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Platelet glycoprotein Ibα provides radiation protection

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Abstract

Background and Purpose: Platelet membrane glycoprotein Ibα (GPIbα), the major ligand-binding subunit of the GPIb-IX-V complex, binds to a number of ligands contributing to hemostasis, thrombosis, and inflammation. Binding to von Willebrand factor (VWF) initiates the process of hemostasis/thrombosis, while binding to the leukocyte receptor Macrophage-1 antigen (Mac-1) has been implicated in modulating the inflammatory response. Thus as GPIbα resides at the nexus of thrombosis and inflammation, we investigated the impact of GPIbα on radiation injury outcomes as this injury triggers both the thrombotic and inflammatory pathways.

Materials and Methods: We used wild-type (WT) C57BL/6J mice and a dysfunctional GPIbα mouse model, in which endogenous GPIbα is replaced with a non-functional α-subunit (hIL-4R/Ibα), to determine whether the impairment of platelet GPIbα alters radiation response. Following exposure to 8.5 Gy total body irradiation (TBI), a series of parameters including radiation lethality, platelet-neutrophil/monocyte interactions, neutrophil/monocyte activation, serum cytokine levels and intestinal injury, were compared between the strains.

Results: The lack of functional GPIbα resulted in higher radiation lethality, greater monocyte activation, increased levels of serum pro-inflammatory cytokines, heightened intestinal damage, and a reduction of intestinal neutrophil recovery.

Conclusion: These data suggest that loss of platelet GPIbα enhances radiation toxicity and that GPIbα-mediated interactions may play a crucial role in limiting radiation damage. Thus,

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Conflict of interest

None

a mechanistic understanding of the biological impact of GPIbα following TBI could provide crucial insights for improving the safety of radiotherapy and minimizing the deleterious effects of accidental or occupational exposure to high-dose radiation.

Keywords

Platelets; Radiation; Inflammation; Intestines; Leukocytes

INTRODUCTION

Radiation toxicity within healthy tissue results from radiotherapy. Within minutes, radiation elicits apoptosis or mitotic death in blood cells and the epithelium of the intestinal mucosa.[1,3] This triggers the loss of intestinal integrity which increases the risk of intestinal flora dissemination, in turn causing inflammation and enhancing the likelihood of sepsis.[4] Additionally, radiation can activate platelets and leukocytes (neutrophils and monocytes) which causes the secretion of pro-inflammatory cytokines,[5] thereby exacerbating inflammation. Persistent inflammation, resulting from radiation injury, can also promote thrombosis (pathological clot formation) which itself causes ischemia, hypoxia and tissue damage in addition to stimulating the inflammatory response thus forming a vicious cycle.[8]

Radiation elicits intestinal damage either directly or via the resulting inflammation it causes. Radiation exposure causes the loss of intestinal epithelial cells, resulting in shortened villi and reduced mucosal surface area (MSA), which compromises the intestinal barrier function.[18] This loss of barrier integrity facilitates the stromal invasion by luminal bacteria of the intestine. Further migration into the circulation triggers an inflammatory response and the possibility of sepsis.[4] Additionally, radiation exposure damages the intestinal crypts that harbor intestinal stem cells. Damage to the stem cell population adversely affect the regeneration of the mucosal epithelium, further exacerbating radiation induced intestinal damage.

Of note, platelets, the primary drivers of hemostasis, actively participate within the inflammatory pathway.[9] Specifically, GPIbα, the ligand binding subunit of GPIb-IX-V complex, has been shown to bind leukocyte Mac-1 (αMβ2, CD11b/CD18) [14,13,15] and exerts either a pro- or anti-inflammatory response depending on the model of inflammation. Previously, the induction of sepsis in GPIbα deficient mouse model demonstrated that the loss of the GPIbα/Mac-1 axis enhanced circulating levels of pro-inflammatory cytokines.[17] Therefore, it is possible that GPIbα plays a similar role in minimizing the cytokine storm and other inflammatory consequences induced by radiation injury. Thus, the role of platelet GPIbα in modulating inflammation caused by radiation warrants investigation. Here we demonstrate that GPIbα disruption causes increased sensitivity to radiation-induced systemic inflammation, intestinal injury, and lethality.

