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Effect of human amnion-derived multipotent progenitor cells on hematopoietic recovery after total body irradiation in C57BL/6 mice

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ABSTRACT

Background: The hematopoietic system is sensitive to the adverse effects of ionizing radiation. Cellular therapies utilizing mesenchymal stem cells or vascular endothelial cells have been explored as potential countermeasures for radiation hematopoietic injuries. We investigated cells cultured from amnion (Amnion-derived Multipotent Progenitor cells, AMPs) for effects on hematopoietic recovery following total body irradiation in mice. ***Materials and Methods***: C57BL/6J mice were sham-irradiated or exposed to ^{60}Co irradiation (7.75 – 7.90 Gy, 0.6 Gy/min). Either AMPs (5×10^6 cells/animal) or vehicle were administered 24 h postirradiation via intraperitoneal injection. ***Results:*** We observed a 13% and 20% improvement in 30-day survival of mice treated with AMPs compared with treatment with vehicle following irradiation at 7.75 and 7.90 Gy, respectively. AMP treatment was characterized by a trend toward accelerated recovery of white blood cells, neutrophils, reticulocytes, and monocytes, measured through day 40 postirradiation after 7.75 Gy. AMP treatment enhanced hematopoietic cell repopulation of spleen and femoral bone marrow as measured by total nucleated cell and hematopoietic progenitor cell counts in comparison to vehicle-treated animals. FACS analysis showed that AMPs treatment significantly mitigated the reduction in $CD11b^+/Gr\text{-}1^{int}$ and $CD11b^+/Gr\text{-}1^{high}$ bone marrow cell populations at the nadir, and improved recovery of these cell types. ***Conclusion:*** Together, our data indicate that AMPs reduced hematopoietic toxicity induced by ionizing radiation when infused within 24 h after radiation injury.

Keywords: *adult stem cells, acute radiation syndrome, ionizing radiation, radiation countermeasure, hematopoietic progenitor.*

INTRODUCTION

High dose total body irradiation (TBI), as the result of a nuclear accident, terrorist event, or as a clinical therapy for cancer, has significant hematopoietic toxicity, resulting in the loss of mature blood cells, hematopoietic progenitors, and the cellular constituents of the bone marrow microenvironment [1-3]. Mortality from acute radiation injury to the hematopoietic system, termed the hematopoietic syndrome, occurs as the result of severe thrombocytopenia, contributing to hemorrhage in multiple organs, accompanied by neutropenia and opportunistic infections [4]. Currently, there are no U.S. Food and Drug Administration-approved medical countermeasures for the hematopoietic effects of ionizing radiation [5].

Mesenchymal stem cells (MSC) and vascular endothelial cells (VEC) have been studied for potential therapeutic use in hematopoietic recovery following ionizing radiation exposure [3,6,7]. MSCs (derived from bone marrow, adipose tissue, or umbilical cord blood) can improve peripheral blood cell counts, increase bone marrow hematopoietic islands, accelerate the recovery of hematopoietic progenitors, and help restore the bone marrow microenvironment after irradiation [3,6,8-11]. Administration of VEC also enhances hematopoietic recovery and survival after lethal irradiation in mice [7, 12]. The hematopoietic effects of MSC and VEC are believed to occur through several mechanisms including decreased apoptosis and necrosis, increased proliferation of endogenous hematopoietic progenitors, and the modulation of potentially harmful radiation-induced cytokines [9, 12, 13].

Amniotic epithelial cells (AEC) have gained attention as potential therapies in regenerative medicine [13, 14]. A subpopulation of AEC, termed Amnion-derived Multipotent Progenitor cells (AMPs) have been specifically investigated for use in wound healing, tissue regeneration, and immune modulation [13, 15, 16]. Phenotypically, AMPs are epithelial cells expressing surface markers CD104, CD49c, and CD49e, but are negative for hematopoietic markers CD34 and CD45, endothelial marker CD31, and mesenchymal markers CD49d and CD140b [15]. AMPs have the capacity to differentiate into multiple lineages and express stem cell marker stage-specific embryonic antigen-4 (SSEA-4) [16]. When activated with pro -inflammatory cytokines, AMPs display increased expression of immunomodulatory molecules, including the non-classical Class I human leukocyte antigen-G (HLA-G) and programmed death ligand 2 (PD-L2) [13, 15, 16]. AMPs also secrete anti-inflammatory factors such as migration inhibitory factor (MIF) and macrophage inhibitory cytokine-1 (MIC-1). AMPs down-regulated mixed lymphocyte reaction (MLR) and peripheral blood mononuclear cell (PBMC) proliferative responses to mitogen, allo-antigen, and recall-antigen stimuli [15]. When co-cultured with purified monocytes, AMPs induce a regulatory dendritic cell phenotype and co-culture supernatants contain cytokines such as interleukin (IL)-10, IL-6, prostaglandin E2 (PGE_2), and soluble HLA-G [15]. In cell culture studies and murine model systems, AMPs are immunologically tolerated, lacking expression of MHC class II antigens and co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86), and do not stimulate allogeneic PBMCs. Recently, we demonstrated in immunologically conditioned mice that AMPs infusion in combination with sub-clinical doses of allogeneic bone marrow cells supported stable multilineage bone marrow cell chimerism and indefinite allogeneic skin graft survival [15].

Here we investigated the efficacy of AMPs for mitigating hematopoietic toxicity after total body irradiation. We hypothesized that AMPs may potentially enhance the survival and/or proliferation of spared hematopoietic progenitor cells following the acute radiation syndrome. We demonstrate that AMPs accelerated multi-lineage hematopoietic recovery in mice following lethal irradiation.

MATERIALS AND METHODS

Amnion-derived Multipotent Progenitor cells (AMPs)

AMPs were obtained from Stemnion, Inc., with study Institutional Review Board approval (IRB #2010.035; Pittsburgh, PA, USA) and characterized for surface marker expression by flow cytometry as previously described [16. 17]. For AMP cell HLA-G and PD-L2 analysis, cryopreserved AMPs were thawed, washed, and plated in proprietary serum-free media (Stemnion, Inc., USA) in Falcon 6-well plates (Fisher Scientific, Pittsburgh, PA, USA) with 10 ng/ml interferon-gamma (IFN-ɣ; R&D Systems, Minneapolis, MN, USA) for 4 days prior to analysis by flow cytometry.

