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Mitigation of Hematopoietic Syndrome of Acute Radiation Syndrome by 1-Palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) is Associated with Regulation of Systemic Inflammation in a Murine Model of Total-Body Irradiation

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The growing risk of accidental radiation exposure due to increased usage of ionizing radiation, such as in nuclear power, industries and medicine, has increased the necessity for the development of radiation countermeasures. Previously, we demonstrated the therapeutic potential of the acetylated diacylglycerol, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG), as a radiation countermeasure by mitigating radiation-associated mortality and hematopoietic acute radiation syndrome (H-ARS) in BALB/c mice after a lethal dose ($LD_{70/30}$) of gamma-ray total-body irradiation (TBI). In this study, we show that PLAG mitigates symptoms of H-ARS, as characterized by mature blood cell recovery and restoration of bone marrow cellularity, by regulating systemic inflammation. Log-rank test demonstrated that high levels of WBCs, lymphocytes and neutrophils on day 10 post-TBI resulted in significantly improved survival rate. PLAG significantly enhanced the nadir values of all major blood cell types as well as bone marrow cellularity. A single TBI at $LD_{70/30}$ induced an immediate increase in the blood levels of CXCL1 (12.5 fold), CXCL2 (1.5 fold), IL-6 (86.9 fold), C-reactive protein (CRP; 1.3 fold) and G-CSF (15.7 fold) at 6 h post-TBI, but the cytokine levels returned to baseline level afterward. When the irradiated mice started to die around 15 days post-TBI, they exhibited a second surge in blood levels of CXCL1 (49.3 fold), CXCL2 (87.1 fold), IL-6 (208 fold), CRP (3.6 fold) and G-CSF (265.7 fold). However, PLAG-treated groups showed a significant decrease in these same blood levels ($P < 0.001$). Considering the inverse correlation between inflammatory cytokine levels and hematological nadirs, PLAG exerts its therapeutic effects on H-ARS by regulating inflammatory cytokine production. These data suggest that PLAG has high potential as a radiation countermeasure to mitigate H-ARS after accidental exposure to radiation.

INTRODUCTION

There is a growing risk of radiation exposure due to rapidly increasing usage of ionizing radiation in various settings such as nuclear power, industries and medicine (*1, 2*). While adequate and well-controlled use of radiation has provided many benefits to humans, accidental overexposure produces detrimental and life-threatening effects to living organisms (*3*). Global exposure of a significant dose of radiation in a short time results in a series of serious clinical complications, which are collectively termed acute radiation syndrome (ARS) (*1, 4*). Hematopoietic syndrome of ARS (H-ARS), which occurs at a dose between 1 and 10 Gy, results from the damage in the hematopoietic system, including the mature blood cells and hematopoietic stem cells in the bone marrow that are responsible for blood cell regeneration (*5*). Without supportive care, the hematopoietic insufficiency caused by radiation eventually leads to death within 30 days in the murine system by allowing a life-threatening opportunistic infection and hemorrhage in the vital organ (*5–7*). To date, there are only three drugs, two granulocyte colony-stimulating factor (G-CSF) biosimilars (filgrastim and pegfilgrastim) and one granulocyte-macrophage colony-stimulating factor (GM-CSF) biosimilar (sargramostim), which have been approved by the U.S. Food and Drug Administration (FDA) as countermeasures against H-ARS (*8, 9*). These drugs increase survival by shortening the time at which blood cell populations are at the nadir. However, a growing body of literature has

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indicated that G-CSF treatment after radiation exposure or cytotoxic chemotherapy aggravates long-term bone marrow damage by exhausting the self-renewal capacity of hematopoietic stem cells (*10–12*). Therefore, there is an urgent need to develop a radiation countermeasure with superior safety profiles as well as ease of distribution and administration.

Radiation has a tremendous effect on the immune system by activating a variety of cytokine cascades (*13, 14*). Radiation-exposed cells and tissues rapidly express pro-inflammatory cytokines, chemokines and growth factors such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, IL-8, the chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2, G-CSF and vascular endothelial growth factor (VEGF) (*15*). Pro-inflammatory cytokines are produced to resist radiation damage and persist until the perceived threats to host integrity are eliminated (*16*). In general, the cytokine production peaks between 4 and 24 h postirradiation and subsequently decreases to baseline levels within 24 h to a few days (*15*). However, cytokine production profiles and their effects at later times have not been fully investigated. In this study, we investigated long-term profiles of cytokine production in mice for 21 days after an $LD_{70/30}$ dose (6.11 Gy) of ^{60}Co total body irradiation (TBI) as well as how the administration of 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) changes the cytokine production profiles after irradiation. Moreover, we performed statistical analyses to identify an association between the cytokine levels and the development of H-ARS.

PLAG, also named EC-18, is a small lipid molecule naturally occurring in the antlers of sika deer and has been shown to enhance hematopoietic activity of stem cells (*17*). It is chemically synthesized from glycerol, palmitic acid and linoleic acid in large quantities (*17*). The formula for synthesizing PLAG is owned by Enzychem Lifesciences Corp. (Daejeon, Republic of Korea). PLAG has been certified and approved as an immune-modulating functional food by the Korean Food and Drug Administration, and was granted Orphan Drug Designation for the treatment of ARS by the FDA in 2018. To test the therapeutic efficacy of PLAG in ARS, we previously performed animal experiments which demonstrated that PLAG (50 or 250 mg/kg/day) improved survival rates in mice that had received an $LD_{70/30}$ dose of TBI, when the PLAG was orally administered from 0–72 h postirradiation through 30 days postirradiation (*18*). We also demonstrated that after $LD_{70/30}$ TBI, PLAG improved mature blood cell recovery, including neutrophils, platelets and red blood cells (RBCs). However, in that previously published study, it was not disclosed whether the improved survival rates in PLAG-treated mice were the consequence of the enhanced recovery of mature blood cells, or if the improvement in survival and blood cell counts had no relevance to each other. We also did not demonstrate whether PLAG protects the depletion of WBC counts, including lymphocytes and monocytes, from radiation despite the fact that these cells constitute the largest population in the blood. Therefore, we conducted further statistical analysis to investigate whether early changes in blood cell counts help determine survival prognosis in irradiated animals. As a result, we found that the levels of WBCs, lymphocytes and neutrophils can be predictors of survival, and hematological nadirs are negatively correlated with inflammatory cytokines/chemokines. Furthermore, we found that PLAG effectively mitigates H-ARS, as characterized by mature blood cell recovery and restoration of bone marrow cellularity, and radiation-induced surge in inflammatory cytokines/chemokines.