MATERIALS AND METHODS

Mouse model, irradiation, tissue collection and euthanasia

Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences (UAMS). Male mice age 8-12 weeks were utilized, as the estrous cycle alters radiation responsiveness. The hIL-4R/Ibα mouse strain is described elsewhere.[17,19]

Mice (not anesthetized) were placed in a custom-made, Plexiglas chamber and exposed to a single 8.5 Gy TBI dose in a Shepherd Mark I ^{137}Cs irradiator (model 25, J. L. Shepherd & Associates, San Fernando, CA, USA, 1 Gy/min). The chamber was placed on a turntable rotating at six rpm for uniform radiation dose distribution.

For non-terminal blood isolation, mice were anesthetized with isoflurane inhalation and blood was collected from the retro-orbital sinus. Platelet counts were determined with a Hemavet 950 (Drew Scientific, Waterbury CT).

For tissue harvesting mice were anesthetized with an intraperitoneal injection of a xylazine (10-12.5 mg/kg) and ketamine (80–100 mg/kg) (Henry Schein, Dublin, OH, USA). Blood was collected from the retro-orbital sinus and intestinal samples were isolated and fixed in methanol–Carnoy's solution. Mice were euthanized by CO_2 asphyxiation followed by cervical cord dislocation.

TPO administration

Recombinant mouse thrombopoietin (TPO) (R&D Systems, 488-TO-025/CF) was dissolved in sterile PBS. A single dose of TPO (20 μg/kg) in 100 μL PBS was administered by intraperitoneal injection, while control mice received the same volume of PBS.

Flow cytometry

Sample preparation for flow cytometry analysis has been described elsewhere.[17]

Cytokine measurement

A Bio Rad 23-plex multiplex kit was used to measure serum cytokine levels with a Luminex-200 dual-laser flow analyzer (Luminex Corp., Austin, TX, USA).

Epithelial mucosal measurements

Fixed intestinal tissues were embedded in paraffin and cut into sections, de-waxed, stained with hematoxylin, washed with diH_2O, stained with 1% eosin solution, dehydrated with alcohol, washed with xylene, and mounted with Permount™ media (Thermo Fisher Scientific, Grand Island, NY, USA).

Mucosal surface area (MSA) was measured via computerized cycloid count method from vertical sections as described elsewhere.[20] The images of hematoxylin and eosin (H&E) stained intestinal sections were superimposed onto a grid system containing cycloids (short

curved lines in a systematic pattern), and the number of intersections between the mucosal surface and cycloids was counted.

H&E stained sections were used to measure villus height and crypt depth using a computer-assisted image analysis platform (Image-Pro Premier, Rockville, MD, USA). Height was measured from the tip to the base of each villus, and crypt depth was measured from the base to the top opening.

Crypt survival was measured by the microcolony assay described by Withers and Elkind.[21]

Immunohistochemical staining for myeloperoxidase (MPO) of proximal segments of the jejunum was obtained 0, 4, and 8 days after TBI to determine intestinal neutrophil recovery. Sections were incubated with MPO primary antibody (Dako, Glostrup, Denmark), followed by an incubation with secondary antibody (Vector laboratories, Burlingame, CA, USA). Slides were then incubated with HRP avidin–biotin complex (Vector Laboratories) before incubation in DAB-HCl solution (Sigma-Aldrich, St. Louis, MO, USA) and 0.003% H_2O_2 in TBS (Cell Signaling Technology). MPO positive cells were quantified using Image-Pro Premier software (Media Cybernetics; Rockville, MD, USA).

Statistical analysis

Data were analyzed with Prism software (version 8.0; GraphPad, San Diego, CA), SAS (version 9.4) and R (version 4.0.2). Following irradiation, Kaplan-Meier estimates were generated to assess survival probabilities over time while Log-rank tests were used to compare survival curves. Platelet-leukocyte associations and CD11b expression were compared using a Student's t-test for the comparison of means. ANOVA was used for comparing multiple groups. A two-sided value of $p < 0.05$ was considered statistically significant.