In vitro AMP cell conditioned media growth factor and cytokine analysis

Cryopreserved amnion-derived cells were

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thawed and cultured in serum-free media at 1×10^6 cells/ml until confluent. Culture conditioned media was taken from cultured AMPs and pooled after 12-15 days and frozen at -20°C until analyzed by RayBio Human Cytokine Antibody Array, G-Series custom 23 analytes (Raybiotech, Norcross, GA, USA) per manufacturer's instructions [18-20]. Conditioned media was diluted 1:2 and 1:40 for analysis. Signal detection was performed using a laser scanner (Axon GenePix 4000B, Molecular Devices, Sunnyvale, CA, USA). Fluorescent intensity values were normalized to positive controls of each measurement. Transforming growth factor (TGF)-β2, hyaluronic acid, SPARC, resolvin-D1, and MIF were measured using enzyme-linked immunosorbent assays (ELISA) (Raybiotech).

Animals and irradiation

All animal experiments were performed in compliance with the Animal Welfare Act, in accordance with the principles in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 2011, and approved by the Armed Forces Radiobiology Research Institute (AFRRI) Institutional Animal Care and Use Committee. Female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were housed in groups of four per cage in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal rooms were maintained at 21 ± 2°C, 50% ± 10% humidity, and 12-h light/dark cycle. Commercial rodent ration (Harlan Teklad Rodent Diet 8604, Harlan Laboratories, Madison, WI, USA) and acidified water (pH = 2.5-3.0), to control opportunistic infections [21], were freely available. Mice, 12–14 weeks of age (17.5-21.5 g) were placed in Lucite jigs for ^{60}Co total body irradiation (TBI) at AFRRI. Controls were sham irradiated. Animals were euthanized using intraperitoneal (i.p.) injection of pentobarbital prior to the collection of tissues.

Animal survival studies

Thirty-day survival studies were performed using 12-14 week-old mice as previously described using 7.75 and 7.9 Gy (0.6 Gy/min) [22]. Four weeks prior to irradiation, mice were randomized for weight and assigned to treatment groups. AMPs (5×10^6 cells/animal) were administered as a single i.p. (100 ml) 24 h after irradiation exposure in vehicle comprised of 100 U/ml preservative-free heparin and 100 U/ml DNase containing 5 mM Mg^{2+}. Treatment groups for 7.75 Gy were: 1) Radiation only (N=16), 2) Radiation + vehicle (N=16), and 3) Radiation + AMPs (N=16). For 7.90 Gy, the same groups were used but the N for groups 1, 2, and 3 were 19, 20 and 20, respectively.

In vivo AMP cell trafficking analysis

C57BL/6J mice were injected with AMPs following 7.75 Gy TBI. This lower dose of radiation was used to ensure sufficient survival of hematopoietic progenitors for analysis. Lung, spleen, bone marrow, thymus, and lymph nodes from 5-12 mice were obtained 7 and 35 days postirradiation. Tissues were stored in RNA Later (Qiagen Science, Germantown, MD, USA). RNA was isolated with TriZol reagent and extracted using Qiagen RNeasy lipid kit (Qiagen). Reverse transcriptase polymerase chain reaction (RT-PCR) was used to convert 1 μg of RNA to cDNA. qRT-PCR for human β-actin was used to detect human AMPs as previously described [13]. Murine β-actin was used for normalization. Spiking of whole tissue with ~1000 AMP cells in total bone marrow allowed the determination of a detection limit of ~1% was obtained [13].

Peripheral blood and bone marrow analysis

Mice were exposed to 7.75 Gy, 0.6 Gy/min TBI. At selected time points, blood was collected in EDTA-containing tubes, as described [22]. Complete blood counts (CBC) with differentials were obtained (Bayer Advia 2120 Hematology Analyzer; Siemens, Tarrytown, NY, USA). Sternebrae were fixed and stored in 10% neutral buffered formalin (pH 7.4), decalcified in 10% formalin/20% EDTA, paraffin embedded, sectioned, and stained with Masson's trichrome or hematoxylin and eosin.

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Hematopoietic progenitor colony-forming cell assays

Mice received 7.75 Gy, 0.6 Gy/min TBI. Femoral bone marrow cells and splenocytes were isolated as described [22]. 1-10 × 10^4 cells per dish were plated in multipotential methylcellulose culture medium (Methocult GF M3434; Stemcell Technologies, Vancouver, BC, Canada). Colony forming unit–granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM), colony forming unit–granulocytic -macrophage (CFU-GM), blast forming unit-erythroid (BFU-e), and total colonies (CFC) were scored 8–10 days of incubation [22]. Clonogenic colony forming cells (CFC) numbers were calculated based on the total viable, nucleated cells per femur or spleen [22].

Flow cytometry for $CD11b^+$ $Gr1^+$ bone marrow cells

Isolated bone marrow (BM) cells were labeled with anti- Ly-6G and Ly-6C - PerCP-Cy™5.5 (clone RB6-8C5; BD Pharmingen, San Diego, CA, USA) and anti-CD11b-PE (clone M1/70; BD Pharmingen) antibodies and analyzed by flow cytometry. 3-7 × 10^5 BM cells were washed and resuspended in ice-cold FACS buffer (1× PBS supplemented with 1% BSA and 0.1% NaN_3). To block non-specific staining, BM cells were preincubated with rat anti-mouse CD16/32 antibody (clone 2.4G2, BD Pharmingen), 30 min on ice, followed by staining with saturating anti-Ly-6G and -Ly-6C and anti-CD11b antibody, 30 min. Cells were then washed twice with ice-cold FACS buffer. 300,000 events were collected using BD Accuri C6 Cytometer (Becton Dickinson, BD, Franklin Lakes, NJ, and USA) and analyzed using FlowJo Software Version 10.0 (Treestar, Ashland, OR, USA).

Statistical analysis

Flow cytometry and colony assay data were analyzed using Student's *t* test, non-paired, unequal variances. A one-tailed Log Rank Test was used for analysis of survival data. Mean survival time (MST) over a 30-day period was also calculated. Hematology and clonogenic CFU assays results are expressed as the mean ± SEM. A value of $P \leq 0.05$ was considered significant.

RESULTS

Phenotypic markers and specific secretory profile of AMPs

AMPs expressed epithelial markers CD9, CD10, CD29, CD49b, CD49c, CD49e, and CD104 and stem cell markers CD90 and SSEA-4 (figure 1). AMPs were negative for endothelial marker CD31, hematopoietic markers CD34 and CD45, and mesenchymal markers CD140b and CD49d. AMPs expressed MHC class I molecules but were negative for MHC class II and co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86). An investigation of immunomodulatory markers showed that IFN-ɣ increased expression of HLA-G and PD-L2 after four days (HLA-G: unstimulated 2.77 ± 1.86; with IFN-ɣ: 55.33 ± 13.3; P = 0.004; PD-L2: unstimulated 1.4 ± 0.29; with IFN-g: 65.13 ± 12.68; P = 0.002).

