Tie2+ Bone Marrow Endothelial Cells Regulate Hematopoietic Stem Cell Regeneration Following Radiation Injury

Phuong L. Doan[1], **J. Lauren Russell**[1], **Heather A. Himburg**[1], **Katherine Helms**[1], **Jeffrey Harris**[1], **Joseph Lucas**[2], **Kirsten Holshausen**[1], **Sarah K. Meadows**[1], **Pamela Daher**[1], **Laura B. Jeffords**[3], **Nelson J. Chao**[1,4], **David G. Kirsch**[3,5], and **John P. Chute**[1,5]

[1]Division of Cellular Therapy, Department of Medicine, Duke University, Durham, NC

[2]Institute for Genome Sciences and Policy, Duke University, Durham, NC

[3]Department of Radiation Oncology, Duke University, Durham, NC

[4]Department of Immunology, Duke University, Durham, NC

[5]Department of Pharmacology and Cancer Biology, Duke University, Durham, NC

Abstract

Hematopoietic stem cells (HSCs) reside in proximity to bone marrow endothelial cells (BM ECs) and maintenance of the HSC pool is dependent upon EC-mediated c-kit signaling. Here, we utilized genetic models to determine if radioprotection of BM ECs could facilitate hematopoietic regeneration following radiation-induced myelosuppression. We developed mice bearing deletion of the pro-apoptotic proteins, BAK and BAX, in Tie2+ endothelial cells (ECs) and HSCs (*Tie2Bak/Bax$^{Fl/-}$* mice) and compared their hematopoietic recovery following total body irradiation (TBI) with mice which retained *Bax* in Tie2+ cells. Mice bearing deletion of *Bak* and *Bax* in Tie2+ cells demonstrated protection of BM HSCs, preserved BM vasculature and 100% survival following lethal dose TBI. In contrast, mice that retained *Bax* expression in Tie2+ cells demonstrated depletion of BM HSCs, disrupted BM vasculature and 10% survival post-TBI. In a complementary study, *VEcadherinBak/Bax$^{Fl/-}$* mice, which lack *Bak* and *Bax* in VEcadherin+ ECs, also demonstrated increased recovery of BM stem/progenitor cells following TBI compared to mice which retained *Bax* in VEcadherin+ ECs. Importantly, chimeric mice which lacked *Bak* and *Bax* in HSCs but retained *Bak* and *Bax* in BM ECs displayed significantly decreased HSC content and survival following TBI compared to mice lacking *Bak* and *Bax* in both HSCs and BM ECs. These data suggest that the hematopoietic response to ionizing radiation is dependent upon

HSC-autonomous responses but is regulated by BM EC-mediated mechanisms. Therefore, BM ECs may be therapeutically targeted as a means to augment hematopoietic reconstitution following myelosuppression.

INTRODUCTION

HSCs have been shown to reside in proximity to osteoblasts within the BM and activation of osteoblasts has been shown to promote the expansion of the HSC pool in vivo [1–3]. BM HSCs have also been found in close association with BM sinusoidal vessels and recent studies have suggested an essential role for BM endothelial cells (ECs) and perivascular CXCL12-abundant reticular cells (CARs) in maintaining the HSC pool in vivo [4–6]. Similarly, nestin$^+$ mesenchymal stem cells (MSCs), as well as the sympathetic nervous system, have been shown to regulate the retention of HSCs in the BM and BM adipocytes have been shown to negatively regulate long-term HSC content within the BM [7–9]. Taken together, these studies suggest a dynamic regulation of the HSC pool during homeostasis via cells which comprise the BM microenvironment.

Whereas much is now known about the signaling mechanisms which regulate HSC homeostasis [10–12], the process of HSC regeneration following myelosuppressive injury is less well understood. We have shown that adult sources of human ECs elaborate soluble growth factors which promote the expansion of murine and human HSCs in vitro and the regeneration of murine and human HSCs in vitro following radiation exposure [13–17]. We have also demonstrated that systemic infusion of autologous or allogeneic ECs accelerates BM HSC reconstitution and hematologic recovery in mice following total body irradiation (TBI) [18, 19]. Conversely, systemic delivery of a neutralizing anti-VEcadherin antibody, which inhibits BM vasculogenesis, significantly delays hematologic recovery following myelosuppression [19, 20]. Recently, Ding et al. demonstrated a requirement for BM EC-mediated stem cell factor (SCF) signaling for the maintenance of the HSC pool during homeostasis [6] and Hooper et al. showed a requirement for VEGFR2$^+$ sinusoidal ECs to allow for normal hematologic recovery following TBI [21]. While these studies have suggested an essential role for BM ECs in regulating HSC maintenance and regeneration in vivo, it remains to be determined whether augmentation of EC function can promote HSC expansion or regeneration in vivo. Here, we utilized the Cre;LoxP system to generate mice bearing a constitutive deletion of *Bak1* and a conditional deletion of *Bax* in Tie2$^+$ cells (Tie2*Bak/Bax*$^{Fl/-}$ mice) or in VEcadherin$^+$ cells (VEcadherin*Bak/Bax*$^{Fl/-}$ mice), along with littermate controls (Tie2*Bak/Bax*$^{Fl/+}$ mice and VEcadherin*Bak/Bax*$^{Fl/+}$ mice), to determine whether deletion of these pro-apoptotic genes in Tie2$^+$ or VEcadherin$^+$ cells promotes HSC regeneration and improves survival following TBI. Using this approach, we show that the hematopoietic response to ionizing radiation is regulated by both HSC-autonomous responses and BM EC-mediated mechanisms.

MATERIALS AND METHODS

Animals

Tie2Cre;Bak1$^{-/-}$*;Bax*$^{+/-}$ mice were bred with *Bak1*$^{-/-}$*;Bax*$^{FL/FL}$ mice bearing a constitutive deletion of *Bak1* and floxed *Bax* alleles to generate *Tie2Bak/Bax*$^{FL/-}$ experimental mice and *Tie2Bak/Bax*$^{FL/+}$ littermate controls. In *Tie2Cre* mice, floxed alleles are recombined by Cre in Tie2-expressing cells and their progeny, which are referred to as Tie2$^+$ cells. *Tie2Bak/Bax*$^{FL/-}$ and *Tie2Bak/Bax*$^{FL/+}$ mice were generated as previously described [22]. To generate chimeric *Tie2Bak/Bax*$^{FL/-}$*;WT-EC* mice, 4×10^6 BM cells from *Tie2Cre;Bak1*$^{-/-}$*;Bax*$^{FL/-}$ (CD45.2$^+$) mice were transplanted into B6.SJL mice (CD45.1$^+$, Jackson Laboratory, Bar Harbor, ME) following 950 cGy TBI (Cs137 irradiator, dose rate

593 cGy/min). Control mice were generated using BM cells from *Tie2Cre;Bak1$^{-/-}$;Bax$^{FL/+}$* mice transplanted into B6.SJL mice to create *Tie2Bak/Bax$^{FL/+}$;WT-EC* mice. Full donor chimerism was verified by flow cytometric analysis at 12 weeks post-transplantation. *VEcadherinCre* mice (Jackson Laboratory, Bar Harbor, ME) were bred with *Bak1$^{-/-}$;Bax$^{+/-}$* mice to generate *VEcadherinBak1$^{-/-}$;Bax$^{+/-}$* mice. These progeny were crossed with *Bak1$^{-/-}$;Bax$^{FL/FL}$* mice to obtain *VEcadherinBak/Bax$^{FL/+}$* mice and *VEcadherinBak/ Bax$^{FL/-}$* mice. *VEcadherinCre (−)* mice, which lack Cre recombinase in VEcadherin$^+$ cells, were also utilized as controls in survival studies. All animal studies described herein were approved by the Duke University Animal Care and Use Committee.

Isolation of BM ECs and Quantitative Real-Time PCR for *Bax* Expression

Mice femurs were collected and BM cells were flushed into PBS (Cellgro, Manassas, VA) with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin-streptomycin (GIBCO, Grand Island, NY). Red blood cells were depleted using RBC lysis buffer (Sigma-Aldrich, St. Louis, MO). Cells were stained with anti-CD45 PE and anti-mouse endothelial cell antigen (MECA-32). Primary anti-MECA was washed and a secondary antibody Streptavidin AlexaFluor 488 (Invitrogen, Carlsbad, CA) was added. cDNA was generated from RNA using RNAeasy kit (Qiagen, Valencia, CA). Real time PCR was performed using Taqman probes for glyceraldehyde-3-phosphate (GAPDH) and *Bax* (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. All primary antibodies were obtained from BD Biosciences (BD, San Jose, CA).