RESULTS

As platelet GPIbα is capable of modulating the inflammatory response,[17] we hypothesized that GPIbα dysfunction would alter radiation-induced inflammation and lethality.[22] Quantification of platelets prior to radiation indicated a ~30% reduction in platelet counts for hIL-4R/Ibα mice relative to WTs, consistent with previous accounts (Figure 1A).[17] A single intraperitoneal administration of TPO raised hIL-4R/Ibα platelet counts to those in WT at 5 days after administration (Figure 1B). WT mice, hIL-4R/Ibα mice with prior TPO treatment, and hIL-4R/Ibα without TPO treatment were exposed to 8.5 Gy TBI. The hIL-4R/Ibα group exhibited 100% (without TPO) and 89% (with TPO) mortality whereas 50% of WT survived until the experimental conclusion (Figure 1C). Since both hIL-4R/Ibα groups showed a significantly higher mortality rate than WT, our results suggest that dysfunctional GPIbα, not the reduced platelet count, is responsible for higher radiation lethality in hIL-4R/Ibα mice.

As TPO treatment did not affect radiation lethality, subsequent experiments with hIL-4R/Ibα mice were performed without TPO treatment.

The GPIbα/Mac-1 interface facilitates platelet-leukocyte interactions under both physiological and pathological states. We investigated if GPIbα dysfunction affected platelet-leukocyte interactions before and after TBI. Prior to TBI, GPIbα dysfunction reduced platelet binding to neutrophils and monocytes. Since TBI causes a drop in leukocyte counts, we investigated alterations in platelet-leukocyte interactions at 4 h and 24 h post-exposure. WT and hIL-4R/Ibα mice did not display any significant change in the percent of platelet-neutrophil interactions when compared to sham at 4 h and 24 h (Figure 2A) with WT samples maintaining higher aggregate levels throughout. Platelet-monocyte interactions were maintained at 4 h post-irradiation for both WT and hIL-4R/Ibα, with WT samples similarly having higher aggregate levels (Figure 2B). Interestingly, monocytes were undetectable at 24 h in both strains after TBI, suggesting monocytes are more susceptible to radiation toxicity than neutrophils. This indicates that GPIbα disruption is responsible for reducing platelet binding to both neutrophils and monocytes before and after TBI.

Leukocyte activation causes an increase in Mac-1 surface expression; therefore Mac-1 is a marker of leukocyte activation.[23] We hypothesized that leukocyte activation/Mac-1 (CD11b) expression would increase following TBI. Though neutrophil activation was less in hIL-4R/Ibα mice before radiation, no difference was observed after exposure, as both strains displayed a reduction in CD11b expression at 4 and 24 h (Figure 3A). Similarly, monocyte activation dropped in WT samples 4 h following irradiation while activation increased at 4 h for hIL-4R/Ibα (Figure 3B). This suggests that GPIbα is capable of diminishing monocyte activation following TBI but has no effect on neutrophils.

As monocytes are highly secretory, we hypothesized that pro-inflammatory cytokine levels would be higher in hIL-4R/Ibα serum as a result of the elevated monocyte activation following TBI. Previous reports indicated maximal serum cytokine levels 3-7 days following radiation so we subjected serum samples collected 4 days following TBI to a panel of 23 cytokines (full panel: Supplemental Figure 1). Among the results, numerous pro-inflammatory cytokines derived primarily from monocytes were increased in hIL-4R/Ibα mice compared to WTs (Figure 4). While under control conditions interleukin (IL)-6, macrophage inflammatory protein (MIP)-1β, and tumor necrosis factor (TNF)-α levels were lower in hIL-4R/Ibα mice compared to WT, at day 4 after TBI, these 3 cytokines were induced in the hIL-4R/Ibα mice to levels significantly higher than in the WT. Moreover, IL-10, keratinocyte chemoattractant (KC), and monocyte chemoattractant protein (MCP)-1 were induced by TBI in hIL-4R/Ibα mice but not in WT (Figure 4).

Higher level of plasma pro-inflammatory cytokines can disrupt homeostasis in various tissues by impairing endothelial barrier integrity. We observed TBI causes significantly higher impairment of endothelial barrier integrity in the lung and intestinal tissues of hIL-4R/Ibα mice compared to WTs. However, we did not observe statistically significant differences in vascular permeability in spleen, kidney, and heart (methods and results presented in Supplemental Figure 2).

Intestinal mucosa epithelial cells are highly sensitive to damage via direct radiation and radiation induced inflammation.[24] Since hIL-4R/Ibα mice exhibit higher levels of pro-inflammatory cytokines, we hypothesized these mice would also exhibit heightened

intestinal damage following TBI. Prior to irradiation we noted the MSA was reduced in hIL-4R/Ibα mice ($p = 0.001$) (Figure 5A) while 4 days following TBI resulted in a recession of the MSA for both strains. Additionally, we noted a decrease in villus height in hIL-4R/Ibα mice, but not in WT (Figure 5B). Crypt depth in both the strains was also increased following TBI (Figure 5C). No difference in the number of surviving crypts was detected in either strain (Figure 5D). This suggests that certain aspects of mucosal damage after TBI are GPIbα dependent.