When cultured in serum-free conditions, AMPs produced growth factors and cytokines involved in tissue regeneration including angiogenin, platelet derived growth factor BB (PDGF-BB), vascular endothelial growth factor (VEGF), transforming growth factor–beta 2 (TGF -β2), amphiregulin, docorin, secreted protein, acidic and rich in cysteine (SPARC), and hyaluronic acid. Anti-inflammatory factors were also detected (macrophage inhibitory cytokine (MIC-1), macrophage migration inhibitory factor (MIF), the protease dipeptidyl peptidase-IV (DPPIV), and the lipid resolving D1)) as well as anti-apoptotic factors (soluble tumor necrosis factor receptor 1 (sTNF-R1), soluble tumor necrosis factor-related apoptosis-inducing ligand receptor-3 (TRAIL-R3), Axl, and tissue inhibitors of metalloproteinases (TIMPs) 1 and 2) (figure 2A, B).

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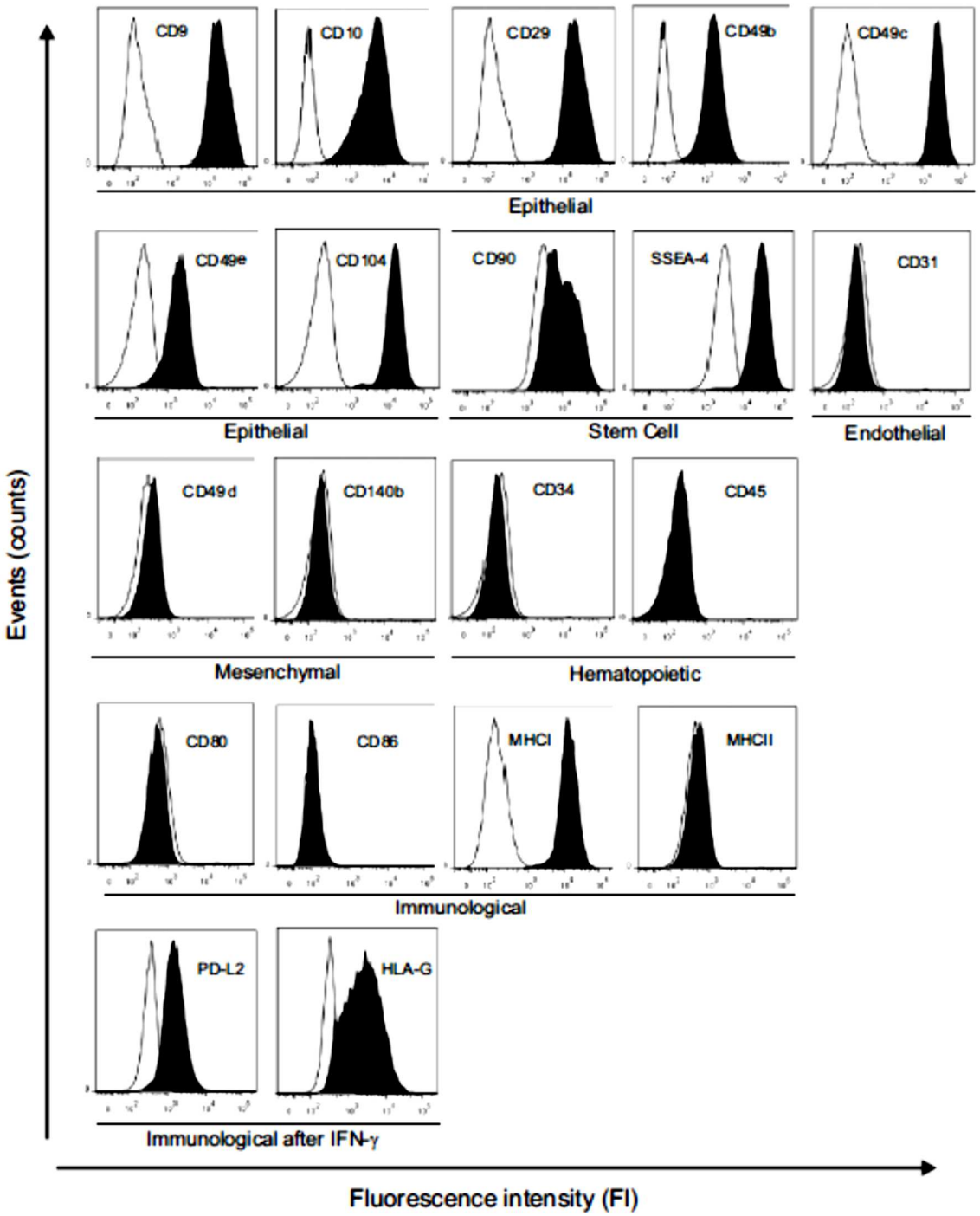

Figure 1. In vitro characterization of AMPs cell surface markers. Representative flow cytometry histogram profiles of cell surface markers of AMP cell compared with antibody isotype controls. Data are a representative of 50 lots of AMP cell cultures, N = 50. Lowest panel: confluent AMPs were treated with IFN-ɣ (10 ng/ml) for 4 days prior to flow cytometry analysis. Representative data are shown for N = 10 lots of AMP cells. CD = Cluster of Differentiation. Cell lineage markers included: Epithelial cell (CD9, CD10, CD29, CD49b, CD49c, CD49e, and CD104); Stem cell (CD90, SSEA-4); Endothelial cell (CD31); Mesenchymal cell (CD49d, CD140b), and Hematopoietic cell (CD34, CD45). Immunology markers included co-stimulatory ligands (CD80, CD86), Major Histocompatibility Complex class I antigen (MHCI) and class II antigen (MHCII), and immunomodulatory ligands: Programmed-death ligand 2 (PD-L2) and Human Leukocyte Antigen G (HLA-G).