Hematopoietic Progenitor Cell Assays

Two hours or 7 days following ionizing radiation, C57Bl6 mice and transgenic mice were sacrificed and BM cells were collected into PBS with 10% serum and 1% penicillin/ streptomycin as described above. Viable BM cells were quantified using trypan blue to exclude dead cells. Cells were then incubated with anti-c-kit, anti-Sca-1 and anti-lineage cocktail antibodies (BD) to measure ckit$^+$sca-1$^+$lin$^-$ (KSL) progenitor cells as previously described [18]. Anti-CD150 and anti-CD48/41 antibodies were utilized to analyze for CD150$^+$CD48/41$^-$ KSL (SLAM$^+$KSL) cells, which are enriched for HSCs [4]. CFU-S12 assays were also performed to measure functional hematopoietic stem/progenitor cell content. 1×10^5 cells were collected from donor mice and injected via tail vein into recipient C57Bl6 mice that had been given 950 cGy TBI. At day +12 post-injection, spleens from recipient mice were harvested and stained with Bouin's fixative solution (Ricca Chemical Company, Arlington, TX), and colonies were counted as previously described [23]. Colony forming cell (CFC) assays for myeloid progenitor cells were performed following manufacturer's guidelines (Stem Cell Technologies, Vancouver, CA).

Competitive Repopulation Assay and Radiation Survival Studies

Donor B6.SJL mice and transgenic mice (all CD45.2$^+$) were irradiated with 300 cGy TBI. Two hours later, BM cells were harvested from the donor mice and $1–3 \times 10^5$ BM cells were injected via tail vein into recipient C57Bl6 or B6.SJL mice (CD45.1$^+$) that had been lethally irradiated with 950 cGy TBI. Host BM cells (1×10^5) were co-injected as competitor cells into recipient mice. Multilineage hematopoietic reconstitution was measured in the PB by flow cytometry at 4, 8, and 12 weeks post-transplant. For competitive repopulating assays using non-irradiated donor mice, a donor cell dose of 3×10^4 BM cells was utilized. For the high dose TBI survival studies, all mice were irradiated with 750 cGy TBI using an X-RAD 320 Biological Irradiator (Precision X-ray, Inc). Mice were treated at 72 cm from the radiation source (SSD) with a dose rate of 104 cGy/min with 320 kVp X-rays, using 12.5 mA and a filter consisting of 2.5 mm Aluminum and 0.1 mm Copper. Mice were evaluated daily to monitor for morbidity and survival through day +30.

Cell Survival and Proliferation Assays

BM ECs of the indicated genotypes were collected over a 70 micron filter (BD Falcon, Bedford, MA) and incubated with 0.25% trypsin-EDTA (GIBCO, Grand Island, NY) at 37oC for 5 minutes. Cells were bound with anti-CD45 microbeads (Miltenyi Biotech, Auburn, CA), then passed through LS MACS columns (Miltenyi Biotech, Auburn, CA) to enrich for ECs. BM ECs and hematopoietic cells were stained with anti-CD45 PE and anti-mouse endothelial cell antigen (MECA-32), and then a secondary antibody Streptavidin AlexaFluor 488 as noted above. Cells were washed and placed in 1X binding buffer, Annexin V-APC, and Propidium Iodide. For the VEcadherin$Bak/Bax^{Fl/-}$ and VEcadherin$Bak/Bax^{Fl/+}$ cell survival studies, BM cells were passed through a lineage depletion column, then labeled with anti-c-kit FITC, anti-sca-1 PE-Cy7, Annexin V-APC, and Propidium Iodide. Cell apoptosis and necrosis were analyzed by flow cytometry according to manufacturer's protocols (BD, San Jose, CA). Cell proliferation was measured in mice of the indicated genotypes following exposure to 300 cGy TBI and administered 5-bromo-2-deoxyridine (BrdU, BD, San Jose, CA) in drinking water from the day of irradiation until the day of collection. BM cells were labeled with anti-cKit PE, anti-sca1 PE-Cy7, anti-lineage APC, and anti-BrdU FITC. Incorporation of BrdU was analyzed by flow cytometry according to the manufacturer's staining protocol (BD, San Jose, CA). All antibodies were obtained from BD Biosciences (BD, San Jose, CA).

BM Immunohistochemical Analyses

Femurs were decalcified and embedded in OCT media (Sakura Finetek, Torrance, CA) as previously described at day +10 following 750 cGy TBI [18, 19]. Ten micrometer sections were cut using the CryoJane tape system (Instrumedics Inc, Hackensack, NJ, USA). Femurs were stained with hematoxylin and anti-mouse endothelial cell antibody (MECA-32) as previously described [18, 24] to assess BM cellularity and vasculature after irradiation. Images were obtained using an Axiovert 200 microscope (Carl Zeiss, Thornwood, NY).

Cytokine Array Analysis

Whole BM was collected from adult, non-irradiated *Tie2Bak/Bax^{FL/-}* mice and *Tie2Bak/Bax^{FL/+}* mice and C57Bl6 mice and at 6 hours following 750 cGy TBI. After centrifugation, BM supernatants were collected into IMDM and analyzed for cytokine concentrations using Quantibody mouse cytokine array 1000, according to manufacturer's guidelines (RayBiotech, Inc., Norcross, GA).

Statistical Analyses

Data are shown as means ± SEM. We performed pair-wise comparisons with the Student's t test (2 tailed distribution). Comparisons of mice survival after TBI were performed using a Fisher's exact test and the Log rank test. Comparisons of cytokine concentrations in the different mice groups were made using a 1-tailed t test.

RESULTS

Deletion of *Bak* and *Bax* in Tie2+ Cells Protects BM HSCs from Radiation Injury

Mice expressing Cre under the control of the Tie2 promoter (Tie2Cre) were crossed with mice carrying floxed alleles for *Bax* in order to create mice bearing a targeted deletion of *Bax* in Tie2+ cells [25]. During development, BAK and BAX have overlapping functions in the vasculature [26], so we utilized mice bearing constitutive deletion of *Bak1* [27] to generate *Tie2Bak/Bax^{FL/-}* mice, which have *Bak* and *Bax* deletion in Tie2+ ECs and HSCs [22]. We compared these mice to *Tie2Bak/Bax^{FL/+}* littermate controls, which retain *Bax* expression in Tie2+ cells. We sought to assess whether deletion of *Bak* and *Bax* in Tie2+

cells could confer protection of the hematopoietic system following radiation exposure. *Tie2Bak/Bax$^{FL/-}$* mice had no detectable levels of *Bax* expressed in BM ECs (CD45$^-$, mouse endothelial cell antigen positive, MECA$^+$) or CD45$^+$MECA$^-$ hematopoietic cells, whereas *Tie2Bak/Bax$^{FL/+}$* mice had detectable *Bax* in both ECs and hematopoietic cells (Fig. 1a). We next examined the hematopoietic profile of non-irradiated *Tie2Bak/Bax$^{FL/-}$* mice and *Tie2Bak/Bax$^{FL/+}$* mice to assess for baseline differences in these mice (Fig. 1b–f). *Tie2Bak/Bax$^{FL/-}$* mice had elevated white blood cell (WBC) counts but a normal WBC differential and normal hemoglobin and platelet counts compared to *Tie2Bak/Bax$^{FL/+}$* mice and C57Bl6 mice (Fig. 1b,c). This phenotype is consistent with that described for mice bearing constitutive deletion of *Bak* and *Bax* [27]. However, we found no significant differences in the percentage or numbers of BM SLAM$^+$KSL cells, which are highly enriched for HSCs [28], between *Tie2Bak/Bax$^{FL/-}$* mice, *Tie2Bak/Bax$^{FL/+}$* mice and C57Bl6 mice (Fig. 1d). Furthermore, functional assays revealed no differences in BM CFU-S12 or HSC competitive repopulating capacity between *Tie2Bak/Bax$^{FL/-}$* mice and *Tie2Bak/Bax$^{FL/+}$* mice (Fig. 1e,f). Taken together, these data demonstrated that *Tie2Bak/Bax$^{FL/-}$* mice did not have increased BM HSC or progenitor content at baseline compared to *Tie2Bak/Bax$^{FL/+}$* mice or C57Bl6 mice.