Neutrophils promote inflammation resolution via the phagocytosis of cell debris accumulated at sites of mucosal wounds, which can be caused by TBI. Since we observed higher inflammation in hIL-4R/Ibα mice, we hypothesized that neutrophil recovery in the intestine would be reduced. We found a significant reduction in the number of intestinal neutrophils at 4 and 8 days following TBI in hIL-4R/Ibα mice when compared to WT (Figure 6).

DISCUSSION

Nearly 3.05 million American cancer survivors were treated with radiation in 2016.[27] Despite the critical nature of this therapy, there is a risk of injury to healthy tissue which exacerbates morbidity and mortality. Additionally, radiation stimulates inflammation which if unchecked can persist and likewise contribute to tissue damage. Considering the number of people at risk of radiation exposure, it is essential to understand the mechanism of radiation-induced injury of healthy tissues. Furthermore, evidence suggests that platelets not only regulate hemostasis/thrombosis but also actively participate in the inflammatory response due to their interactions with leukocytes.[9] This positioning at the interface of inflammation warranted investigation into the potential of platelets to alter radiation-induced inflammation, tissue damage and mortality.

Platelet-neutrophil and platelet-monocytes aggregates correlate to inflammation and inflammatory disorders.[28,29] Platelet GPIbα and leukocyte Mac-1 are well-documented binding partners and promote the formation of platelet-leukocyte aggregates.[14,17] Though well characterized, the GPIbα/Mac-1 axis significance within radiation induced inflammation is unknown. While irradiation did not alter platelet-leukocyte aggregates in WT and hIL-4/Ibα mice after irradiation, the percentage of aggregates remained reduced in GPIbα dysfunctional mice at all-time points. Moreover, we observed a significant decrease in neutrophil activation (Mac-1) for both strains and in WT monocytes following TBI. Interestingly, hIL-4/Ibα mice had increased monocyte activation post-irradiation indicating GPIbα deficiency may contribute to heightened inflammation.

Monocytes secrete numerous pro-inflammatory cytokines that elicit systemic inflammation. Previous reports found septic hIL-4R/Ibα mice had elevated serum cytokine levels which resulted from reduced platelet-monocyte associations.[17] This suggests GPIbα modulates systemic inflammation and as radiation elicits systemic inflammation, we predicted GPIbα dysfunction potentially increases radiation induced inflammation. Indeed, we observed an increase in pro-inflammatory cytokines in hIL-4R/Ibα mice relative to WT after TBI, indicating GPIbα disruption enhances radiation-induced inflammation. In future studies,

we will address the hypothesis that the pro-inflammatory cytokines that are upregulated in irradiated hIL-4R/Ibα mice may lead to leukocyte activation that contribute to tissue damage and thereby the increased radiation mortality seen in these mice.

These heightened levels of cytokines could contribute to the observed intestinal mucosal epithelium damage following TBI[4]; however, it is difficult to discern whether mucosal damage is a cause or consequence of elevated cytokine levels. Also, we did not observe strain-dependent changes in crypt depth and number of surviving crypts following TBI. This implies that the mucosal epithelial layer, but not the crypt region, is differentially affected by GPIbα disruption. This region-specific variation in radiation damage could be due to differences in radiation sensitivity between two intestinal regions, as mucosal epithelial cells are more radiosensitive than crypt cells. Intestinal stromal neutrophils also play a crucial role in the inflammatory resolution.[3132,33] We observed a reduction in intestinal neutrophil recovery in hIL-4/Ibα mice relative to WT after TBI, and this diminished rate of neutrophil recovery potentially contributes to greater intestinal damage. While further studies are required to ascertain the role of GPIbα in modulating the extent of healthy tissue damage after irradiation, our evidence suggests a GPIbα component to intestinal toxicity, which is a major contributor to radiation induced mortality. Future studies will address the relative contribution of each of the immunological and intestinal events to radiation lethality in this mouse model. Lastly, since hIL-4R/Ibα mice show curtailed clot formation compared to WT, it will be important to assess the effects of TBI on thrombosis in this mouse model.