MATERIALS AND METHODS

Animals and Ethics Statement

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (The ethic code is KRIBB-AEC-20051) and were performed in compliance with the Guide for the Care and Use of Laboratory Animals by the National Research Council and Korean national laws for animal welfare. Details about mice and their housing and care are described elsewhere (*18*).

Animal Experiment

Irradiation and PLAG administration were performed as previously described elsewhere (*18*). Briefly, male or female BALB/c mice (11 weeks old) were placed in single chambers of a lead-shielded irradiation apparatus and received a single, uniform dose of total-body γ-ray irradiation from a ^{60}Co source (J.L. Shepherd & Associates, San Fernando, CA) at a dose rate of 0.833 Gy/min. Dose rates were measured using a EPD Mk2+ electronic dosimeter (Thermo Scientific™, Waltham, MA). PLAG was obtained from Enzychem Lifesciences Corp. (Jecheon, South Korea) and re-suspended in sterile phosphate buffered saline (PBS). Mice received PLAG [50, 100, 250 or 500 mg/kg, oral (p.o.) administration] or vehicle (sterile PBS; 0.1 ml/mouse, p.o.) beginning 24 h postirradiation and continuing daily until 21 days postirradiation. Approximately 200 μl of whole blood was collected from the orbital sinuses using EDTA-free capillary tubes (Kimble Chase Life Science and Research Products LLC, Vineland, NJ) and collection tubes containing K3EDTA (Greiner Bio-One International, Kremsmünster, Austria). Due to the relatively large amount of blood sampling, blood collection was performed on different animals for each time point. The blood cells were counted and classified by complete blood count (CBC) analysis using Mindray BC-5000 Vet auto-hematology analyzer (Shenzhen Mindray Biomedical Electronics, Guangdong Sheng, China). After CBC analysis, plasma was separated from the whole blood by centrifugation and stored at –80°C for further analysis.

Histological Analysis

The femurs of each mouse were removed after euthanasia, and then placed in 10% formalin overnight. The samples were transferred to Korea Pathology Technical Center (Cheongju, South Korea) for decalcification, paraffin embedding and hematoxylin and eosin (H&E) staining. The images of femur sections were captured using a Leica ICC50W microscope (Wetzlar, Germany). Histological analysis of bone marrow was conducted using ImageJ software version 2 (National Institutes of Health, Bethesda, MD) by selecting hematoxylin-stained cells in the bone marrow using a color threshold.

TABLE 1
Comparison of Hematologic Nadirs in Irradiated mice Treated with PLAG (50, 100, 250 and 500 mg/kg)

		PLAG-treated (mg/kg)			
No. of animals	Control 13/40 (32.5%)	50 24/40 (60%)	100 27/40 (67.5%)	250 34/40 (85%)	500 34/40 (85%)
WBC ($\times 10^3$/μl)					
Mean (SE)	0.26 (0.01)	0.29 (0.01)	0.26 (0.01)	0.39 (0.02)	0.31 (0.01)
Median	0.26	0.27	0.26	0.37	0.3
Minimum, maximum	0.13, 0.4	0.13, 0.54	0.13, 0.39	0.19, 0.71	0.13, 0.49
P value		0.0858	0.8586	<0.0001***	0.0139*
Lymphocytes ($\times 10^3$/μl)					
Mean (SE)	0.15 (0.01)	0.16 (0.01)	0.15 (0.01)	0.19 (0.01)	0.16 (0.01)
Median	0.15	0.17	0.15	0.18	0.16
Minimum, maximum	0.03, 0.22	0.07, 0.27	0.06, 0.24	0.11, 0.32	0.06, 0.28
P value		0.1245	0.9773	0.0001***	0.1412
Neutrophils ($\times 10^3$/μl)					
Mean (SE)	0.03 (0.003)	0.04 (0.004)	0.03 (0.003)	0.065 (0.004)	0.04 (0.004)
Median	0.02	0.03	0.03	0.06	0.03
Minimum, maximum	0.01, 0.09	0.001, 0.12	0.001, 0.11	0.01, 0.11	0.001, 0.14
P value		0.0811	0.794	<0.0001***	0.1072
Monocytes ($\times 10^3$/μl)					
Mean (SE)	0.017 (0.002)	0.017 (0.002)	0.015 (0.002)	0.024 (0.002)	0.016 (0.001)
Median	0.02	0.02	0.01	0.02	0.02
Minimum, maximum	0.001, 0.04	0.001, 0.04	0.001, 0.04	0.001, 0.06	0.001, 0.04
P value		0.9756	0.2964	0.0116*	0.6505
Platelets ($\times 10^3$/μl)					
Mean (SE)	36.4 (2.5)	37.1 (2.1)	36.2 (1.7)	57.5 (2.7)	38.7 (1.6)
Median	32	34	32.5	57	41
Minimum, maximum	8, 74	21, 79	21, 69	24, 103	15, 57
P value		0.8251	0.9545	<0.0001***	0.4456
RBCs ($\times 10^6$/μl)					
Mean (SE)	3.85 (0.23)	3.26 (0.15)	3.77 (0.13)	4.55 (0.14)	3.71 (0.17)
Median	3.78	3.18	3.63	4.59	3.69
Minimum, maximum	1.1, 6.93	1.41, 6.33	1.76, 6.36	2.27, 6.05	1.71, 5.61
P value		0.0395*	0.7486	0.0128*	0.6198

Measurement of Plasma Cytokine Levels

As described elsewhere (*19*), the concentration of murine CXCL1, CXCL2, IL-6 and G-CSF in mouse plasma was measured using the Mouse Premixed Multi-Analyte Kit (R&D Systems™, Minneapolis, MN) according to the manufacturer's instructions. Fluorescence intensities were measured using a MAGPIX® multiplexing system (Luminex® Inc., Austin, TX). The concentration of murine C-reactive protein (CRP) in mouse plasma was measured using a CRP ELISA kit (R&D Systems) according to the manufacturer's instructions. Optical densities were measured at 450 nm using a Bio-Rad® Model 550 microplate reader (Hercules, CA). The concentration of analytes in plasma were calculated from standard curves generated by a curve-fitting program.