We next tested whether deletion of *Bak* and *Bax* in Tie2$^+$ cells altered the response of the hematopoietic system to ionizing radiation injury. At 2 hours following 300 cGy TBI, BM ECs (CD45$^-$ MECA$^+$) in *Tie2Bak/Bax$^{FL/-}$* mice contained significantly less Annexin$^+$ cells compared to BM ECs from *Tie2Bak/Bax$^{FL/+}$* mice (Fig. 2a). BM hematopoietic cells (CD45$^+$MECA$^-$) from *Tie2Bak/Bax$^{FL/-}$* mice also displayed significantly decreased Annexin$^+$ cells compared *Tie2Bak/Bax$^{FL/+}$* mice. To test whether deletion of *Bak* and *Bax* in Tie2$^+$ cells protected BM HSCs from depletion following TBI, we compared BM HSC and progenitor cell content in *Tie2Bak/Bax$^{FL/-}$* mice versus *Tie2Bak/Bax$^{FL/+}$* mice following 300 cGy TBI. At 2 hours after 300 cGy, *Tie2Bak/Bax$^{FL/-}$* mice contained significantly increased numbers of BM KSL progenitor cells and BM CFU-S12 compared to irradiated *Tie2Bak/Bax$^{FL/+}$* controls (Fig. 2b–d). Importantly, recipient mice that were competitively transplanted with BM cells from irradiated *Tie2Bak/Bax$^{FL/-}$* mice contained 2.5-fold and 18-fold increased donor hematopoietic cell engraftment at 12 weeks post-transplant compared to mice transplanted with an equal dose of BM cells from irradiated *Tie2Bak/Bax$^{FL/+}$* mice or C57Bl6 mice, respectively (Fig. 2e,f). Of note, B cell and T cell reconstitution was also significantly increased in recipient mice transplanted with BM cells from *Tie2Bak/Bax$^{FL/-}$* mice compared to mice transplanted with HSCs from *Tie2Bak/Bax$^{FL/+}$* mice (Fig. 2e,f)

Tie2$^+$ BM ECs Regulate the Hematopoietic Response to Radiation Injury

Tie2Cre mice express Cre recombinase in both ECs and a majority of hematopoietic cells. Therefore, it is not possible to determine the relative contributions of BM ECs versus HSC-autonomous mechanisms to the radiation protection of HSCs observed in *Tie2Bak/Bax$^{FL/-}$* mice. In order to discriminate the specific contributions of Tie2$^+$ BM ECs and Tie2$^+$ HSCs in mediating the observed radioprotection in *Tie2Bak/Bax$^{FL/-}$* mice, we generated chimeric mice. We transplanted 4×10^6 BM cells from *Tie2Bak/Bax$^{FL/-}$* mice into lethally irradiated (950 cGy) B6.SJL mice such that, at 12 weeks post-transplant, the recipient mice were fully reconstituted with donor hematopoietic cells (mean 97.0 ± 2.0 donor CD45.2$^+$ cells; Fig. 3a,b). These recipient mice demonstrated deletion of *Bax* in BM CD45$^+$ hematopoietic cells while retaining expression of *Bax* in BM ECs (*Tie2Bak/Bax$^{FL/-}$*; wild type ECs (WT-EC), Fig. 3c). We then exposed the chimeric *Tie2Bak/Bax$^{FL/-}$; WT-EC* mice to 300 cGy TBI and compared their HSC and progenitor cell content to that of irradiated *Tie2Bak/Bax$^{FL/-}$* mice. At 2 hours after 300 cGy TBI, *Tie2Bak/Bax$^{FL/-}$;WT-EC* mice demonstrated significant depletion of BM KSL cells and CFU-S12 compared to irradiated *Tie2Bak/Bax$^{FL/-}$* mice

(Fig. 3d,e). As an additional control for the effects of TBI conditioning in the generation of the *Tie2Bak/Bax*$^{FL/-}$*;WT-EC* mice, we also irradiated *Tie2Bak/Bax*$^{FL/-}$ mice with 950 cGy TBI and transplanted these mice with BM cells from *Tie2Bak/Bax*$^{FL/-}$ mice, to generate *Tie2Bak/Bax*$^{FL/-}$;*FL-* mice. Following 300 cGy TBI, *Tie2Bak/Bax*$^{FL/-}$*;WT-EC* mice also had significantly less BM KSL and CFU-S12 content than *Tie2Bak/Bax*$^{FL/-}$;*FL-* mice (Fig. 3d,e), suggesting that the prior TBI conditioning did not account for the decreased BM progenitor cell content in *Tie2Bak/Bax*$^{FL/-}$*;WT-EC* mice compared to *Tie2Bak/Bax*$^{FL/-}$ mice. Importantly, recipient mice that were transplanted competitively with BM cells from irradiated *Tie2Bak/Bax*$^{FL/-}$*;WT-EC* mice displayed 5-fold lower donor CD45.2$^+$ hematopoietic cell engraftment at 12 weeks compared to mice transplanted with the identical dose of BM from irradiated *Tie2Bak/Bax*$^{FL/-}$ mice (Fig. 3f,g). Taken together, these data suggest that Tie2$^+$ BM ECs regulate the response of HSCs to radiation injury and that deletion of the intrinsic pathway of apoptosis in BM ECs contributes to the radioprotection of the HSC pool.

Deletion of *Bak* and *Bax* in Tie2$^+$ ECs Improves the Survival of Irradiated Mice

In order to determine if the radioprotection of BM HSCs observed in *Tie2Bak/Bax*$^{FL/-}$ mice was relevant at radiation dose levels that would affect survival, we examined the BM cellular response and survival of *Tie2Bak/Bax*$^{FL/-}$ mice, *Tie2Bak/Bax*$^{FL/+}$ mice, *Tie2Bak/ Bax*$^{FL/-}$*;WT-EC* mice and C57Bl6 mice following a lethal dose of TBI (750 cGy). At day +10 following 750 cGy, C57Bl6 mice and *Tie2Bak/Bax*$^{FL/+}$ mice demonstrated severe BM hypocellularity and disruption of the BM vasculature (Fig. 4a). In contrast, *Tie2Bak/Bax*$^{FL/-}$ mice demonstrated nearly normal BM cellularity and preserved sinusoidal vasculature at this time point. *Tie2Bak/Bax*$^{FL/-}$*;WT-EC* mice demonstrated substantially decreased BM cellularity and increased disruption of the BM vasculature compared to *Tie2Bak/Bax*$^{FL/-}$ mice, confirming the contribution of Tie2$^+$ BM ECs in regulating the hematopoietic response to radiation injury (Fig. 4a). However, *Tie2Bak/Bax*$^{FL/-}$*;WT-EC* mice retained qualitatively increased BM cellularity compared to *Tie2Bak/Bax*$^{FL/+}$ mice and C57Bl6 mice, suggesting that the HSC response to ionizing radiation was regulated in a cell-autonomous and an EC-dependent manner.

Tie2Bak/Bax$^{FL/-}$ mice also displayed significantly increased survival compared to control mice following lethal dose TBI. Following 750 cGy TBI, 90% of C57Bl6 mice and 91% of the *Tie2Bak/Bax*$^{FL/+}$ mice died by day +30 (Fig. 4b). In contrast, 100% of the *Tie2Bak/ Bax*$^{FL/-}$ mice remained alive and well at day +30 post-irradiation (*P*<0.001 vs. B6 and *FL/+* mice). Interestingly, *Tie2Bak/Bax*$^{FL/-}$*;WT-EC* mice demonstrated an intermediate survival of 42% compared to *Tie2Bak/Bax*$^{FL/-}$ mice (*P*=0.005), suggesting that retention of the intrinsic pathway of apoptosis in BM ECs significantly increased mortality after TBI (Fig. 4b). Of note, none of the *Tie2Bak/Bax*$^{FL/+}$*;WT-EC* mice, which retained *Bax* in BM hematopoietic cells and BM ECs, survived past day +30. Therefore, these results indicate that deletion of the intrinsic pathway of apoptosis in BM HSCs is sufficient to protect a subset of mice from death due to hematopoietic failure after TBI, but significantly increased radioprotection is conferred by deletion of *Bak* and *Bax* in both ECs and HSCs.

Deletion of *Bak* and *Bax* in VEcadherin$^+$ ECs Promotes Hematopoietic Recovery in Mice Following TBI

In order to test our hypothesis that BM ECs regulate the response of the HSC pool to ionizing radiation in a 2nd model, we utilized *VEcadherinCre* mice that express Cre in ECs and only a small minority of hematopoietic cells [29, 30]. We then generated *VEcadherinBak/Bax*$^{FL/-}$ mice in which Cre-mediated deletion of *Bax* occurs in VEcadherin$^+$ cells and *Bax* is retained in CD45$^+$MECA$^-$ hematopoietic cells (Fig. 5a). We compared functional hematopoietic progenitor content in non-irradiated *VEcadherinBak/*

$Bax^{FL/-}$ mice and $VEcadherinBak/Bax^{FL/+}$ mice and determined that there were no baseline differences in BM CFC and CFU-S12 content (Fig. 5b). Furthermore, these mice displayed comparable cellularity and vascular density at baseline (Fig. 5c). However, at 7 days following 300 cGy TBI, $VEcadherinBak/Bax^{FL/+}$ mice displayed decreased BM cellularity and increased disruption of vascular integrity compared to $VEcadherinBak/Bax^{FL/-}$ mice (Fig. 5c). Interestingly, 300 cGy TBI caused significant depletion of BM CFCs and CFU-S12 in both $VEcadherinBak/Bax^{FL/-}$ mice and $VEcadherinBak/Bax^{FL/+}$ mice at +2 hours post-TBI (Fig. 5d). However, at day +7 post-TBI, $VEcadherinBak/Bax^{FL/-}$ mice displayed significantly increased recovery of BM CFCs and CFUS-12 compared to $VEcadherinBak/Bax^{FL/+}$ mice (Fig. 5d). At this same time point post-TBI, $VEcadherinBak/Bax^{FL/-}$ mice also displayed markedly increased survival of BM lin⁻ hematopoietic cells and increased BrdU incorporation in BM KSL cells in vivo compared to $VEcadherinBak/Bax^{FL/+}$ mice (Fig. 5e,f). These results suggest that BM ECs may not affect the immediate survival of HSCs following irradiation, but can promote the regeneration of HSCs and progenitor cells subsequent to radiation injury. While it is possible that the observed regeneration of HSCs and progenitors in $VEcadherinBak/Bax^{FL/-}$ mice is contributed to by a small number of hematopoietic cells with deletion of Bak and Bax, these results suggest a role for BM ECs in regulating HSC regeneration following radiation injury.