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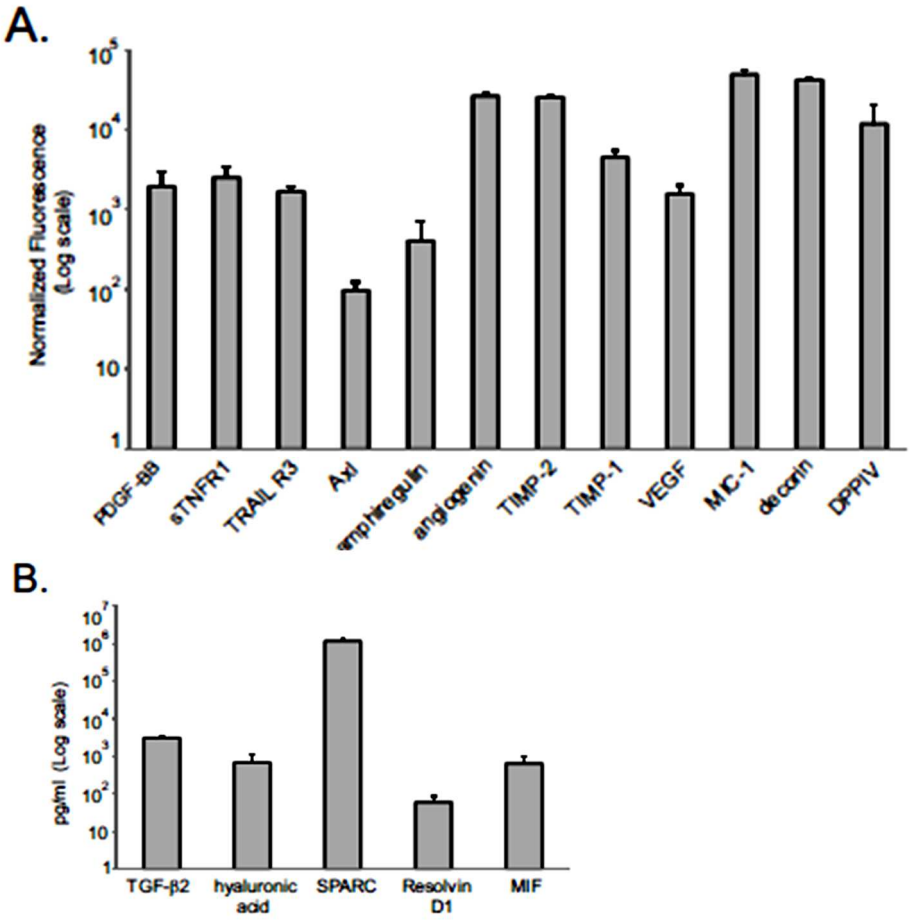

Figure 2. In vitro characterization of AMPs conditioned medium. AMPs were grown to confluence and after 12-15 days the culture conditioned medium was removed and analyzed for the presence of cytokines and growth factors. (A) Detection of growth factors and cytokines using antibody array. The data presented are representative from 30 separate pools of 20 individual AMP cell culture supernatants. (B) Detection of growth factors and cytokines using individual ELISAs. Bar graphs show means ± SD, N=3 separate pools of 20 individual AMP cell culture supernatants.

AMPs improved survival from total body irradiation in mice

We examined the effects of a single i.p. injection of AMPs (5×10^6) given 24 h postirradiation on the survival of mice exposed to 7.75 Gy or 7.90 Gy TBI. At 7.75 Gy, AMPs administration resulted in 69% survival at 30 days and a MST of 28 days. This compared with 56% survival and MST of 26 days for vehicle-treated animals, and 38% survival with MST of 27 days for untreated animals irradiated at 7.75 Gy (figure 3A). At 7.90 Gy, survival for AMP-treated mice at 30-days postirradiation was 75% (figure 3B) with a MST of 29 days. This compared with 55% survival and MST of 26 days for vehicle-treated animals, and 42% survival and a MST of 25 days for untreated irradiated mice. At 7.90 Gy there was a significant improvement in survival for AMPs-treated animals compared to untreated irradiated mice ($P < 0.03$), but not when compared with vehicle-treated mice.

In vivo AMP trafficking

We evaluated AMPs trafficking to hematopoietic tissues or the lung following 7.75 Gy irradiation to allow sufficient survival of hematopoietic progenitors for analysis in later studies. AMPs were administered by i.p. injection 24 h postirradiation and tissues were evaluated 7 and 35 days postirradiation. AMPs were not detected in the lung, spleen, bone marrow, or thymus at either 7 or 35 days (N=12 for both; data not shown). Evaluation of the

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lymph nodes at 7 and 35 days also showed no detection of AMPs (N=8 and N=5, respectively; data not shown).

Effect of AMPs on hematopoietic cell recovery in irradiated mice

To study the effects of AMPs on hematopoietic cell recovery, mice were irradiated at 7.75 Gy and injected i.p. with AMPs or vehicle at 24 h postirradiation. At days 7, 14, 20, 34, and 41 postirradiation, peripheral blood was collected. Baseline total bone marrow cellularity was determined for comparison at day 0 in untreated, non-irradiated mice. Blood hematology showed no differences between the AMPs and vehicle-treated control mice over the first 14 days postirradiation (figure 4). We observed trends toward improved early recovery of white blood cells (WBC), neutrophils, reticulocytes, and monocytes in AMPs-treated mice compared with vehicle treatment that did not reach significance (figure 4A, B, E, G). At day 20, AMPs-treated mice displayed a modest increase in platelets (PLT) and reticulocytes compared with vehicle-treated animals, but these differences did not reach significance. This was followed by a rapid increase at 40 days in WBC, red blood cells (RBC), hematocrit (HCT), reticulocytes, PLT, neutrophil, and monocyte counts in both vehicle - and AMPs-treated mice.

We evaluated the effects of AMPs on spleen total cellularity and hematopoietic progenitor recovery (figure 5A-E). Although there were higher levels of total splenic cellularity at days 30 and 40 with AMPs compared to vehicle, these values were not statistically significant (figure 5A). At 14 days, AMPs improved the recovery of total splenic CFC vs. vehicle controls 179 ± 69 cells vs 29 ± 13 cells, $P < 0.05$) and CFU-GEMM (21 ± 10 cells vs. 0 ± 0 cells, $P < 0.05$) (figure 5B and C, respectively). Mean values for these groups showed a marked improvement with AMP cells also at day 40 as well, but due to the high variability within the AMP treated groups, failed to reach statistical significance. We observed trends toward improved recovery of Splenic CFU-GM and BFU-e at 30-40 days with AMPs, but these did not reach statistical significance again due to high standard deviations (figure 5D and E).

In the bone marrow, we observed that AMPs treatment resulted in a trend toward accelerated femoral cellularity and recovery of several progenitors: CFU-GEMM, CFU-GM, and BFU-e at 30-40 days (figure 6). The increased recovery of the bone marrow progenitors were reflected in histological sections of the bone marrow. Increased overall cellularity was observed between 7-14 days in the sternabrae of mice treated with AMPs (figure 7).