Assessment of the Association of Blood Cells and Day 30 Survival Status

For the extended analysis on the association of blood cells and day 30 survival status, a portion of the data presented was based on the same cohort of mice as previously reported (*18*), and the remaining data were from unpublished PLAG dose efficacy data (PLAG 100 and 500 mg/kg). Table 1 summarizes the study disposition used for the meta-analysis. Distributions of the blood cell values on day 10 or 12 post-TBI were assessed and $\log_{10}$ transformed to render the parametric statistical analysis. The blood cell values on day 10 or 12 post-TBI were chosen because all mice appeared to develop hematological nadirs during this critical period. The blood cells were then categorized at the level of 0.38×10^3 cells/μl, 0.23×10^3 cells/μl, 0.07×10^3 cells/μl, 0.04×10^3 cells/μl, 45×10^3 cells/μl and 6.52×10^6 cells/μl for WBCs, lymphocytes, neutrophils, monocytes, platelets and RBCs, respectively. These cutoff values were chosen based on the median/interquartile range (IQR) for each blood cell distribution, and high versus low values for each blood cell type were separated using the cutoff above/below the median. Then, Kaplan-Meier plots along with log-rank tests were conducted to assess how survival probabilities differ between the high and low levels of each blood cell type over the follow-up period, with the error band indicating the corresponding two-sided 95% confidence interval. The Cox proportional hazards model was used to estimate the relative death risk of low blood cell levels compared to that of high blood cell levels. This analysis was performed using GraphPad Prism version 8.4.3 (La Jolla, CA).

Other Statistical Analyses

For statistical analysis of hematologic and cytokine data, unpaired Student's *t* test or one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test were performed using GraphPad Prism version 8.4.3. $P < 0.05$ was considered statistically significant, and the results are expressed as the mean ± SD. The correlation between two continuous variables was determined by Spearman's correlation coefficient.

RESULTS

Association between Blood Cell Levels and Risk of Death

The hematopoietic insufficiency caused by radiation increases mortality by increasing the risk of opportunistic

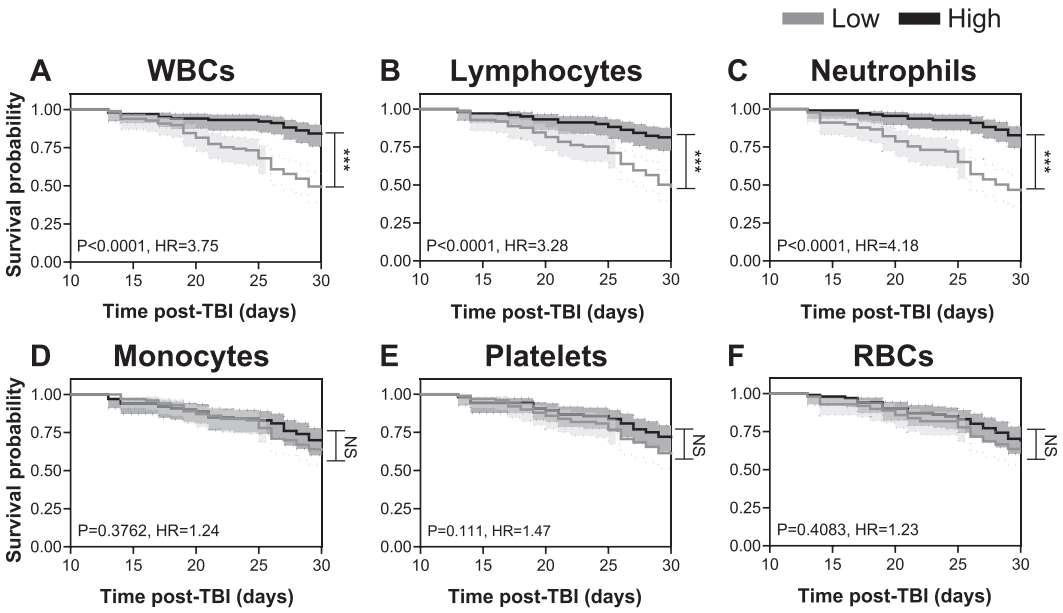


FIG. 1. Blood cells and survival. Survival curves were based on each blood cell measured (n = 200). Cox regression model shows overall survival with confidence intervals (CIs) for each blood cell based on time from TBI to last follow-up date (death or still survived), with significance indicated by *P* value and hazard ratio (HR). The number of cells were measured using CBC analyzer, and on day 10 or 12 post-TBI, when all blood cell types except RBCs reached the nadir, the high versus low cell numbers were determined using a cutoff above the median for each blood cell. Survival was lower if the number of circulating (panel A) WBCs, (panel B) lymphocytes and (panel C) neutrophils was low (gray line, below cutoffs of 0.38×10^3 cells/μl for WBCs, 0.23 $\times 10^3$ cells/μl for lymphocytes, 0.07×10^3 cells/μl for neutrophils) versus high (black line, above cutoffs). However, there was little relationship between survival and (panel D) monocytes, (panel E) platelets and (panel F) RBCs (cutoff value of 0.04×10^3 cells/μl for monocytes, 45×10^3 cells/μl for platelets, and 6.52×10^6 cells/μl for RBCs). Each line indicates the predicted survival probability over follow-up time, with the error band indicating the corresponding two-sided 95% CI.

infection and vascular permeability leading to hemorrhage (*5–7*). To further characterize the relationship between blood cell levels and survival in our H-ARS model, each blood cell was classified into high versus low values based on the cutoff above the median value, and then survival curves were re-drawn to assess how survival prognosis differs depending on the levels of each blood cell types. The blood cell values on day 10 or 12 post-TBI were chosen because most blood cell populations, except RBCs, reached the nadir while no mice had yet died at that time. Stratifying mice by blood cell levels of high versus low using the cutoffs as described in Materials and Methods, we found that low numbers of WBCs [hazard ratio (HR) = 3.75, $P < 0.0001$), lymphocytes (HR = 3.28, $P < 0.0001$) and neutrophils (HR = 4.18, $P < 0.0001$)] were significantly associated with poor survival (Fig. 1A–C). On the other hand, the cell counts for monocytes (HR = 1.24, $P = 0.3762$), platelets (HR = 1.47, $P = 0.111$) and RBCs (HR = 1.23, $P = 0.4083$) on day 10 or 12 postirradiation did not predict the overall survival of the mice (Fig. 1D–F). These findings suggest that the peripheral blood levels of WBCs, lymphocytes and neutrophils are strong predictors of radiation-associated death.