CONCLUSIONS

We demonstrated the significance of platelet GPIbα in modifying the pathophysiological response to radiation. Utilizing a model of GPIbα dysfunction we demonstrated that GPIbα disruption causes a reduction in circulating platelet-leukocyte aggregates, enhanced monocyte activation, elevated serum cytokines, increased intestinal damage, reduced intestinal neutrophil recovery, and heightened mortality after TBI. As such, our study provides insight into a previously unexplored role for platelet GPIbα in modulating radiation toxicity. These findings establish a foundation to develop platelet-centric strategies to limit damage to healthy tissues that arises from radiotherapy or accidental overexposures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

CD	Cluster of differentiation
GP	Glycoprotein
IL	Interleukin
IR	Ionizing radiation
KC	Keratinocyte chemoattractant
Mac-1	Macrophage-1 antigen
MCP-1	Monocyte Chemoattractant Protein 1
MFI	Mean fluorescence intensity
MIP-1β	Macrophage inflammatory protein-1β
MSA	Mucosal surface area
SEM	Standard error of the mean
TBI	Total body irradiation
TNFα	Tumor necrosis factor alpha
TPO	Thrombopoietin
VWF	von Willebrand factor
WT	Wild type

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Essentials

- Platelet glycoprotein Ibα (GPIbα) dysfunction enhances radiation lethality in mice.
- GPIbα dysfunction reduces the formation of platelet-leukocyte aggregates prior to and following total-body irradiation.
- Reduced platelet association with monocytes correlates with increased monocyte activation following radiation.
- GPIbα dysfunction increases serum cytokine levels and intestinal damage and decreases neutrophil recovery in the intestine in response to radiation exposure.

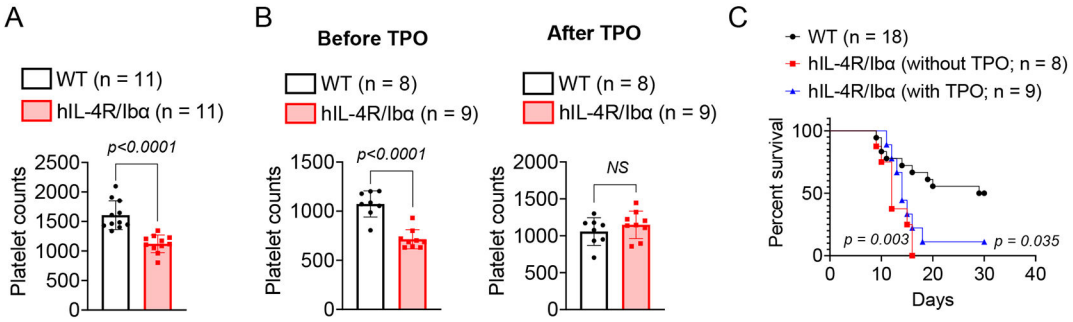

Figure 1. Platelet counts prior to TBI and radiation lethality following TBI.

(A) Platelet counts in WT and hIL-4R/Ibα mice prior to radiation. (B) Platelet counts in WT and hIL-4R/Ibα mice before and 5 days after a single dose of TPO (20 μg/kg body weight) administration by intraperitoneal injection. Kaplan–Meier survival curves after exposure to 8.5 Gy TBI. Log-rank tests confirmed that both hIL-4R/lbα with TPO (p=0.035) and without TPO (p=0.003) had significantly lower percent survival as compared to the WT group. WT n = 18; hIL-4R/Ibα without TPO (n = 8); hIL-4R/Ibα with TPO (n = 9).

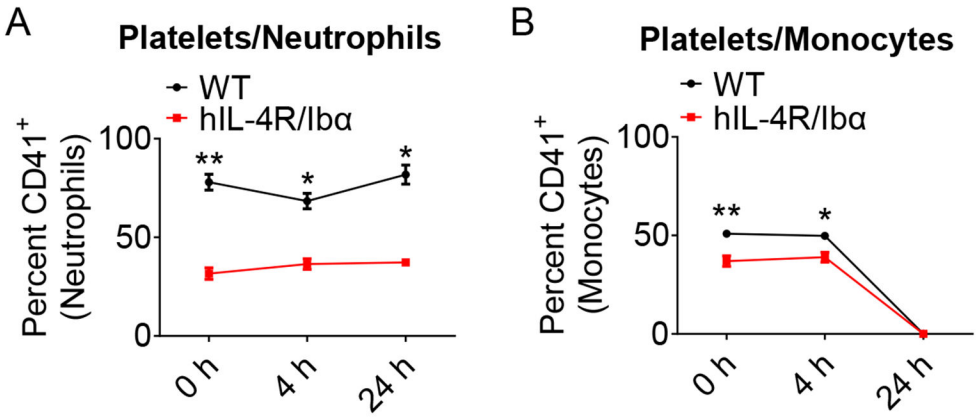

Figure 2. Platelet–neutrophil/monocyte interactions prior to and following TBI.