In order to determine if the deletion of Bak and Bax in VEcadherin⁺ ECs could also protect mice from lethal dose total body irradiation (TBI), we irradiated $VEcadherinBak/Bax^{FL/-}$ mice, $VEcadherinBak/Bax^{FL/+}$ mice and $VEcadherinCre\ (-)$ mice with 750 cGy TBI and compared the survival of each group. $VEcadherinBak/Bax^{FL/-}$ mice demonstrated a significant increase in 15-day survival compared to $VEcadherinBak/Bax^{Fl/+}$ mice and $VEcadherinCre\ (-)$ mice following 750 cGy TBI (P=0.04 and P=0.004, respectively, Figure 5g). However, the differences in 30-day survival following lethal dose TBI between these groups did not reach statistical significance. Taken together, these data suggest that deletion of Bak and Bax in VEcadherin⁺ ECs alone can contribute to an earlier recovery of BM hematopoietic progenitor cells after sublethal irradiation and an early survival benefit following lethal dose TBI, but this effect is incomplete in the absence of protection of HSCs.

Deletion of *Bak* and *Bax* in Tie2⁺ Cells Alters Cytokine Concentrations in BM Serum

In order to begin to identify candidate mechanisms through which deletion of Bak and Bax in Tie2⁺ cells promotes the radioprotection of mice, we isolated BM serum from non-irradiated $Tie2Bak/Bax^{FL/-}$ mice, $Tie2Bak/Bax^{FL/+}$ mice and C57Bl6 mice and at 6 hours following 750 cGy TBI and analyzed the concentrations of cytokines within the BM serum of the different groups of mice. Interestingly, numerous cytokines were substantially increased or decreased in $Tie2Bak/Bax^{FL/-}$ mice compared to $Tie2Bak/Bax^{FL/+}$ mice and C57Bl6 mice at baseline and at 6 hours following 750 cGy TBI (Figures 6, 7 and Tables S1–S4). IGFBP2, epidermal growth factor (EGF), VEGF-D, and CD30T were upregulated more than 20-fold in the BM of non-irradiated $Tie2Bak/Bax^{FL/-}$ mice compared to C57Bl6 mice (P<0.05, Figure 6). VEGF-D, CD30T and IGFBP2 were also upregulated more than 10-fold in $Tie2Bak/Bax^{FL/-}$ mice compared to $Tie2Bak/Bax^{FL/+}$ mice. Interestingly, granulocyte-monocyte colony stimulating factor (GMCSF) and monocyte chemotactic protein 1 (MCP1) were more than 10-fold downregulated in the BM of $Tie2Bak/Bax^{FL/-}$ mice compared to $Tie2Bak/Bax^{FL/+}$ mice and C57Bl6 mice at baseline (Figure 6). At 6 hours following 750 cGy TBI, EGF, IL12p40, IL-21, IL-7, IL-15 and hepatocyte growth factor (HGF) were more than 20-fold increased in the BM of $Tie2Bak/Bax^{FL/-}$ mice compared to C57Bl6 mice (Figure 7). EGF and GITR were also significantly increased in the BM of $Tie2Bak/Bax^{FL/-}$ mice compared to $Tie2Bak/Bax^{FL/+}$ mice, although the fold differences were smaller. As we observed in the analysis of non-irradiated mice, GMCSF and MCP1 were also the most significantly downregulated proteins in the BM of irradiated $Tie2Bak/Bax^{FL/-}$ mice

compared to *Tie2Bak/Bax$^{FL/+}$* mice and C57Bl6 mice following 750 cGy. Taken together, these data identify candidate secreted proteins and signaling mechanisms that may contribute to the radioprotection of the hematopoietic system observed in *Tie2Bak/Bax$^{FL/−}$* mice. Functional studies are now underway to determine if any of these proteins, alone or in combination, can mediate radiation protection or mitigation in vivo.

DISCUSSION

Recent studies have suggested that BM ECs play an essential role in the maintenance of the HSC pool during homeostasis and perhaps in regulating hematologic recovery following myelosuppression [6, 19–21]. However, the precise contribution of BM ECs, either independently or in concert with HSC-autonomous effects, toward regeneration of the HSC pool or the hematopoietic system has yet to be delineated. Here, using a combination of the Cre-LoxP system and BM transplant models, we show that targeted deletion of *Bak* and *Bax* in Tie2$^+$ cells protects BM HSCs from radiation-induced depletion compared to mice that retained *Bax* expression in Tie2$^+$ cells. Moreover, chimeric *Tie2Bak/Bax$^{Fl/−}$;WT-EC* mice, bearing deletion of *Bak* and *Bax* in BM HSCs and retention of *Bak* and *Bax* in BM ECs, displayed a significant depletion of BM HSCs following TBI compared to *Tie2Bak/Bax$^{Fl/−}$* mice in which *Bak* and *Bax* were deleted in both BM ECs and HSCs. These results suggest that BM ECs regulate the response of the HSC pool to radiation. In order to test our hypothesis further using a different model, we also examined the hematopoietic response to ionizing radiation in *VEcadherinBak/Bax$^{FL/−}$* mice, which have deletion of *Bak* and *Bax* in VEcadherin$^+$ ECs. *VEcadherinCre* mice, unlike *Tie2Cre* mice, demonstrate Cre activation in only a small subset of hematopoietic cells in the adult mice, so it provides a more EC-specific deletion of target genes [29][30]. Deletion of *Bak* and *Bax* in VEcadherin$^+$ ECs did not protect BM HSCs from radiation-induced depletion immediately (+ 2 hrs) after exposure, but HSC and progenitor cell recovery was accelerated in *VEcadherinBak/Bax$^{Fl/−}$* mice at day +7 following 300 cGy irradiation compared to *VEcadherinBak/Bax$^{Fl/+}$* control mice. Taken together, these results suggest an important contribution from BM ECs in regulating the regeneration of hematopoietic stem/progenitor cells following ionizing radiation.

In order to determine whether radioprotection of BM ECs could also improve the survival of mice following lethal dose TBI, we examined the survival of *Tie2Bak/Bax$^{Fl/−}$* mice, *Tie2Bak/Bax$^{Fl/−}$;WT-EC* mice and control mice following 750 cGy TBI. The results of these studies suggested that protection of the BM vasculature may improve survival following TBI, since mice bearing deletion of *Bak* and *Bax* in BM HSCs and BM ECs had 100% survival following TBI, whereas only 42% of mice which retained *Bak* and *Bax* in BM ECs survived, despite deletion of *Bak* and *Bax* in BM HSCs. When *VEcadherinBak/Bax$^{Fl/−}$* mice were exposed to lethal dose TBI, these mice demonstrated a significant increase in 15-day survival compared to *VEcadherinBak/Bax$^{Fl/+}$* mice and *VEcadherinCre (−)* mice, but the effect was not durable and the magnitude of this effect was less than that observed in *Tie2Bak/Bax$^{FL/−}$;WT-EC* mice. These results highlight several interesting findings. First, deletion of *Bak* and *Bax* in both Tie2$^+$ HSCs and BM ECs provides 100% protection from lethal dose TBI. Second, deletion of *Bak* and *Bax* solely in BM HSCs, in the absence of protection of BM ECs, provides incomplete radioprotection which confers significantly lower survival following lethal dose TBI compared to mice with deletion of *Bak* and *Bax* in both HSCs and ECs. Third, deletion of *Bak* and *Bax* primarily in BM ECs, in the absence of protection of BM HSCs, provides early but incomplete radioprotection of mice following lethal dose TBI. Taken together, these data demonstrate that the hematopoietic response to ionizing radiation is dependent upon HSC-autonomous responses, but is regulated by BM EC-mediated mechanisms.

The results presented here provide important new information about the role of BAK and BAX in regulating radiation-induced injury to HSCs and ECs. Our data also suggest that targeted inhibition of BAK- and BAX-mediated apoptosis in BM ECs has therapeutic potential as a means to promote hematopoietic reconstitution following radiation injury. Since *Tie2Bak/Bax*[FL/+] mice displayed comparable radiosensitivity of the hematopoietic system to C57Bl6 mice, and increased radiosensitivity compared to *Tie2Bak/Bax*[FL/−] mice, this suggests that BAK and BAX play a redundant role within the hematopoietic system in initiating the intrinsic pathway of apoptosis. Our findings relating to bone marrow ECs complement studies in other systems which have suggested redundant roles for BAK and BAX in mediating the intrinsic apoptotic pathway [22, 31], but do not support conclusions from a prior study which suggested that BAK and BAX do not exhibit functional redundancy in radiation-induced EC apoptosis in the intestinal mucosa [32]. One possible explanation for these divergent observations is that BAK and BAX may fill overlapping or non-overlapping functions depending upon the organ system. However, in the present study, we provide compelling genetic data that in bone marrow ECs, deletion of both BAK and BAX are required to block radiation-induced apoptosis.