Administration of PLAG Improves Peripheral Blood Cell Recovery in Irradiated Mice

Since it was verified that peripheral blood cell counts, especially WBCs, lymphocytes and neutrophils, could be predictive biomarkers for overall survival in H-ARS, the radio-mitigating efficacy of PLAG on H-ARS was tested in mice that received an $LD_{70/30}$ dose of TBI. PLAG was previously demonstrated to have mitigating effects on radiation-induced neutropenia, thrombocytopenia and anemia (*18*), but the efficacy of PLAG on WBC and lymphocyte counts has not been described, despite the fact that these cells constitute the largest population in the total blood. We first analyzed the nadirs of all blood cell types in irradiated mice treated with PLAG 50, 100, 250 and 500 mg/kg for 30 days post-TBI, as shown in Table 1. PLAG 250 mg/kg most effectively and significantly improved the nadirs of all blood cell types, which resulted in the most improved survival status. Next, the peripheral blood cell levels between 6.11 Gy TBI-only mice and irradiated PLAG 250 mg/kg-treated mice were compared from day 10 through day 21 because all irradiated mice developed hematological nadirs during this period, which corresponds to the time of highest mortality in our H-ARS model. In

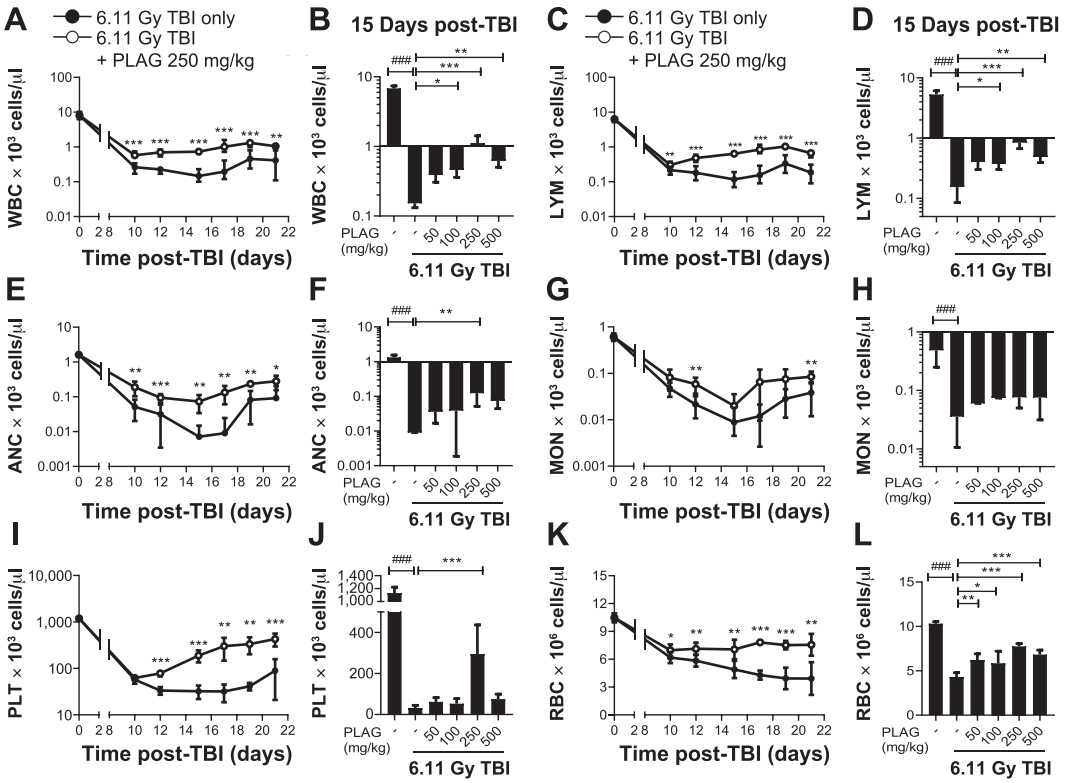


FIG. 2. PLAG mitigates hematopoietic syndrome in irradiated mice. Mice (n = 6–7 males per group) received an $LD_{70/30}$ (6.11 Gy) of γ-ray TBI and 50, 100, 250 and 500 mg/kg/day of PLAG by oral gavage on days 1–21 beginning ~24 h postirradiation. The following panels show the effects of PLAG on the kinetics of: (panel A) WBCs, (panel C) lymphocytes, (panel E) absolute neutrophil counts (ANC), (panel G) monocytes, (panel I) platelets and (panel K) RBCs on days 10–21 post-TBI. The following panels show the dose effect of PLAG on circulating levels of: (panel B) WBCs, (panel D) lymphocytes, (panel F) ANC, (panel H) monocytes, (panel J) platelets and (panel L) RBCs on day 15 post-TBI. #Negative control vs. 6.11 Gy TBI only; *6.11 Gy TBI only vs. 6.11 Gy + PLAG-treated groups. $^{\#/*}P < 0.05$, $^{\#\#/**}P < 0.01$, $^{\#\#\#/***}P < 0.001$. The complete blood count (CBC) data are representative of five independent experiments with 6–7 mice per group.

PLAG 250 mg/kg-administered and irradiated mice, WBCs and lymphocyte levels were significantly recovered compared to 6.11 Gy TBI-only mice (Fig. 2A and C), and PLAG attenuated the reduction of WBC and lymphocyte counts in a dose-dependent manner (Fig. 2B and D). Consistent with the previously published results (*18*), peripheral neutrophil, platelet and RBC counts in PLAG 250 mg/kg-treated mice were significantly and dose-dependently higher than in controls at the observed time points (Fig. 2E, F and I–L). In addition, monocyte counts in PLAG 250 mg/kg-treated mice were significantly higher than in controls at a few time points (Fig. 2G), and PLAG tended to increase monocyte counts in a dose-dependent manner (Fig. 2H). These results indicate that PLAG is a very promising therapeutic agent for hematopoietic recovery in ARS.

Administration of PLAG Improves Bone Marrow Cellularity in Irradiated Mice

Previously, our laboratory showed that PLAG improved bone marrow recovery, especially megakaryocyte/erythrocyte progenitors (MEP), in a murine model of 5-fluorouracil-induced thrombocytopenia (*20*). In this study, we investigated the effect of PLAG administration on bone marrow recovery in TBI mice. The irradiated mice displayed a significant bone marrow cellularity in the femur compared to the nonirradiated mice, showing ~8 and ~4% cellularity on days 7 and 15 postirradiation, respectively. PLAG-treated mice showed ~42% cellularity at 7 days post-TBI and ~52% cellularity at 15 days post-TBI (Fig. 3). Similar to our previous study, we observed an increased MEP population in the bone marrow of PLAG-treated mice (Fig. 3A). These data demonstrate that PLAG has the capacity for accelerating bone marrow recovery after irradiation.