Whole blood was collected before 8.5 Gy TBI and at 4 h or 24 h after TBI from WT and hIL-4R/Ibα mice by retro-orbital puncture. Platelet–neutrophil/monocyte interactions were determined by measuring the percentage of platelet-positive ($CD41^+$) events in the neutrophil ($Gr\text{-}1^+/CD115^-$) or monocyte ($CD115^+$) population. To compare the difference in platelet associations with neutrophils and monocytes between the WT and hIL-4R/Ibα mice before and after TBI, a Student's t-test was performed on mean values. * and ** represent p value <0.01, and <0.001, respectively. WT n = 17 (sham), 5 (TBI); hIL-4R/Ibα n = 17 (sham), 5 (TBI).

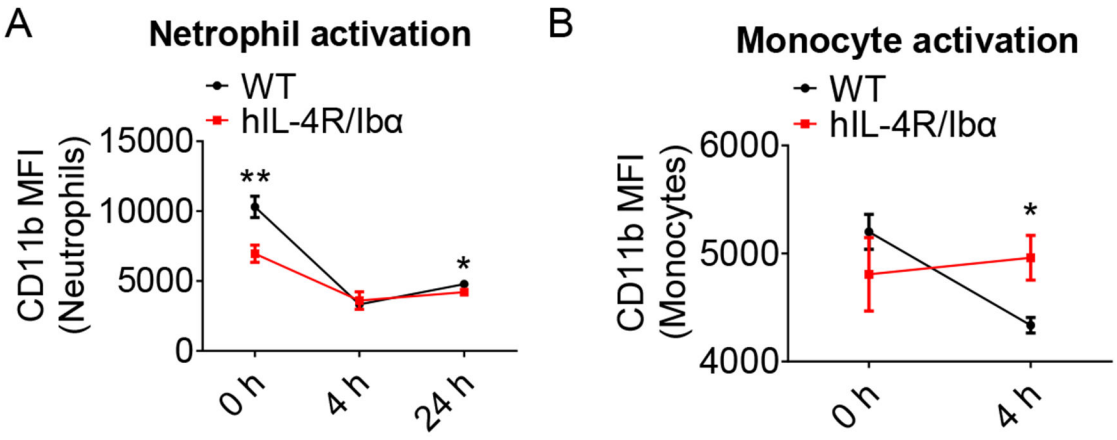

Figure 3. Activation of leukocytes and monocytes following TBI.

The expression of Mac-1 (CD11b/CD18), a marker of leukocyte activation was measured before and after 8.5 Gy TBI. The mean fluorescence intensity (MFI) of Mac-1 was determined for the neutrophil and monocyte population in WT and hIL-4R/Ibα mice. To compare the difference in activation of neutrophils and monocytes between the WT and hIL-4R/Ibα mice before and after TBI, a Student's t-test was performed on mean values. * and ** signify p value <0.01, and <0.001, respectively. WT n = 17 (sham), 5 (TBI); hIL-4R/Ibα n = 17 (sham), 5 (TBI).