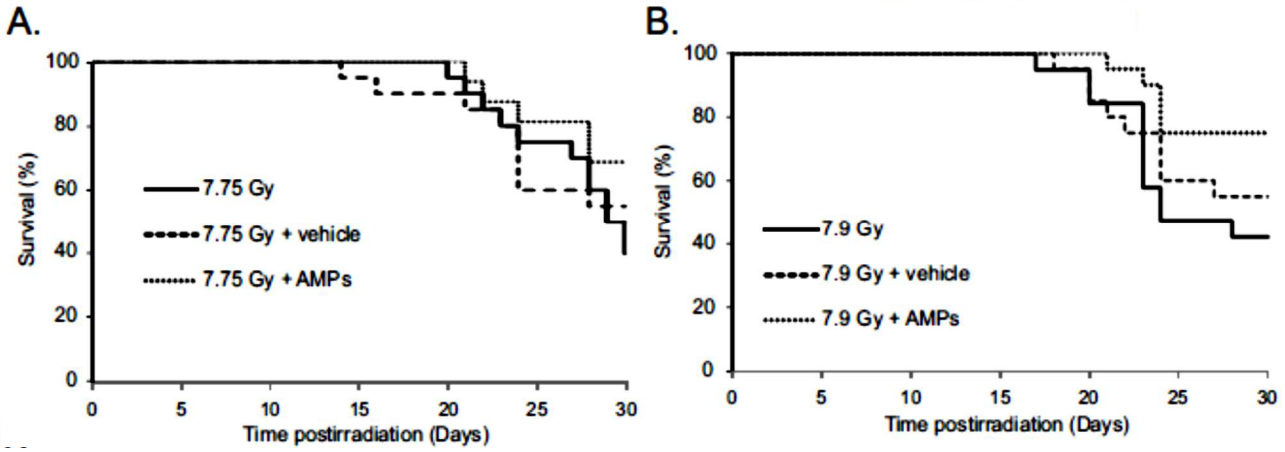

Figure 3. AMPs effects on survival following total body irradiation. Mice were exposed to 7.75 (A) or 7.90 Gy (B) total body irradiation. Mice were divided into three groups: untreated (7.75 Gy, N=16; 7.90 Gy, N=19), treated with vehicle (7.75 Gy, N=16; 7.90 Gy, N=20), or treated with AMPs (7.75 Gy, N=16; 7.90 Gy, N=20). Vehicle alone and AMP cells were administered by i.p. injection 24 h postirradiation. Kaplan-Meir survival curves are shown.

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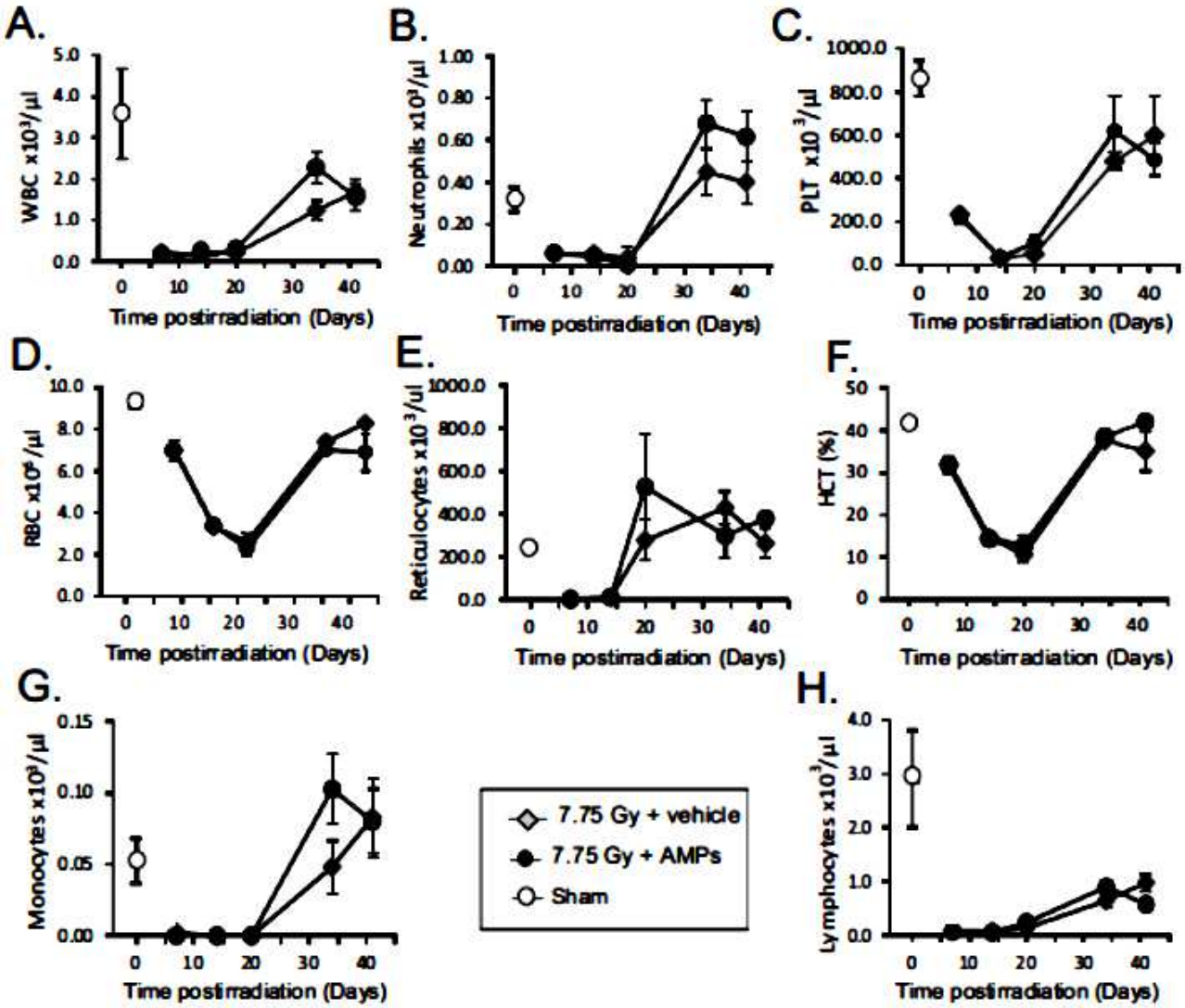

Figure 4. AMPs effects on mature blood cell recovery after 7.75 Gy total body irradiation. Effects of a single infusion of AMPs on peripheral blood cell recovery in high dose irradiated mice. 24 h after 7.75 Gy irradiation mice were infused i.p. with vehicle containing no cells or AMPs (5 x 106). At the indicated time points, blood was analyzed for mature blood cell populations: (A) white blood cells (WBC); (B) neutrophils; (C) platelets (PLT); (D) red blood cells (RBC); (E) reticulocytes; (F) hematocrit (HCT); (G) monocytes; (H) lymphocytes. The data represent the mean of 6 mice per time point ± SEM, N = 4-5 mice.