Our observation that deletion of *Bak* and *Bax* in Tie2^{+} cells confers radioprotection on HSCs is consistent with prior studies which demonstrated that deletion of PUMA (p53 upregulated mediator of apoptosis), a member of the BH3 family of pro-apoptotic molecules, protected HSCs from radiation-induced apoptosis [33, 34]. PUMA, along with other BH3 family members (BID and BIM), promotes apoptosis via activation of BAK and BAX or inhibition of anti-apoptotic BCL-2/BCL-XL/MCL-1 proteins [35]. The unique finding here is the demonstration that genetic deletion of *Bak* and *Bax* expression in BM ECs modulated BM hematopoietic stem and progenitor cell regeneration following TBI. Therefore, targeted inhibition of pro-apoptotic proteins in BM ECs may have therapeutic potential as a means to augment the regeneration of the hematopoietic system after radiation-induced failure.

In recent years, it has become increasingly evident that cells within the BM microenvironment regulate HSC homeostasis in vivo [1, 3–9]. A role for BM ECs and osteoblasts in regulating hematopoietic regeneration following myelosuppression has also been suggested [2, 21]. However, the precise mechanisms through which BM microenvironmental cells regulate HSC regeneration and hematopoietic reconstitution remain unclear. Nonetheless, the translational potential of targeting niche elements as a means to augment hematopoiesis and hematopoietic reconstitution has been demonstrated [1, 2, 14]. Calvi et al. [1] showed that systemic administration of parathyroid hormone (PTH), which activates the PTH receptor on BM osteoblasts, to wild type mice for 5 weeks caused a significant increase in BM HSCs in vivo. Adams et al. [2] showed that PTH-mediated augmentation of BM osteoblast function amplified GCSF-induced mobilization of BM hematopoietic progenitors into the PB [2], and protected the BM progenitor cell compartment from the toxic effects of recurrent exposure to cyclophosphamide treatment [2]. Therefore, pharmacologic targeting of the HSC niche represents a promising approach for augmentation of the HSC pool and hematopoietic regeneration in vivo. Here, we demonstrate via a genetic approach that BM ECs modulate HSC regeneration, hematopoietic reconstitution and overall survival in mice following TBI. Utilizing cytokine array analysis of BM supernatants from *Tie2Bak/Bax*[FL/−] mice and *Tie2Bak/Bax*[FL/+] mice and C57Bl6 mice, we have identified several soluble proteins and candidate signaling pathways which can now be interrogated to determine their specific role in regulating the hematopoietic response to ionizing radiation. We anticipate that these studies will reveal novel mechanisms through which BM ECs regulate HSC regeneration after injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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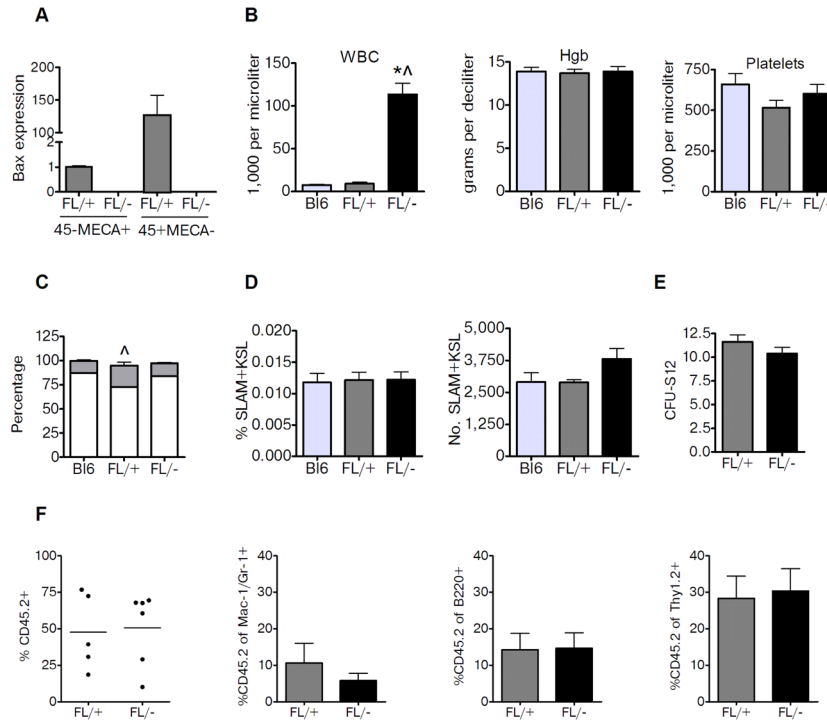
Figure 1.
Deletion of *Bak* and *Bax* in Tie2$^+$ cells does not alter baseline HSC content or function. (**A**) Endothelial cells (CD45$^-$MECA$^+$) and hematopoietic cells (CD45$^+$MECA$^-$) were isolated from the BM. Relative expression of *Bax* is shown in these cell populations from *Tie2Bak/Bax$^{FL/-}$* mice (FL/−), *Tie2Bak/Bax$^{FL/+}$* mice (FL/+)(*n*=4–7/group). (**B**) Peripheral blood cells from 8 week old C57Bl6 (Bl6) mice, FL/+ mice and FL/− mice were analyzed for WBCs, hemoglobin (Hgb) concentration, and platelet counts. FL/− mice had significantly higher PB WBCs compared to control mice (*n*=5/group). *P<0.001 and ^P<0.001 versus FL/+ and Bl6 mice; means ± SEM. (**C**) Percentage of PB lymphocytes (gray) and neutrophils (white) is shown between non-irradiated Bl6 mice, FL/+ mice and FL/− mice (*n*=5/group). ^P=0.02 for lymphocyte percentage between FL/+ and FL/− mice. (**D**) Percentages of CD150$^+$CD48$^-$CD41$^-$KSL cells (SLAM$^+$KSL cells) and total numbers of SLAM$^+$KSL cells per femur in the BM of non-irradiated Bl6, FL/+ and FL/− mice are shown (*n*=3–4/group). (**E**) Numbers of BM CFU-S12 in non-irradiated FL/+ and FL/− mice are shown (*n*=6–8/group). (**F**) Scatter plots show the donor CD45.2$^+$ cell engraftment in recipient CD45.1$^+$ mice at 12 weeks following competitive transplantation with 3×10^4 BM cells from non-irradiated FL/− or FL/+ mice with 1×10^5 competing B6.SJL (CD45.1$^+$) BM cells (*n*=5–6/group). Mean levels of engraftment are represented by the horizontal lines (mean 50.8% vs. 47.5% CD45.2$^+$ cells in PB at 12 weeks. The mean percentages of donor CD45.2$^+$ cells within the myeloid (Mac-1/Gr-1), B cell (B220) and T cell (Thy 1.2) cell populations at 12 weeks are shown at right.
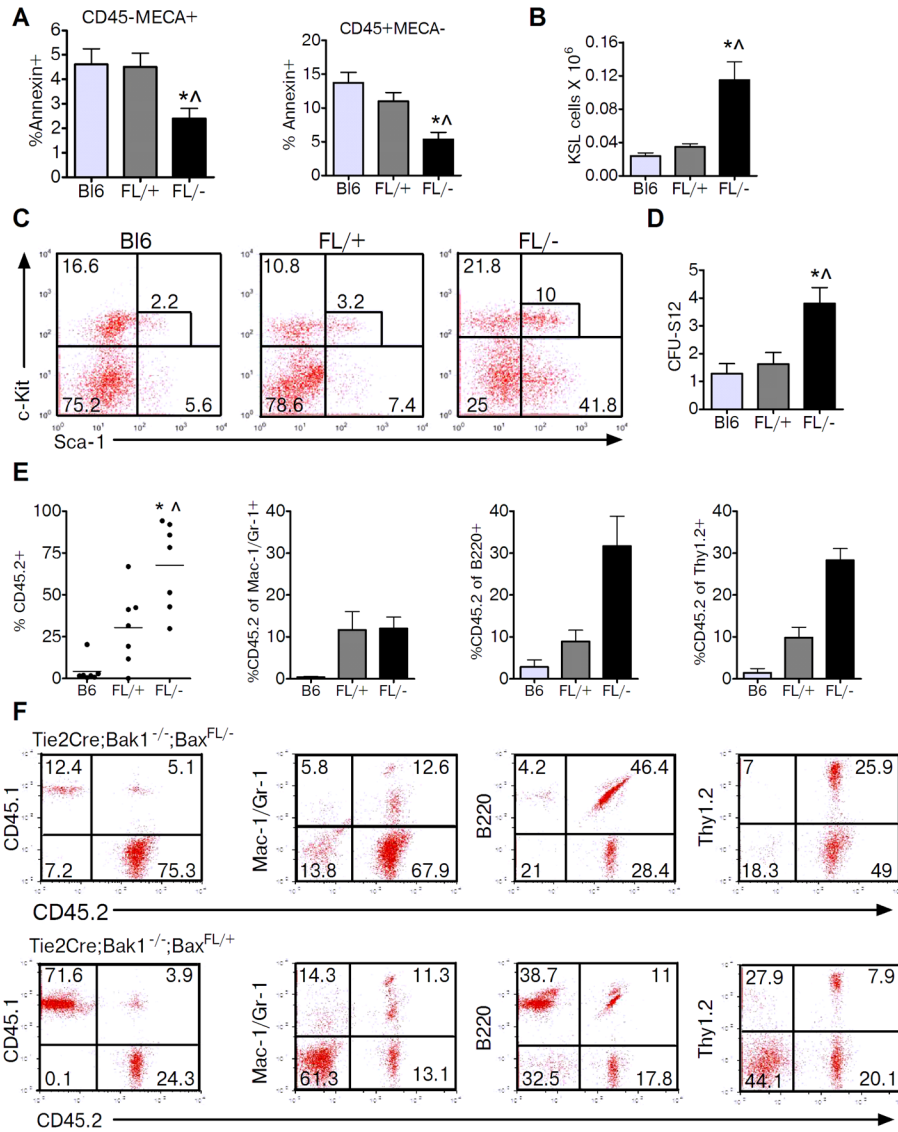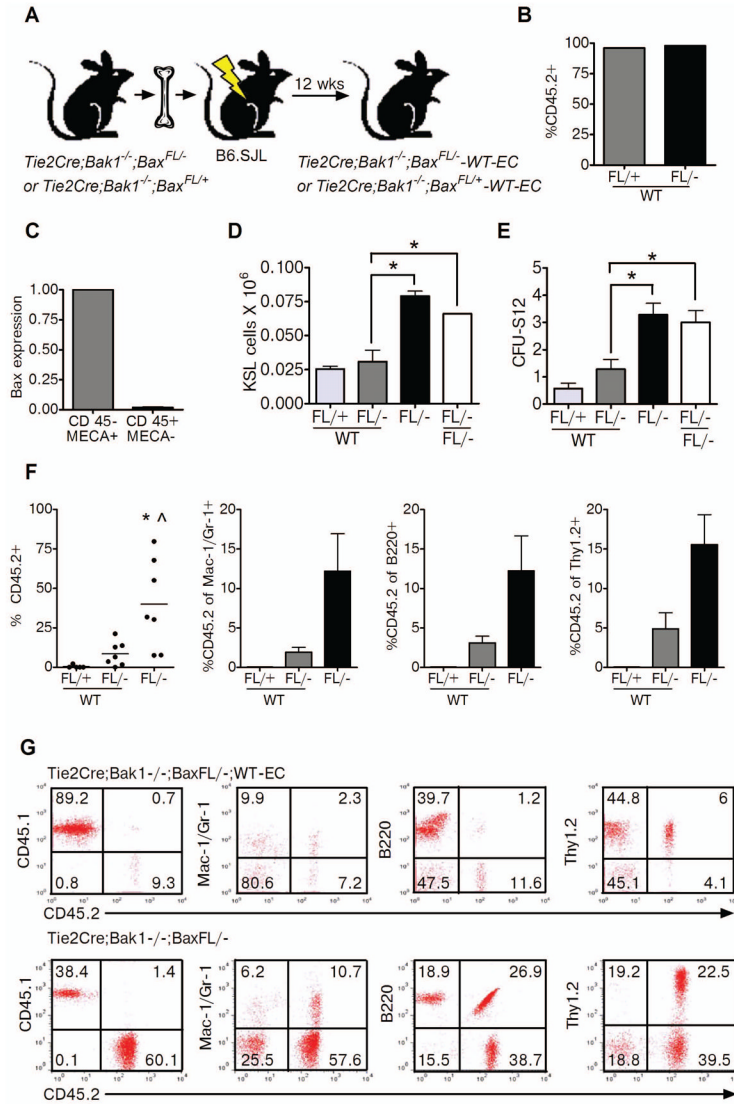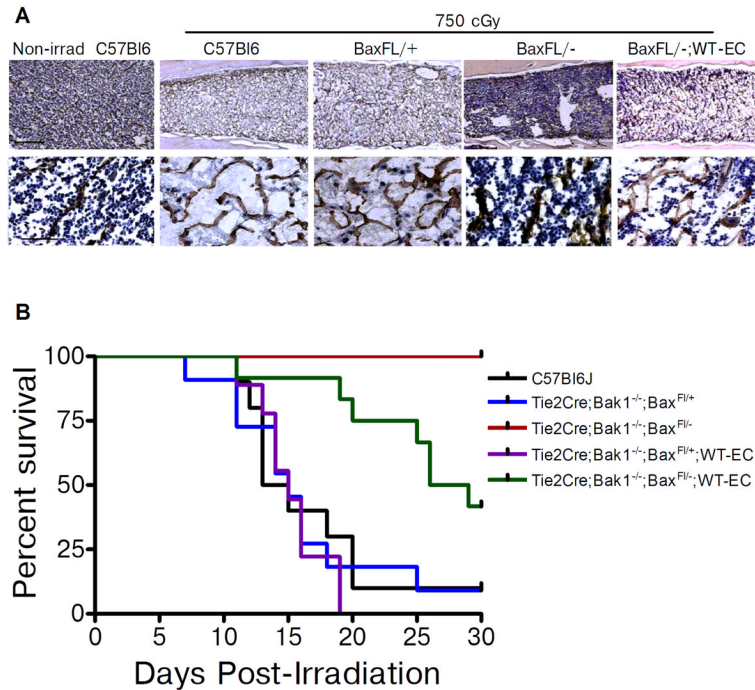
Figure 2.
Deletion of *Bak* and *Bax* in Tie2$^+$ cells protects BM HSCs and progenitor cells from radiation injury. (**A**) Percentages of Annexin$^+$ cells are shown for BM ECs (CD45$^-$MECA$^+$, left) and BM hematopoietic cells (CD45$^+$MECA$^-$, right) in BL6, FL/+ and FL/− mice at 2 hours after 300 cGy TBI (*n*=3–5/group). *P=0.04 and ^P=0.04 versus Bl6 and FL/+ for CD45$^-$MECA$^+$ cells, respectively. *P=0.009 and ^P=0.03 versus Bl6 and FL/+ for CD45$^+$MECA$^-$ cells, respectively. (**B**) Bl6, FL/+ and FL/− mice were irradiated with 300 cGy TBI and BM cells were collected at +2 hours post-irradiation. BM KSL cells per femur were increased in FL/− mice compared to control mice (*n*=3–7/group). *P=0.006 and ^P=0.006 versus Bl6 and FL/+ mice, respectively. (**C**) Representative flow cytometric analysis of BM KSL cells in Bl6, FL/+, and FL/− mice following 300 cGy TBI. (**D**) BM CFU-S12 content was increased in FL/− mice compared to control mice. *P=0.003 and ^P=0.01 versus Bl6 and FL/+ mice, respectively (means ± SEM, *n*=6–8/group). (**E**) Scatter plots show the PB engraftment at 12 weeks post-transplant of donor BM CD45.2$^+$ cells that were harvested from FL/− or FL/+ mice at +2 hours following 300 cGy and competitively transplanted at a dose of 3×10^5 BM cells into lethally irradiated CD45.1$^+$ recipient mice