Association between Hematological Nadirs and Inflammatory Cytokines/Chemokines

Many studies have demonstrated that systemic inflammatory response syndrome (SIRS), also referred to as cytokine storm, induced by radiation, is a major cause of disease severity and death (*6, 21*). It is well known that the expression of pro-inflammatory cytokines/chemokines is closely associated with the function and migration of blood

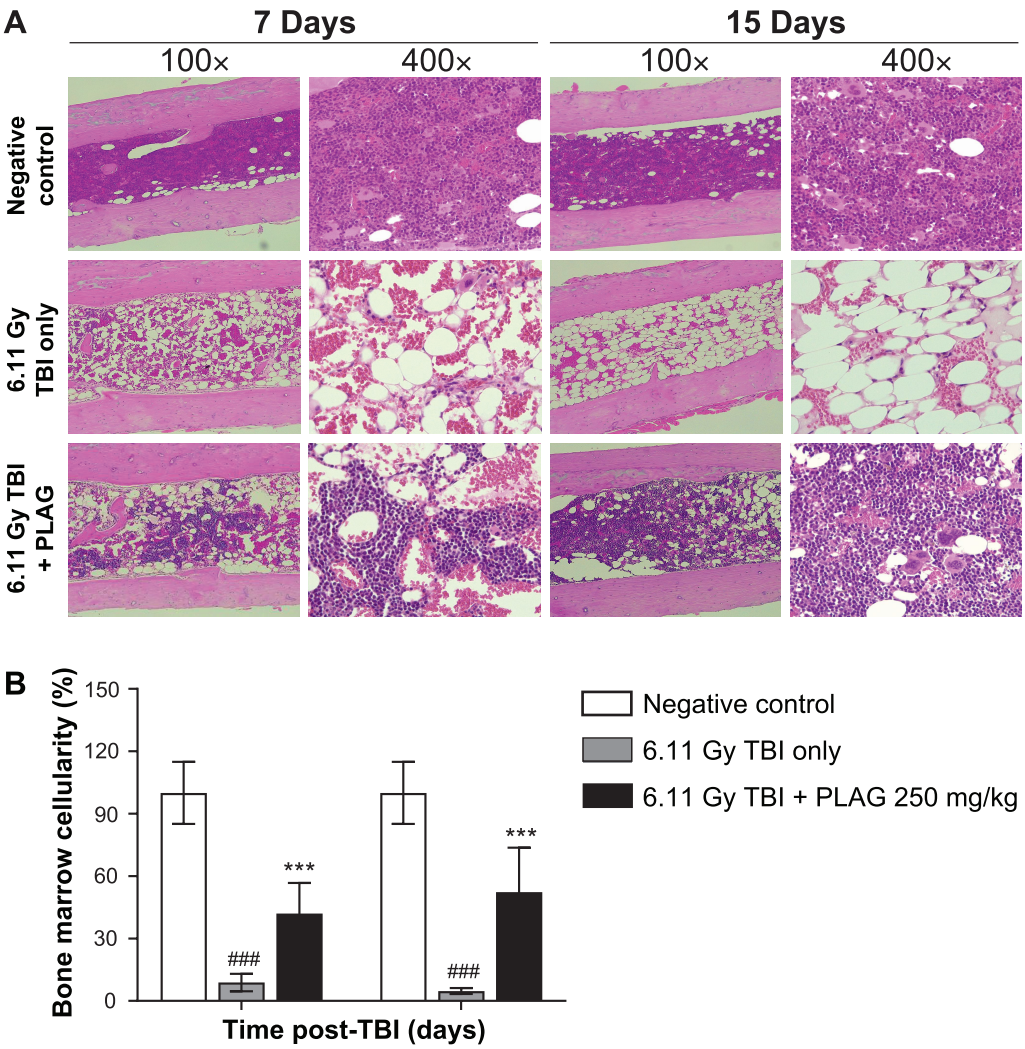


FIG. 3. PLAG improves bone marrow cellularity in irradiated mice. Male BALB/c mice (n = 5 per group), 11 weeks of age, received 6.11 Gy TBI of ^{60}Co γ rays (0.833 Gy/min) or no irradiation (negative control). Mice received PBS as a vehicle or PLAG (250 mg/kg) beginning ~24 h post-TBI. Panel A: Femurs were obtained from mice at 7 and 15 days post-TBI and processed for H&E staining. Images shown are representative of five mice in each group. Panel B: Bone marrow cellularity was analyzed using ImageJ software by selecting hematoxylin-stained cells in the bone marrow using a color threshold. For determination of bone marrow cellularity, ~4–5 fields of view per bone marrow section were assessed. #Negative control vs. 6.11 Gy TBI only; *6.11 Gy TBI only vs. 6.11 Gy + PLAG-treated groups. $^{\#/*}P < 0.05$, $^{\#\#/**}P < 0.01$, $^{\#\#\#/***}P < 0.001$.

cells (*22*). However, the association between hematological nadirs and inflammation in the ARS model has not been sufficiently elucidated. Therefore, we evaluated correlations between hematological nadirs and plasma levels of pro-inflammatory cytokines/chemokines, including CXCL1, CXCL2 and IL-6, between day 10 and 21 post-TBI using Spearman's rank correlation. As shown in Fig. 4, the decreasing number of each blood cell type exhibited a tendency toward negative correlations with elevated levels of the cytokines/chemokines. Spearman's correlation coefficient r_s values for CXCL2 were between –0.4 and –0.65 with high statistical significance ($P < 0.0001$), indicating that CXCL2 was moderately and negatively correlated with the nadirs of all blood cell types. The r_s values for CXCL1 were between –0.4 and –0.6 with high statistical significance ($P < 0.0001$), indicating that CXCL1 was also moderately and negatively correlated with the hematological nadirs. However, the r_s values for IL-6 were between –0.1 and –0.4, indicating a weak and negative correlation with the hematological nadirs. From these results, we concluded that the development of H-ARS may have a correlation with inflammatory responses.