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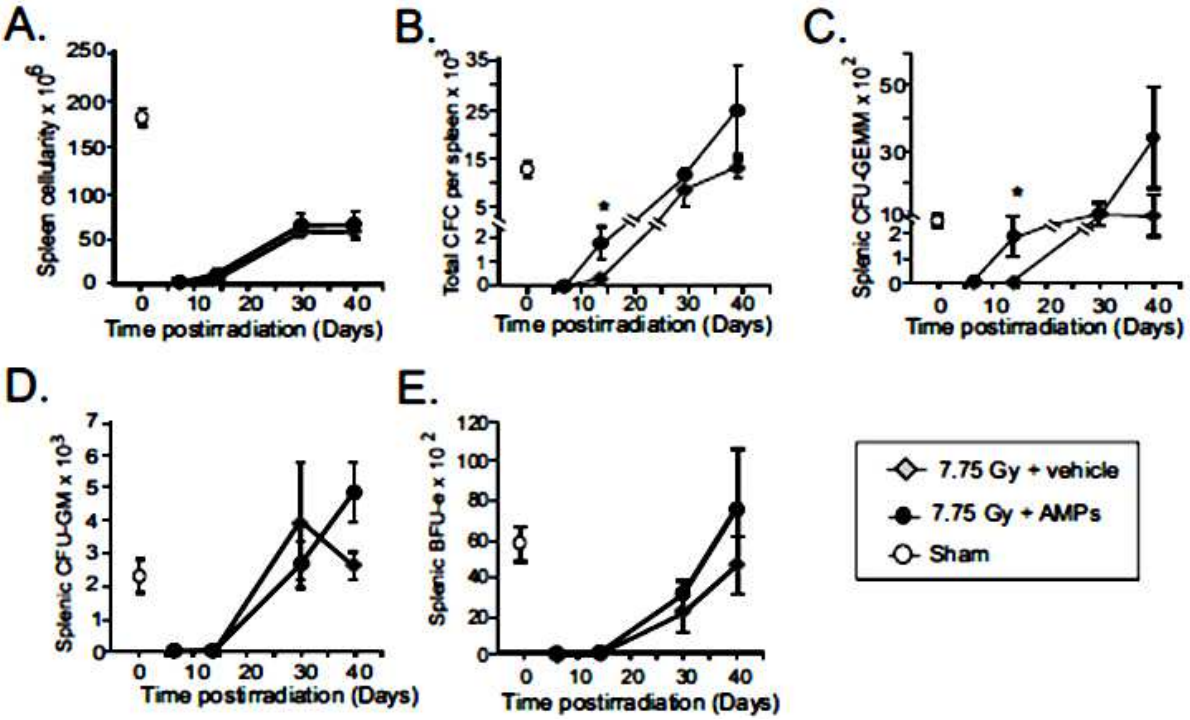

Figure 5. Extramedullary splenic hematopoiesis following AMPs treatment in irradiated mice. C57BL/6J mice were exposed to 7.75 Gy TBI. Mice were administered vehicle (control) or AMP (i.p.) 24 h postirradiation. A-E. Spleens were removed at the indicated times postirradiation and cellularity and CFC progenitor cell content was determined: (A) total cellularity; (B) total colony forming cells (CFC); (C) splenic CFU-GEMM; (D) splenic CFU-GM; (E) splenic BFU-e. Data are nucleated cell counts or CFU per spleen, and show means ± SEM. * indicates statistical significance from vehicle control. N = 10.

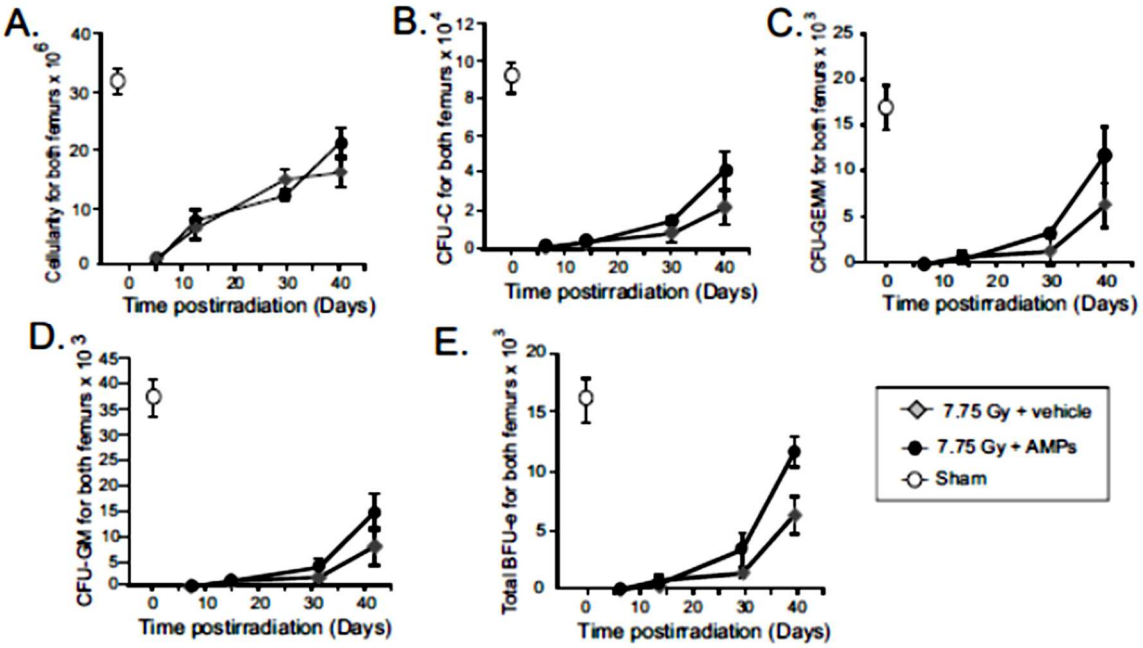

Figure 6. Hematopoietic progenitor cell recovery following AMPs treatment in irradiated mice. C57BL/6J mice were exposed to 7.75 Gy TBI. Mice were administered vehicle or AMPs (i.p.) 24 h postirradiation. Bone marrow was removed at the indicated time points, and cellularity or progenitor cell content for both femurs was determined: (A) total cellularity; (B) total CFC; (C) CFU-GEMM; (D) CFU-GM; (E) BFU-e. Data indicate total nucleated cells or CFU per animal, and show means ± SEM, N = 4-5. One of two independent experiments is shown with similar results.