with 1×10^5 non-irradiated host competitor BM cells. PB engraftment of the same dose of donor BM CD45.2^{+} cells harvested from irradiated Bl6 mice following transplant into lethally irradiated CD45.1[+] recipient mice is shown at left. Each dot represents the engraftment of an individual mouse. Lines represent the mean levels of engraftment in each group (B6: 4.2%, FL/+: 30.4%, FL/−: 67.7%, *n*=7/group). The mean percentages of donor CD45.2^{+} cells within the myeloid, B cell and T cell populations at 12 weeks are shown at right. (**F**) Representative flow cytometric analysis of CD45.2[+] donor cell engraftment at 12 weeks in recipients ofTie2Bak/Bax[FL/−] or Tie2Bak/Bax[FL/+] BM cells.
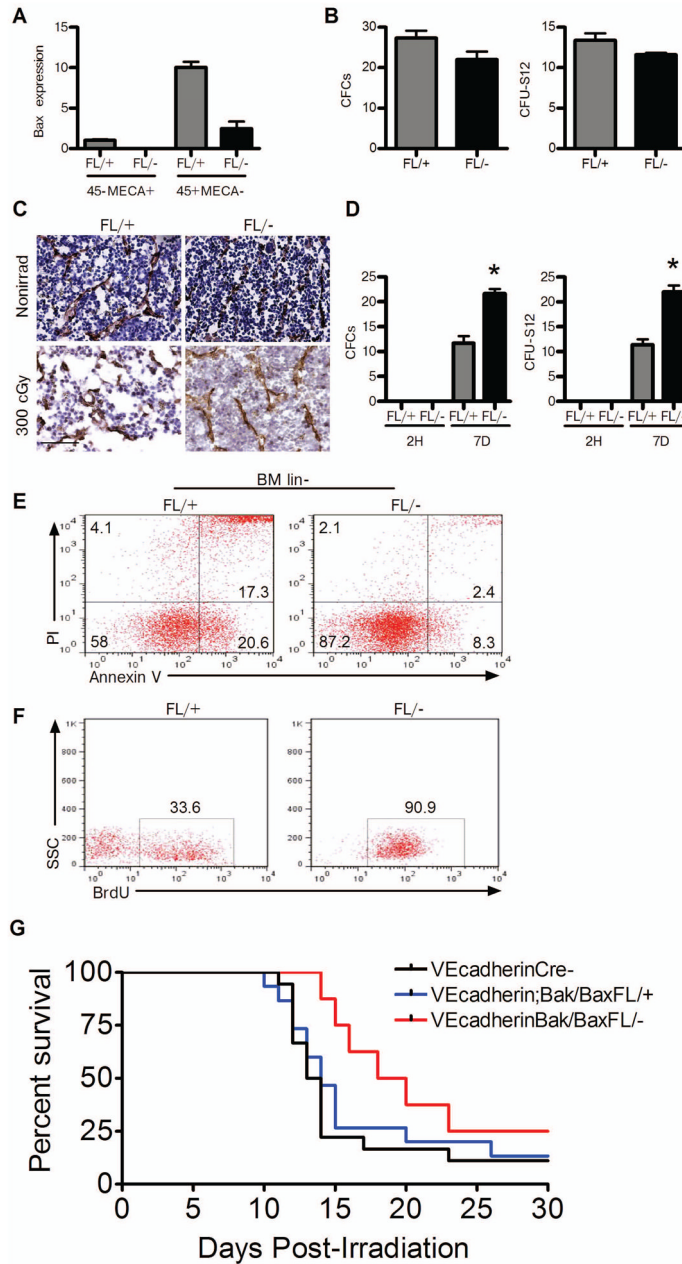
Figure 3.
(**A**) Schematic representation of the BM transplant model utilized to generate chimeric mice bearing deletion of *Bak* and *Bax* in Tie2+ hematopoietic cells while retaining *Bak* and *Bax* in BM ECs (*Tie2Bak/Bax^{FL/−};WT-EC* mice), along with control mice. (**B**) Mean levels ± SEM of CD45.2+ donor cell engraftment are shown in the PB of recipient CD45.1+ mice at 12 weeks following transplantation of BM cells from FL/+ and FL/− mice (*n*=4–5 mice/group). (**C**) Relative expression of *Bax* is shown in FACS-isolated CD45−MECA+ BM ECs and CD45+MECA− BM hematopoietic cells in recipient B6.SJL mice at 12 weeks following transplant with BM cells from *Tie2Bak/Bax^{FL/−}* mice (means ± SEM, *n*=2). (**D**) BM KSL cells per femur were decreased in *Tie2Bak/Bax^{FL/−};WT-EC* mice (FL/−;WT) compared to *Tie2Bak/Bax^{FL/−}* mice (FL/−) and *Tie2Bak/Bax^{FL/−};FL-EC* (FL/−;FL/−) mice following 300 cGy TBI. *P=0.007 and *P=0.046 for KSL cells in FL/−;WT-EC mice versus FL/− and FL/−;FL/− mice, respectively (means + SEM, *n*=2–3). (**E**) BM CFU-S12 were also significantly decreased in FL/−;WT mice compared to FL/− and FL/−;FL/− mice. *P=0.004 and *P=0.01 for CFU-S12 in FL/−;WT mice versus FL/− and FL/−;FL/− mice, respectively (means ± SEM, *n*=7). No significant differences were noted in BM KSL cells or CFU-S12