Administration of PLAG Attenuates Blood Levels of Inflammatory Cytokines/Chemokines in Irradiated Mice

Since there was an inverse correlation between radiation-induced hematological nadirs and the increase in pro-inflammatory cytokines/chemokines, we next investigated the kinetics of plasma CXCL1, CXCL2 and IL-6 over 21 days post-TBI. Radiation induced an immediate and rapid

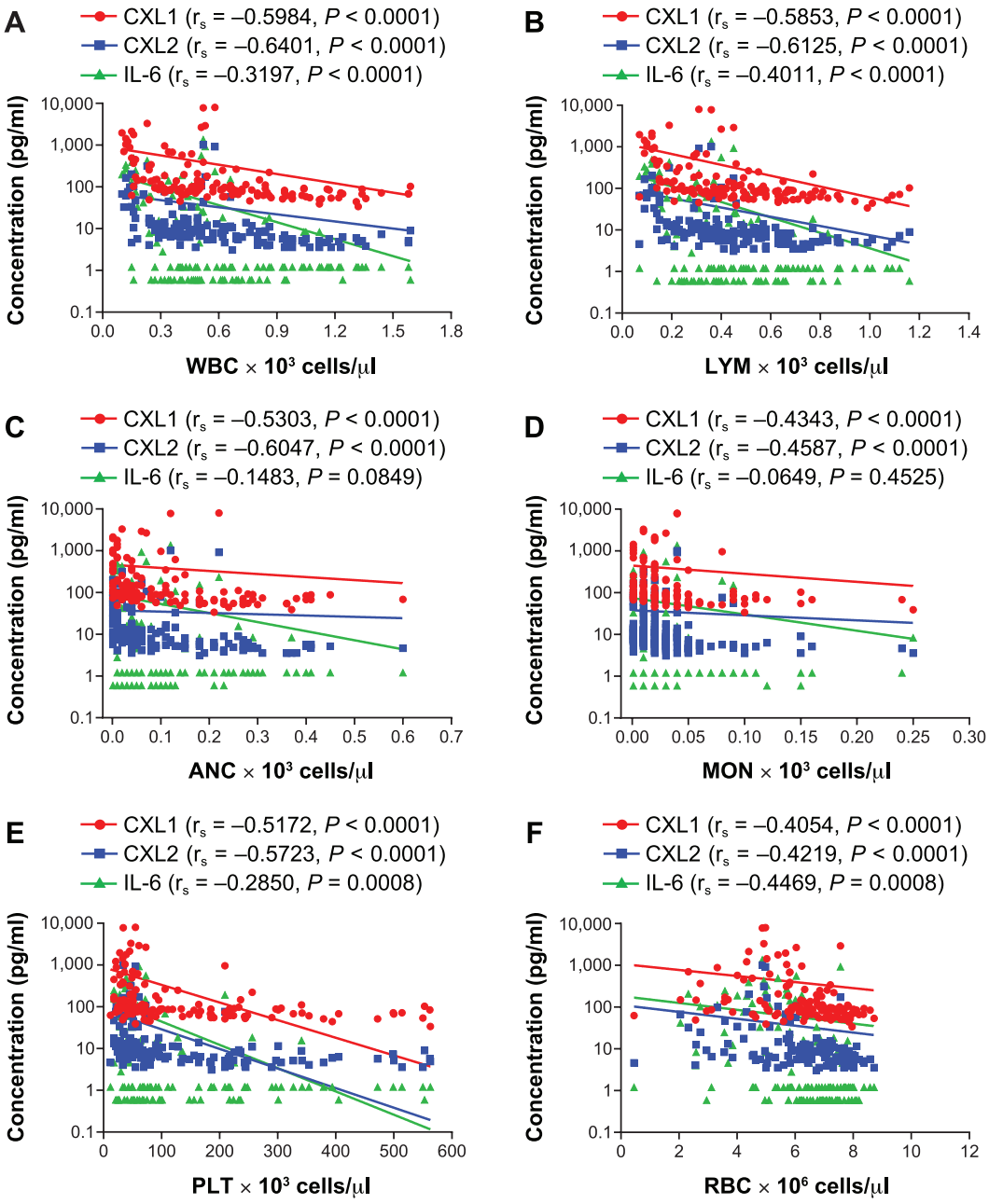
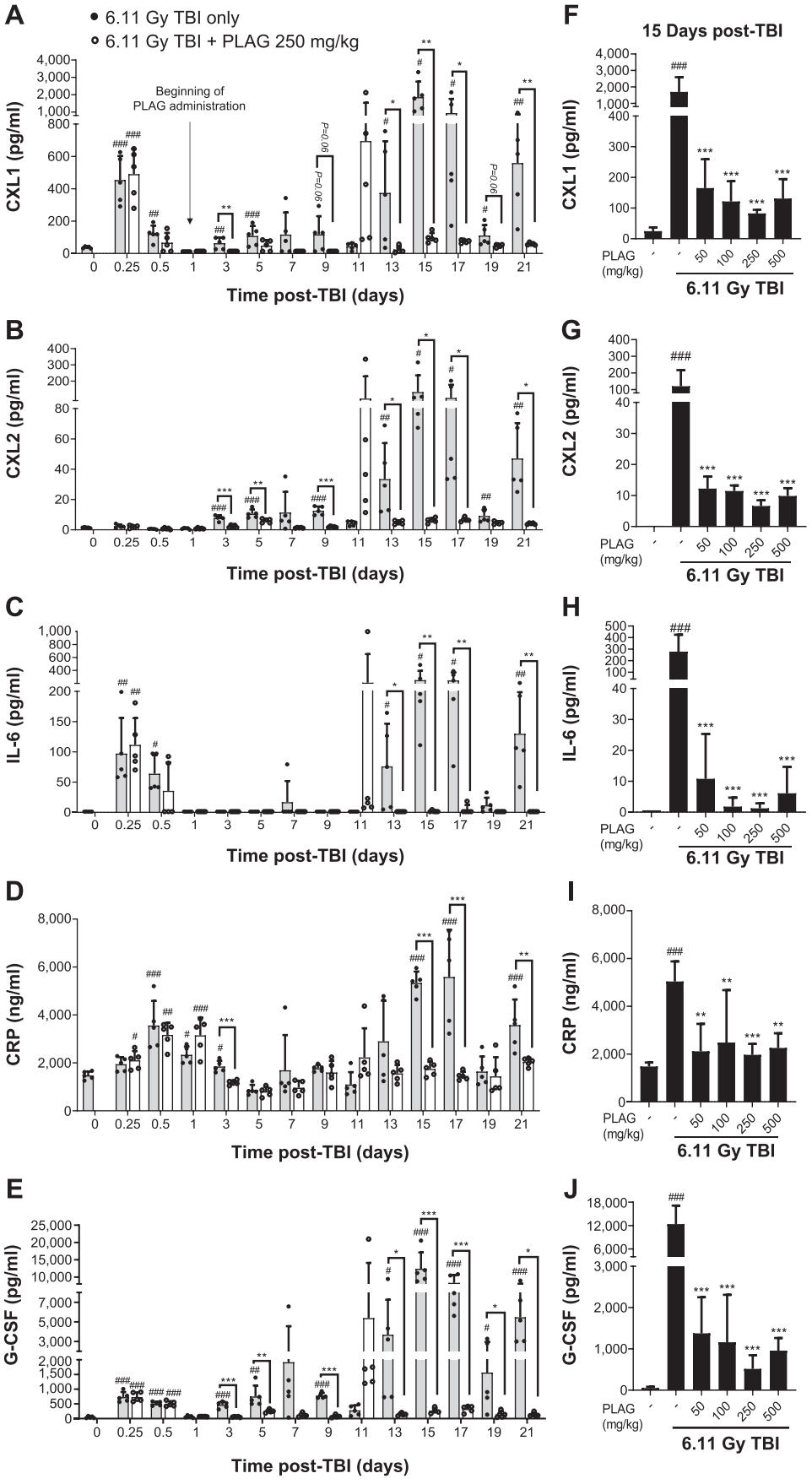