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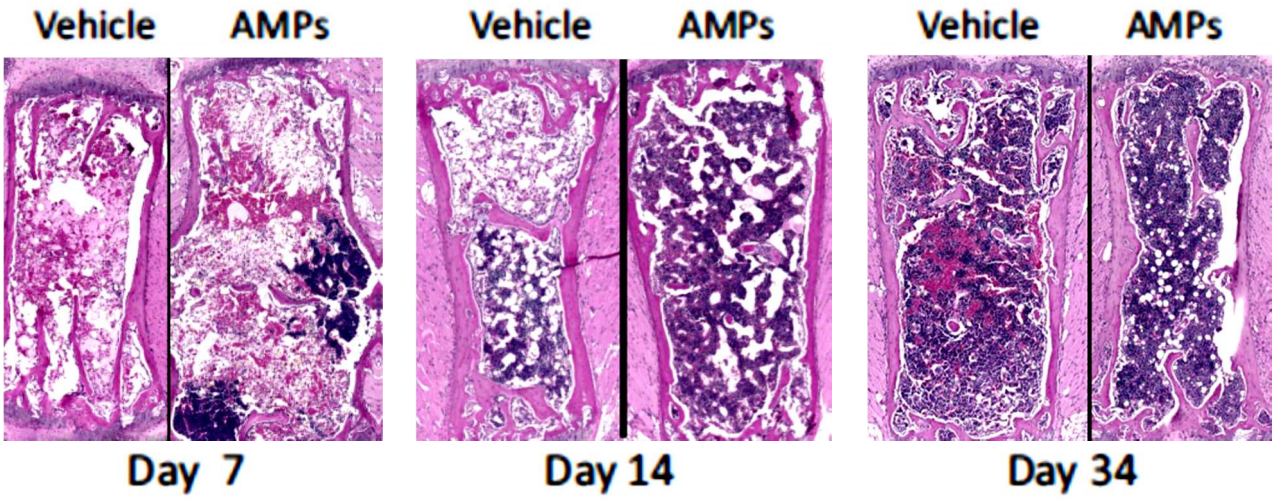

Figure 7. Bone marrow hematopoietic repopulation following high dose radiation exposure with AMPs treatment. Representative histology sections of sternebrae excised at day 7, 14 and 34 postirradiation (7.75 Gy). Tissues were fixed in 10% neutral buffer formalin, decalcified, embedded in paraffin, sectioned at 5 µm, and then stained with Masson's trichome. Representative images are shown.

We conducted further analysis of specific progenitors. Gr-1^{+}CD11b^{+} cells represent about 20-30% of normal bone marrow cells, and are a heterogeneous population of myeloid cells in various maturation states (23). Based on the Gr-1 expression intensity the myeloid cells can be divided into 3 subsets based on high, intermediate or negligible Gr-1 expression (CD11b^{+}/Gr-1^{high}, CD11b^{+}/Gr-1^{int}, and CD11b^{+}/Gr-1^{-}, respectively)(24). The CD11^{+}/Gr-1high cells are mostly granulocytes and neutrophils; CD11b^{+}/Gr-1^{int} cells are mainly monocytes and myeloid precursors; CD11b^{+}/ Gr-1^{-} cells are primarily cells with monocytic morphology [24]. The gating strategy is shown in Figure 8A. TBI resulted in a nadir for CD11b^{+}/Gr-1^{-}, CD11b^{+}/Gr-1^{int}, and CD11^{+}/Gr-1^{high} populations by day 14 (figure 8). AMPs treatment significantly mitigated the reduction in both the CD11b^{+}/Gr-1^{int} and the CD11b^{+}/Gr-1^{high} populations at the nadir, and improved recovery of both of these populations at 40 days (figure 8C, D). In the CD11b^{+}/Gr-1^{high} population at day 14 AMPs treatment resulted in 17.1 ± 6.3% vs 2.1 ± 0.6% ($p<0.05$); at day 40, AMPs treatment resulted in 43.1 ± 7.3% vs 28.2 ± 5.1% ($p<0.05$). In the CD11b^{+}/Gr-1^{int} population at day 7 postirradiation AMPs treatment resulted in 2.5 ± 0.1% vs 2.0 ± 0.1% ($p<0.05$); at day 40, AMPs treatment resulted in 10.0 ± 1.7% vs 5.2 ± 1.9% ($p<0.05$).

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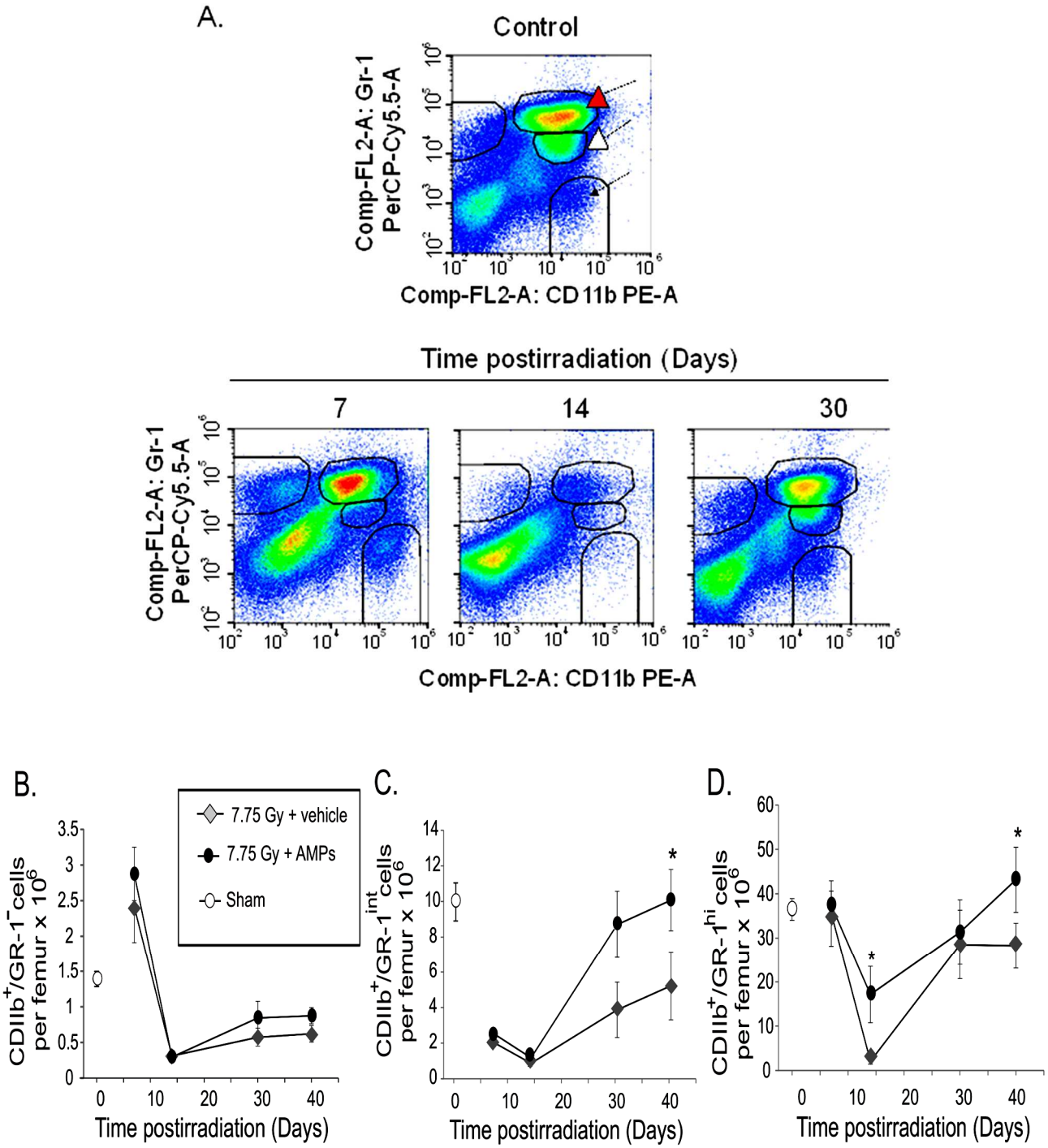