in FL/− mice compared to FL/−;FL/− mice. (**F**) The scatter plots show donor CD45.2$^+$ cell engraftment in the PB at 12 weeks following competitive transplantation of 1×10^5 BM cells from irradiated FL/+;WT mice, FL/−;WT mice or FL/− mice into lethally irradiated CD45.1$^+$ recipient mice. Each dot represents the engraftment of an individual mouse. Horizontal lines represent the mean levels of engraftment (FL/+;WT-EC: 0.1%, FL/−;WT-EC: 8.5%, FL/−: 40.0%, *n*=5–7/group). The mean percentages of donor CD45.2$^+$ cells within the myeloid, B cell and T cell populations are shown at right. (**G**) Representative flow cytometric analysis of donor CD45.2$^+$ cell engraftment and differentiation in recipient mice after transplantation with BM from irradiated FL/−;WT mice or FL/− mice.
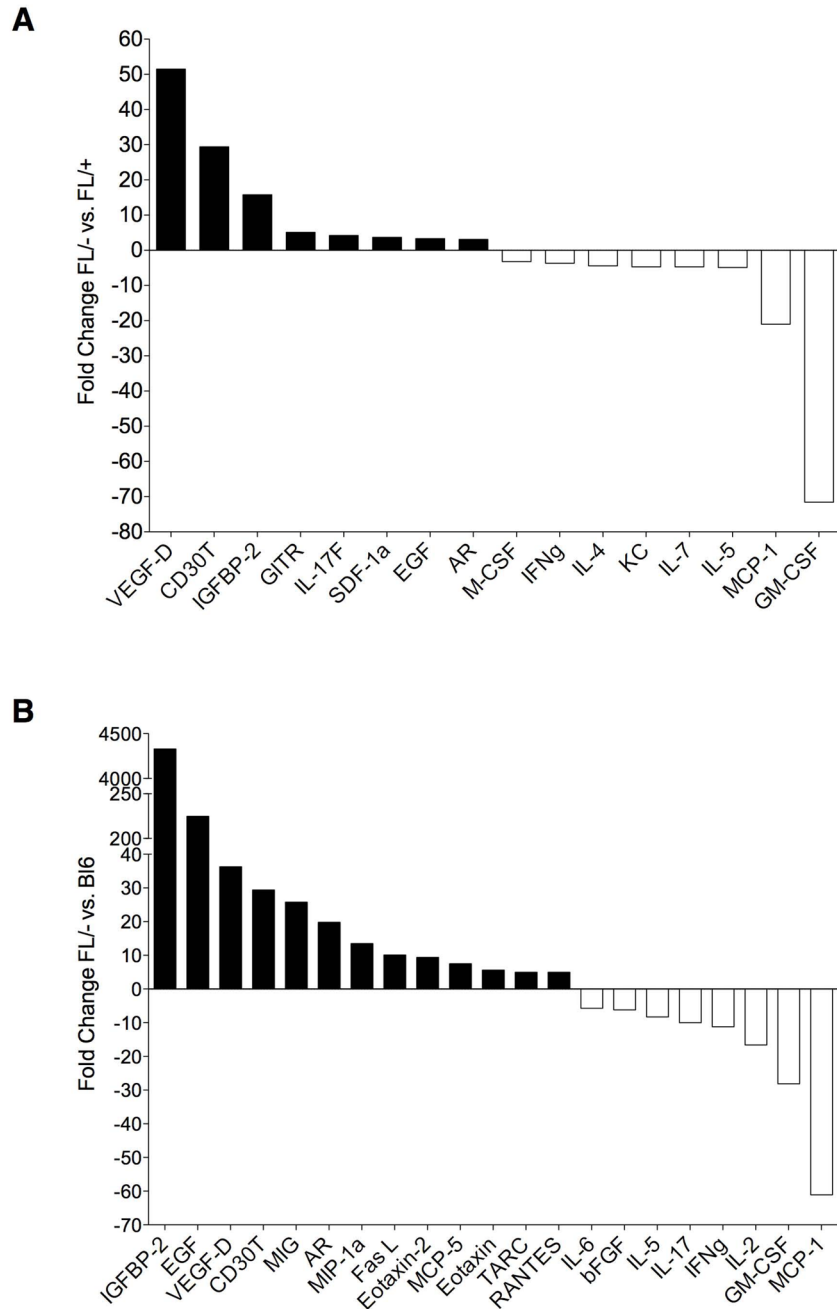
Figure 4.
Deletion of *Bak* and *Bax* in Tie2^{+} ECs preserves BM cellularity and improves survival following high dose TBI. (**A**) Representative cross sections of femurs from non-irradiated C57Bl6 mice and from C57Bl6 mice, *Tie2Bak/Bax*[FL/+] mice (BaxFL/+), *Tie2Bak/Bax*[FL/−] mice (BaxFL/−) and *Tie2Bak/Bax*[FL/−];*WT-EC* mice (BaxFL/−;WT-EC) at day +10 following 750 cGy TBI. Hematoxylin and mouse endothelial cell antigen (MECA) staining was performed. MECA-positive vessels are shown in brown (top, scale bar 250 microns; bottom, 50 microns), demonstrating preservation of BM sinusoidal vasculature in BaxFL/− mice and disruption of the vasculature in BaxFL/−;WT-EC mice, BaxFL/+ mice and C57Bl6 mice. The BaxFL/− mice also demonstrated substantially increased BM cellularity compared to BaxFL/−;WT-EC mice, C57Bl6 mice and BaxFL/+ mice. (**B**) Deletion of *Bak* and *Bax* in Tie2^{+} ECs improved the survival of mice exposed to lethal dose TBI. Adult C57Bl6 mice and mice bearing deletions of *Bak* and *Bax* (identified at top) were irradiated with 750 cGy TBI and subsequently followed for 30 days (*n*=9–12/group). *Tie2Bak/Bax*[FL/−] mice demonstrated 100% survival (10 of 10), compared to 10% survival (1 of 10) for C57Bl6 mice (*P*<0.0001) and 9% survival (1 of 11) for *Tie2Bak/Bax*[FL/+] mice (*P*<0.0001). *Tie2Bak/Bax*[FL/−];*WT-EC* mice (BaxFL/−;WT-EC) demonstrated significantly decreased survival (42%, 5 of 12) compared to *Tie2Bak/Bax*[FL/−] mice (**P*=0.005), but significantly increased survival compared to *Tie2Bak/Bax*[FL/+];*WT-EC* mice (BaxFL/+;WT EC) (0 of 9, *P*<0.0001). Log rank test was performed for all comparisons.

