\pm$ SEM. Data from all time points were pooled for statistical analyses: † $p < 0.05$ comparing non-TBI to TBI-G34, ‡ $p < 0.05$ comparing non-TBI to TBI-cont. $n = 1-3$ mice per group per time point.

Table 1

Summary of individual H-ARS experiments presented in Figures 1 and 2.

Experiment	Rad dose (Gy, LDXX/30 estimate)	Group	%Survival (# live/total)
06.2/3	7.96, LD50	16 doses Neupogen , days 1 to 16	93% (28/30)
06.2/3	7.96, LD50	16 doses vehicle, days 1 to 16	60% (18/30)
07.002	7.96, LD50	16 doses Neupogen , days 1 to 16	96% (25/26)
07.002	7.96, LD50	1 dose vehicle, day 1	53% (16/30)
07.003	7.96, LD50	16 doses Neupogen , days 1 to 16	96% (25/26)
07.003	7.96, LD50	1 dose vehicle, day 1	70% (21/30)
07.009	7.96, LD50	16 doses Neupogen , days 1 to 16	90% (27/30)
07.009	7.96, LD50	1 dose vehicle, day 3	80% (24/30)
07.010	7.96, LD50	16 doses Neupogen , days 1 to 16	83% (25/30)
07.010	7.96, LD50	11 dose vehicle, days 1 to 11	67% (20/30)
08.008	8.42, LD50	16 doses Neupogen , days 1 to 16	85% (17/20)
08.008	8.42, LD50	2 doses vehicle, days 1+7	55% (11/20)
11.219	8.53, LD50	16 doses Neupogen , days 1 to 16	65% (11/17)
11.219	8.53, LD50	16 doses vehicle, days 1 to 16	24% (4/17)
11.219	8.72, LD70	16 doses Neupogen , days 1 to 16	59% (10/17)
11.219	8.72, LD70	16 doses vehicle, days 1 to 16	0% (0/17)
11.219	9.04, LD90	16 doses Neupogen , days 1 to 16	13% (2/16)
11.219	9.04, LD90	16 doses vehicle, days 1 to 16	0% (0/17)
08.008	7.96, LD50	1 dose Neulasta , day 1	95% (19/20)
08.008	7.96, LD50	2 doses Neulasta , days 1+7	95% (19/20)
08.008	7.96, LD50	2 doses vehicle, days 1+7	55% (11/20)
08.001	7.76, LD50	2 doses G34 , days 1+7	95% (19/20)
08.001	7.76, LD50	3 doses G34 , days 1+7+14	95% (19/20)
08.001	7.76, LD50	3 doses vehicle, days 1+7+14	80% (16/20)
08.001	7.96, LD70	2 doses G34 , days 1+7	75% (15/20)
08.001	7.96, LD70	3 doses G34 , days 1+7+14	85% (17/20)
08.001	7.96, LD70	3 doses vehicle, days 1+7+14	55% (11/20)
11.201	7.96, LD70	1 dose G34 , day 1	100% (30/30)
11.201	7.96, LD70	1 dose vehicle, day 1	73% (22/30)
11.201	7.96, LD70	2 doses G34 , days 1+8	100% (30/30)
11.201	7.96, LD70	2 doses vehicle, days 1+8	83% (25/30)
11.201	7.96, LD70	3 doses G34 , days 1+8+15	100% (30/30)
11.201	7.96, LD70	3 doses vehicle, days 1+8+15	70% (21/30)
11.215	8.06, LD70	1 dose G34 , day 1	95% (19/20)
11.215	8.06, LD70	1 dose vehicle, day 1	85% (17/20)
11.215	8.06, LD70	2 doses G34 , days 1+8	95% (19/20)

Experiment	Rad dose (Gy, LDXX/30 estimate)	Group	%Survival (# live/total)
11.215	8.06, LD70	2 doses vehicle, days 1+8	90% (18/20)
11.215	8.29, LD90	1 dose G34 , day 1	100% (20/20)
11.215	8.29, LD90	1 dose vehicle, day 1	60% (12/20)
11.215	8.29, LD90	2 doses G34 , days 1+8	90% (18/20)
11.215	8.29, LD90	2 doses vehicle, days 1+8	75% (15/20)
11.218	8.70, LD90	1 dose G34 , 0.1 mg/kg, day 1	47% (14/30)
11.218	8.70, LD90	1 dose G34 , 0.3 mg/kg, day 1	37% (11/30)
11.218	8.70, LD90	1 dose G34 , 1.0 mg/kg, day 1	47% (14/30)
11.218	8.70, LD90	1 dose Neulasta , 0.1 mg/kg, day 1	57% (17/30)
11.218	8.70, LD90	1 dose Neulasta , 0.3 mg/kg, day 1	43% (13/30)
11.218	8.70, LD90	1 dose Neulasta , 1.0 mg/kg, day 1	47% (14/30)
11.218	8.70, LD90	1 dose vehicle, day 1	0% (0/30)

Table 2

Mean Survival Time (MST) of decedent mice in Neupogen, Neulasta, and Maxy-G34 survival studies.

Growth factor	Dose ($\mu\text{g kg}^{-1} \text{ day}^{-1}$)	Injections	MST ^a (Days $\pm$ SD)
Neupogen^b	125.0	16	14.3 $\pm$ 3.7
Vehicle	N/A	1–16 ^c	17.6 $\pm$ 4.7
Neulasta^d	1.0	1	18.0 ^e
		2	13.0 ^e
Vehicle	N/A	2	19.4 $\pm$ 3.5
		1	12.0 ^e
Maxy-G34^f	1.0	2	15.2 $\pm$ 3.7 ^g
		3	10.2 $\pm$ 2.6 ^g
		1	21.9 $\pm$ 4.4
Vehicle	N/A	2	20.7 $\pm$ 3.2
		3	17.1 $\pm$ 5.6

^a Average of all studies.

^b Significantly decreased compared to vehicle when the MST of all studies are compared ($p < 0.001$).

^c Varied between 1 and 16 injections.

^d Statistical analysis of MST could not be done since only one mouse died in the Neulasta-treated groups.

^e No SD, since only one mouse died in this group.

^f Significantly decreased compared to vehicle, when the MST of all Maxy-G34-treated groups were combined and compared to all vehicle groups combined ($p = 0.001$).

^g Significantly decreased compared to corresponding vehicle group ($p = 0.01$).

Table 3

Mean Survival Time (MST) of decedent mice in Maxy-G34 and Neulasta dose reduction study

Growth factor	Dose (mg kg ⁻¹ day ⁻¹)	Injections	MST (Days)
Maxy-G34	0.1	1	17.6
	0.3	1	14.3
	1.0	1	14.8
Neulasta	0.1	1	16.3
	0.3	1	15.5
	1.0	1	15.8
Vehicle	N/A	1	16.2