Figure 8. Bone marrow granulopoiesis in AMP-treated irradiated mice. Bone marrow collected from vehicle-treated or AMP-treated mice irradiated at 7.75 Gy was analyzed for expression of CD11b and GR-1 expression using polychromatic flow cytometric analysis. (A) Dot plot analysis of bone marrow of sham irradiated bone marrow and bone marrow obtained at specific time points postirradiation. FSC-A versus SSC-A gating strategy was designed to exclude debris and aggregates and examine the expression of $CD11b^{+}Gr\text{-}1^{+}$ cells. $CD11b^{+}Gr\text{-}1^{lo}$ (black arrow); $CD11b^{+}Gr\text{-}1^{int}$ (white arrow); $CD11b^{+}Gr\text{-}1^{hi}$ (red arrow). (B) granulocyte-macrophage progenitors ($CD11b^{+}/Gr\text{-}1^{-}$); (C) monocytes and myeloid precursors ($CD11b^{+}Gr\text{-}1^{int}$); (D) mature granulocytes/neutrophils ($CD11b^{+}Gr\text{-}1^{hi}$) per femur. Data indicate number of cells per mouse, and show means ± SEM. * indicates statistical significance from vehicle-treated animals at the same time point, N = 4-5.

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DISCUSSION

In radiation-induced acute hematopoietic syndrome the loss of mature blood cells is a significant part of radiation morbidity, and mortality is believed to occur due to prolonged myelosuppression from the loss of hematopoietic progenitors and stem cells [25-28]. Here we examined the effects of AMPs on survival and hematopoietic recovery in a murine model of acute radiation injury. AMPs improved the recovery of hematopoietic progenitor populations $CD11b^{+}/Gr\text{-}1^{int}$ (monocytes and myeloid precursors) and the $CD11b^{+}/Gr\text{-}1^{high}$ (granulocytes and neutrophils) populations in the bone marrow as determined by FACS analysis, and total splenic and splenic CFU-GEMM colony formation assays.

Our findings indicate that AMPs enhanced the recovery of narrow subset of hematopoietic progenitors following total body irradiation. We observed a trend toward improved survival, improved bone marrow cellularity, and recovery of other hematopoietic progenitors following total body irradiation, but these trends did not reach significance. This suggests that although specific hematopoietic cell recovery is enhanced by AMPs, recovery of this cellular subset alone is not sufficient to induce full hematopoietic recovery after total body irradiation. Other laboratories have examined the mitigation of radiation-induced hematopoietic injury by the injection of MSC, MSC-like populations, or VEC. Infusion of MSC was demonstrated to improve recovery of CFU-GM and bone marrow fibroblasts following 5.5 Gy TBI [3]. This investigation also showed trends toward improved recovery of peripheral blood cells (WBC, HGB, and PLT) that did not reach significance [3]. In a separate study examining the effects of MSCs following 7 Gy TBI in mice, improved recovery was observed for WBC, total lymphocytes, and total monocytes [9]. VEC treatment improved total bone marrow cellularity and the recovery of WBC and PLT following 7 Gy TBI in mice [7]. An intramuscular injection of adherent placental stromal cells increased hematopoietic progenitors and improved the recovery of WBC, PLT, and RBC at ~24 days following an $LD_{70/30}$ dose of TBI in mice [18]. These studies, combined with our findings, suggest that different hematopoietic populations may be protected or have enhanced recovery depending upon the therapeutic stem cell type administered, with some adult stem cells having more potent effects on hematopoietic recovery.

The effects we observed for AMPs did not require their engraftment. Similar results were obtained with MSCs, where the expansion, self-renewal, and differentiation of spared marrow HSC following lethal TBI did not require MSCs to engraft in the recipient marrow [6, 18, 29, 32]. The lack of evidence for engraftment has led to the hypothesis that cellular therapies function through indirect regulatory mechanisms. Secreted factors, growth factors and/or immune modulators, have been hypothesized to contribute to the survival/repopulation of marrow progenitors and cells in the marrow microenvironment. *In vitro*, MSCs produce essential hematopoietic factors including IL-6, FGF-7, MCP-3, IL-11, LIF, SCF, Flt3 ligand, and SDF [3, 18]. VECs were demonstrated to produce VEGF, PDGF-AA, SDF-1, and IL-6 [7]. Cultured AMPs produce detectable levels of angiogenin, PDGF-BB, VEGF, TGF-β2, amphiregulin, decorin, and hyaluronic acid, as well as anti-apoptotic and protective factors. However, the administration of cytokines associated with VEC-induced hematopoietic recovery (without VECs), was ineffective [7, 12]. This suggests that secreted factors alone may not be responsible for the survival benefit observed with cellular therapies. The ability of AMPs, and other therapeutic cells, to modulate peripheral hematopoietic cells, potentially via cell-cell contacts, may be a critical component for hematopoietic progenitor survival and expansion.

The placenta plays an important role in hematopoiesis and development, and is a source of pluripotent HSCs [33-39]. The placenta harbors a large pool of pluripotent HSCs with the capacity to self-renew and repopulate the entire hematopoietic system in irradiated hosts, but the *in vivo* potential of placenta-derived AMPs was not previously explored [33-37]. Our findings

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suggest that AMPs may provide enhanced recovery for only a narrow range of hematopoietic cell populations.

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