Figure 5.
Deletion of *Bak* and *Bax* in VEcadherin^{+} ECs promotes BM hematopoietic stem/progenitor cell regeneration in vivo. (**A**) Relative expression of *Bax* is shown in BM ECs (CD45^{−}MECA^{+}) and hematopoietic cells (CD45^{+}MECA^{−}) in *VEcadherinBak/Bax[FL/+]* mice (FL/+) and *VEcadherinBak/Bax[FL/−]* mice (FL/−) (*n*=3/group). *Bax* was not detected in CD45^{−}MECA^{+} ECs from FL/− mice but was detected in CD45^{+}MECA^{−} hematopoietic cells from FL/− mice. (**B**) Non-irradiated FL/+ and FL/− mice demonstrated comparable BM CFC and CFU-S12 content. (**C**) Representative cross sections of femurs are shown from non-irradiated *VEcadherinBak/Bax[FL/+]* mice (FL/+) and *VEcadherinBak/Bax[FL/−]* mice (FL/−) and at day +7 following 300 cGy TBI. Mouse endothelial cell antigen (brown) and hematoxylin (blue) stained femurs showed similar BM vasculature and cellularity in non-

irradiated mice. After 300 cGy, the FL/+ mice had significant disruption of the BM sinusoidal vasculature and decreased BM cellularity compared to FL/− mice. Scale bar is 50 microns. (**D**) FL/− and FL/+ mice were irradiated with 300 cGy TBI and BM cells were collected at 2 hours post-TBI and at day +7 for measurement of hematopoietic progenitor cell content. No differences were observed at 2 hours post-TBI in BM CFCs or CFU-S12 (*n*=3–5/group). At day +7 post TBI, mean numbers of BM CFCs and BM CFU-S12 were significantly increased in FL/− mice compared to FL/+ mice (*n*=3–5/group). *P=0.004 versus FL/+ for CFCs and *P=0.0002 versus FL/+ for CFU-S12. (**E**) Flow cytometric analysis demonstrated decreased apoptotic (Annexin$^+$PI$^−$) and necrotic (Annexin$^+$PI$^+$) BM lin$^−$ hematopoietic cells in FL/− mice at day +7 post TBI compared to FL/+ mice. (**F**) BrdU incorporation was increased in BM KSL progenitor cells in FL/− mice compared to FL/+ mice at day +7 following TBI. (**G**) Survival of *VEcadherinBak/Bax$^{Fl/−}$* mice (n=8), *VEcadherinBak/Bax$^{Fl/+}$* mice (n=15) and *VEcadherinCre (−)* mice (n=18) following lethal dose TBI. Survival curves of *VEcadherinBak/Bax$^{FL/−}$* mice (red line), *VEcadherinBak/Bax$^{FL/+}$* mice (blue line) and *VEcadherinCre (−)* mice (black line) are shown following 750 cGy TBI (15-day survival: *P*=0.04 and *P*=0.004 for *VEcadherinBak/Bax$^{Fl/−}$* mice vs. *VEcadherinBak/Bax$^{Fl/+}$* mice and *VEcadherinCre (−)* mice, respectively, Fisher's exact test; 30-day survival analysis: *P*=0.07 and *P*=0.06 for *VEcadherinBak/Bax$^{Fl/−}$* mice vs. *VEcadherinBak/Bax$^{Fl/+}$* mice and *VEcadherinCre (−)* mice, respectively, Log Rank analysis.

A

B

Figure 6.
Cytokine concentrations in the BM of non-irradiated *Tie2Bak/Bax[Fl/−]* mice.Cytokines are shown which were upregulated (filled bars) or downregulated (unfilled bars) 3-fold in the BM of non-irradiated *Tie2Bak/Bax[Fl/−]* mice (FL/−) versus *Tie2Bak/Bax[Fl/+]* mice (FL/+) (**A**) and 5-fold in non-irradiated FL/− versus C57Bl6 mice (**B**, *P*<0.05 for each cytokine). VEGF-D=vascular endothelial growth factor-D, IGFBP2=insulin like growth factor binding protein 2, GITR=glucocorticoid-induced TNFR-related protein, IL-17f=interleukin-17f, SDF1a=stromal derived factor 1a, AR=amphiregulin, IFNg=interferon gamma, KC=keratinocyte chemoattractant, MIG=migration inhibitory factor, MIP1a=macrophage inflammatory protein 1a, FasL=Fas ligand, TARC=thymus and activation regulated

chemokine, RANTES=regulated and normal T cell expressed and secreted protein, bFGF=beta fibroblast growth factor
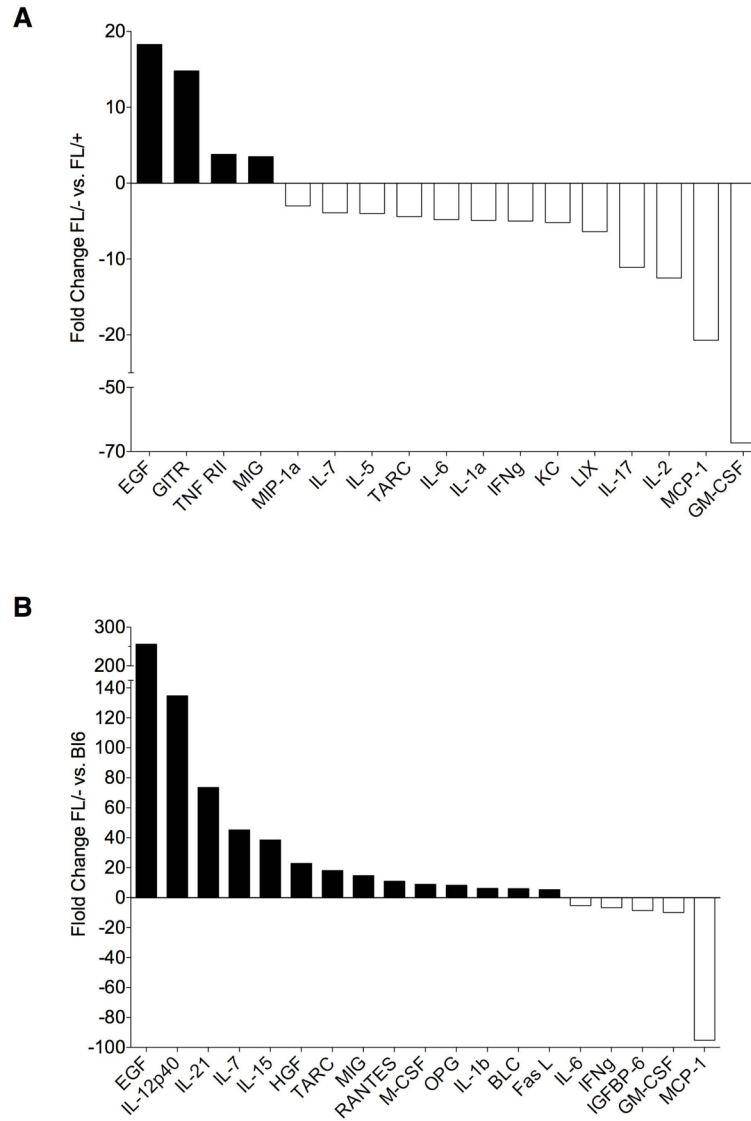
Figure 7.
Cytokine concentrations in the BM of irradiated *Tie2Bak/Bax*^{Fl/−} mice. Cytokines which were upregulated or downregulated   3-fold at 6 hours following 750 cGy in the BM of FL/−-versus FL/+ mice (**A**) and   5-fold in FL/− versus C57Bl6 mice (**B**) are also shown. TNF RII=tumor necrosis factor receptor II, LIX=lipopolysaccharide-induced CXC chemokine, HGF=hepatocyte growth factor, OPG=osteoprotegerin, BLC=B lymphocyte chemoattractant