FIG. 4. Spearman's rank correlation for hematology and cytokines in irradiated mice. Blood samples were harvested from mice (n = 136 males per day) on days 15–21 after a single dose, $LD_{70/30}$ (6.11 Gy), of TBI. Panels A–F: Correlation plots between WBCs, lymphocytes, ANCs, monocytes, platelets and RBCs, respectively, and pro-inflammatory cytokines/chemokines CXCL1, CXCL2 and IL-6 (Spearman's rank correlations). r_s indicates the Spearman's correlation coefficient.

rise in plasma levels of CXCL1, CXCL2 and IL-6 by approximately 12.5 fold ($P < 0.001$), 1.5 fold ($P = 0.1622$), and 86.9 fold ($P = 0.0052$) on day 0.25 post-TBI, respectively (Fig. 5A–C). At this time point, PLAG did not influence the cytokine/chemokine levels since it was administered to the mice beginning 24 h post-TBI. When the irradiated mice began to die at around 15 days post-TBI, they exhibited an extra surge in the plasma levels of CXCL1 (49.26 fold, $P = 0.0103$), CXCL2 (87.13 fold, $P = 0.0477$) and IL-6 (208.05 fold, $P = 0.018$). On the other hand, PLAG-treated groups showed a sharp increase in the cytokine/chemokine levels on day 11 post-TBI, which occurred earlier than the TBI-only group, followed by a significant and immediate recovery to the baseline levels from day 13 and day 21 post-TBI (Fig. 5A–C). Moreover, PLAG attenuated the plasma levels of the cytokines/chemokines in a dose-dependent manner on day 15 post-TBI (Fig. 5F–H). CRP is a well-known inflammatory


A
6.11 Gy TBI only
6.11 Gy TBI + PLAG 250 mg/kg
Beginning of PLAG administration
CXL1 (pg/ml)
Time post-TBI (days)
B
CXL2 (pg/ml)
Time post-TBI (days)
C
IL-6 (pg/ml)
Time post-TBI (days)
D
CRP (ng/ml)
Time post-TBI (days)
E
G-CSF (pg/ml)
Time post-TBI (days)
F
15 Days post-TBI
CXL1 (pg/ml)
PLAG (mg/kg)
6.11 Gy TBI
G
CXL2 (pg/ml)
H
IL-6 (pg/ml)
I
CRP (ng/ml)
J
G-CSF (pg/ml)

marker used for many health conditions in both clinical and research settings, and many studies have demonstrated an elevated serum CRP level in cancer patients after radiotherapy (*23, 24*). Therefore, we examined the plasma level of CRP. As with the pro-inflammatory cytokines/chemokines, CRP had approximately two significant upward tendencies, around day 0.5 and day 15 post-TBI. PLAG significantly and dose-dependently attenuated radiation-induced CRP production at around day 15 post-TBI (Fig. 5D and I). Next, we examined the kinetics of endogenous G-CSF, a hematopoietic cytokine involved in proliferation, differentiation and mobilization of the neutrophil lineage (*25, 26*). As a result, we observed a radiation-induced increase in endogenous G-CSF that was significantly modulated by PLAG treatment (Fig. 5E). The kinetics of radiation-induced endogenous G-CSF production was very similar to that of CXCL1, CXCL2 and IL-6, as shown in Fig. 5A–C. Radiation induced an approximately ~265-fold increase in endogenous G-CSF at day 15 post-TBI ($P < 0.001$), while PLAG significantly and dose-dependently attenuated radiation-induced G-CSF production at day 15 post-TBI ($P < 0.001$) (Fig. 5J). In conclusion, PLAG was shown to be very effective in attenuating radiation-induced pro-inflammatory cytokines/chemokines, CRP and G-CSF, which are closely associated with the occurrence of H-ARS.

DISCUSSION

During the past few decades, there has been growing public concern regarding accidental or deliberate radiation exposure to a large population caused by a large-scale radiation event, such as nuclear plant disasters or nuclear terrorism, which has escalated the necessity for the development of radiation countermeasures (*1–3*). We previously showed that a 30-day course of PLAG (50 or 250 mg/kg/day), first orally administered 0–72 h post-TBI, improved survival rates and H-ARS, especially radiation-induced neutropenia, thrombocytopenia and anemia, in mice (*18*). The current study serves to complement the results in our previously published work by examining a statistical relationship between early changes in mature blood cell counts and the risk of mortality in the murine H-ARS model. Based on the statistical relationship, we concluded that the recovery of mature blood cells in PLAG-treated animals correlated with the improved survival. Our current study also demonstrated the inverse correlation between the hematological nadirs and the degree of pro-inflammatory cytokine production induced by radiation as well as the mitigating effect of PLAG administration on radiation-induced cytokine levels in peripheral blood.

Hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) are rapidly dividing, which makes them highly sensitive to radiation (*5*). HSCs have a D_0 of approximately 1.15 ± 0.08 Gy of Co^{60} γ rays, which means that a dose of 1.15 ± 0.08 Gy is required to reduce the population of HSCs to 37% (*27*). This is why H-ARS is observed with radiation exposures exceeding 1 Gy. The severity and duration of H-ARS symptoms and probability of recovery are dependent on the absorbed radiation dose (*5*). If there is no regeneration, pancytopenia will be prolonged with a high predisposition to infection and hemorrhage, ultimately leading to death within 30 days (*5–7*). Many previously published studies have concluded that recovery of specific hematopoietic cell types was closely correlated with overall survival after irradiation (*28–30*). Correspondingly, our data confirmed that early hematopoietic recovery, especially leukocytes, correlated with increased survival. Thus, to mitigate the lethality of H-ARS, it is critical to restore the impaired hematopoietic function in the bone marrow. In the current study, we reported that PLAG administration significantly increased the mean nadirs of all types of mature blood cells and bone marrow cellularity. Therefore, as our current findings suggest, the enhanced early bone marrow recovery resulting in increases in nadirs of all blood cell types has led to the improved survivability in the irradiated PLAG-treated animals.

G-CSF is a hematopoietic cytokine that stimulates proliferation, maturation, survival and mobilization of various hematopoietic progenitors (*25, 26*). Exogenous recombinant G-CSF treatment has been applied as a prophylaxis to myelosuppressive patients who received cytotoxic chemotherapy or radiotherapy so as to induce hematopoietic progenitor proliferation (*31, 32*). In addition, as previously mentioned, biosimilars of recombinant human G-CSF are one of the few FDA-approved countermeasures for the treatment of H-ARS (*9*). For this reason, we posited that radiation exposure would decrease endogenous production of G-CSF and that PLAG might exert its mitigating effect on H-ARS by increasing the endogenous G-CSF levels. However, contrary to our expectation, the serum concentration of endogenous G-CSF was immediately and rapidly increased in response to radiation, and the elevated levels were mostly maintained for 11 days post-TBI. Furthermore, extra surges in endogenous G-CSF concentration were observed between 13 and 21 days post-TBI

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FIG. 5. PLAG attenuates blood levels of inflammatory cytokines/chemokines, C-reactive protein (CRP) and granulocyte colony stimulating factor (G-CSF) in irradiated mice. Mice (n = 6–7 males per group) received an $LD_{70/30}$ (6.11 Gy) of γ-ray irradiation and 50, 100, 250 and 500 mg/kg/day of PLAG by oral gavage on days 1–21 beginning ~24 h post-TBI. Panels A–E: Effect of PLAG administration on the kinetics of the chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2, interleukin-6 (IL-6), CRP and G-CSF, respectively, in blood post-TBI. Panels F–J: Dose effect of PLAG administration on the blood level of CXCL1, CXCL2, IL-6, CRP and G-CSF on day 15 post-TBI. #Negative control vs. 6.11 Gy TBI only; *6.11 Gy TBI only vs. 6.11 Gy + PLAG-treated groups. $^{\#/*}P < 0.05$, $^{\#\#/**}P < 0.01$, $^{\#\#\#/***}P < 0.001$. Data are representative of three independent experiments with five mice per group.

despite the absence of external stimuli. We realized that this is not a new finding, and has already been reported elsewhere (*33, 34*). Moreover, through a review of relevant literature, we determined that this result is in line with other clinical studies of myelosuppressive patients after high-dose cytotoxic chemotherapy in which endogenous G-CSF levels in serum are inversely correlated with absolute neutrophil count (ANC) (*35–38*). In other words, myelosuppressive patients with a high level of endogenous G-CSF concentration are at higher risk of severe neutropenia. On the other hand, endogenous G-CSF levels in the irradiated PLAG-treated animals were significantly lower compared to those in the irradiated non-treated animals, and the extra surges of serum G-CSF levels between 13 and 21 days post-TBI, which were seen in the TBI-only animals, were not observed in the TBI PLAG-treated animals. Instead, the endogenous G-CSF level in the TBI PLAG-treated group was sharply increased on day 11, followed by a speedy recovery to the baseline level on day 13 post-TBI. A putative cause of this phenomenon may be that internal unknown stimuli induce the production of endogenous G-CSF, as well as pro-inflammatory cytokines/chemokines, to promote the release of neutrophils from the bone marrow. Moreover, the improvement of bone marrow cellularity by PLAG would enable the release of enough neutrophils into circulation for the removal of the unknown stimuli. Therefore, judging from the findings of previously reported research, the lower levels of endogenous G-CSF concentration seen in the irradiated PLAG-treated animals are closely associated with the improvement in bone marrow cellularity and mature blood cell levels, particularly neutrophils and monocytes. However, to analyze the exact distinction between PLAG and G-CSF biosimilars in terms of therapeutic effectiveness and occurrence of side effects, a direct comparison test should be performed in an H-ARS model.

In addition to G-CSF, we examined radiation-induced changes in the plasma level of CRP, which is an acute-phase inflammatory protein mainly produced in the liver in response to trauma, infection, inflammatory reactions and cancers (*39, 40*). CRP acts as an opsonin by binding to pathogens, degenerating cells and cell debris. It also activates the classical C1q pathway of complement system (*40*). CRP has been perceived as a marker of systemic inflammation in several disorders. This study did not disclose the mechanism of CRP suppression by PLAG, but we hypothesized that the suppression might result from direct protection of the liver tissues or suppression of its upstream inflammatory cytokines. Indeed, PLAG significantly decreased the plasma level of IL-6, which is a primary upstream cytokine responsible for producing CRP in the liver.

Many published studies have described the rapid rise in the plasma levels of pro-inflammatory cytokines and chemokines as a defense strategy against radiation-induced tissue damage (*16*). In the same manner, we observed the immediate and rapid increase in the plasma levels of CXCL1, CXCL2 and IL-6 after a $LD_{70/30}$ of TBI. In addition, statistical analysis revealed that the extra surges of cytokine expressions seen in between 13 and 21 days post-TBI had a decisive role, not only in developing hematological nadirs, but also in determining life or death of the irradiated animals, and PLAG remarkably downregulated the surges of cytokine production. However, in our current study, we did not investigate why the cytokines were explosively increased between 13 and 21 days post-TBI and which factors were associated with this phenomenon. Further studies to address these questions are required to better understand the mechanism of action of PLAG in ARS.

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