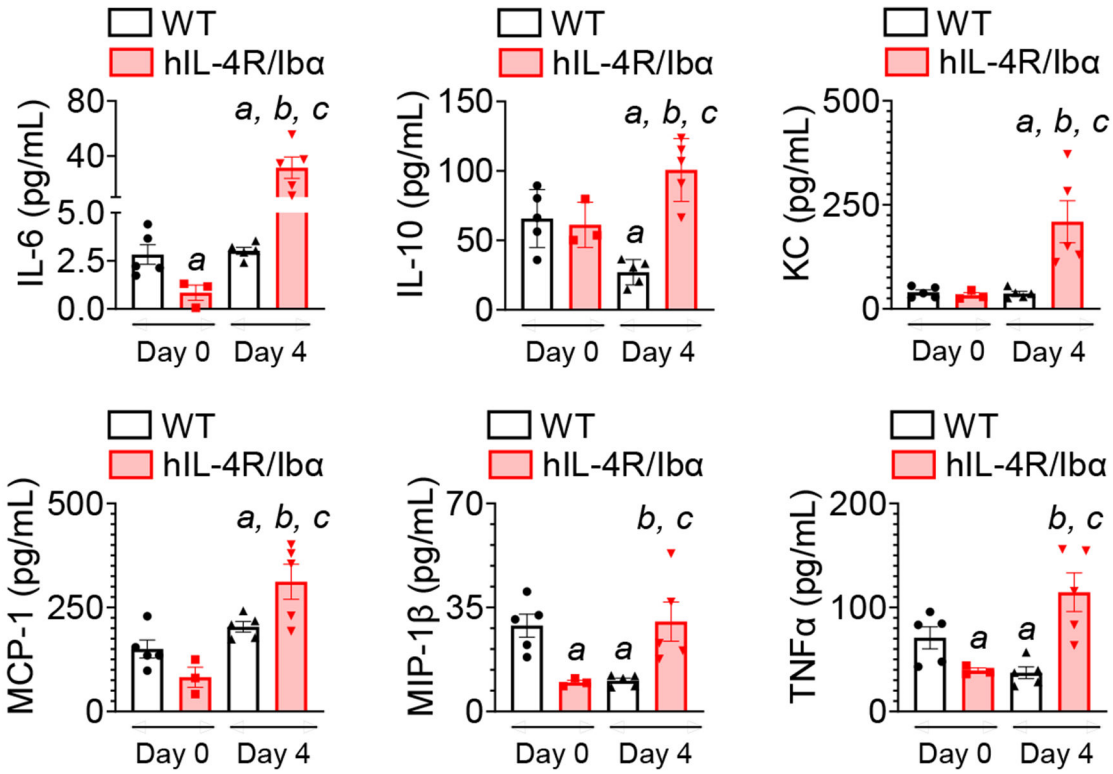

Figure 4. Serum cytokines level following TBI.

Circulating serum cytokine levels at days 0 and 4 after 8.5 Gy TBI in WT and hIL-4R/Ibα mice were tested using a multiplex Luminex assay panel for 23 inflammatory analytes. The cytokine data shown are plotted as the means ± SEM for n = 3 to 5 mice/group and include only those cytokines primarily produced by monocytes. a, b, and c, represent P(value) < 0.05 from sham irradiated WT, sham irradiated hIL-4R/Ibα, and irradiated WT, respectively. WT n = 5; hIL-4R/Ibα n = 5.

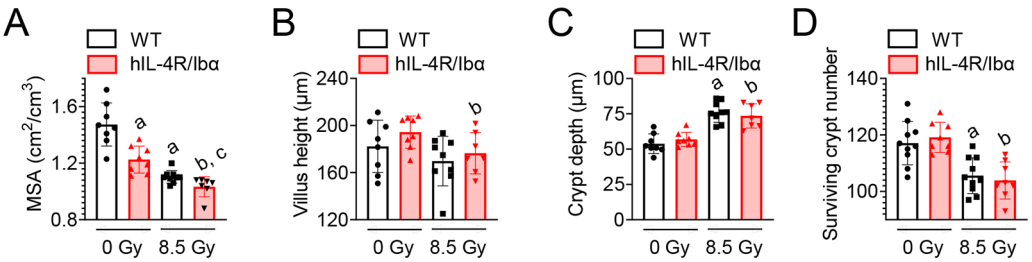

Figure 5. Histomorphometric alterations in the intestine.

Alterations in (A) MSA, (B) villus height, (C) crypt depth, and (D) surviving crypt number were measured in hIL-4R/Ibα mice and WTs 4 days after TBI. a, b, and c, represent P(value) < 0.05 from sham irradiated WT, sham irradiated hIL-4R/Ibα, and irradiated WT, respectively. To compare the differences in intestinal histomorphometric alteration between the WT and hIL-4R/Ibα mice before and after TBI, a Student's t-test was performed on mean values. Additionally, one way ANOVA was performed to assess the presence of statistically significant differences among four groups followed by Tukey's multiple comparison test. WT n = 5; hIL-4R/Ibα n = 5.

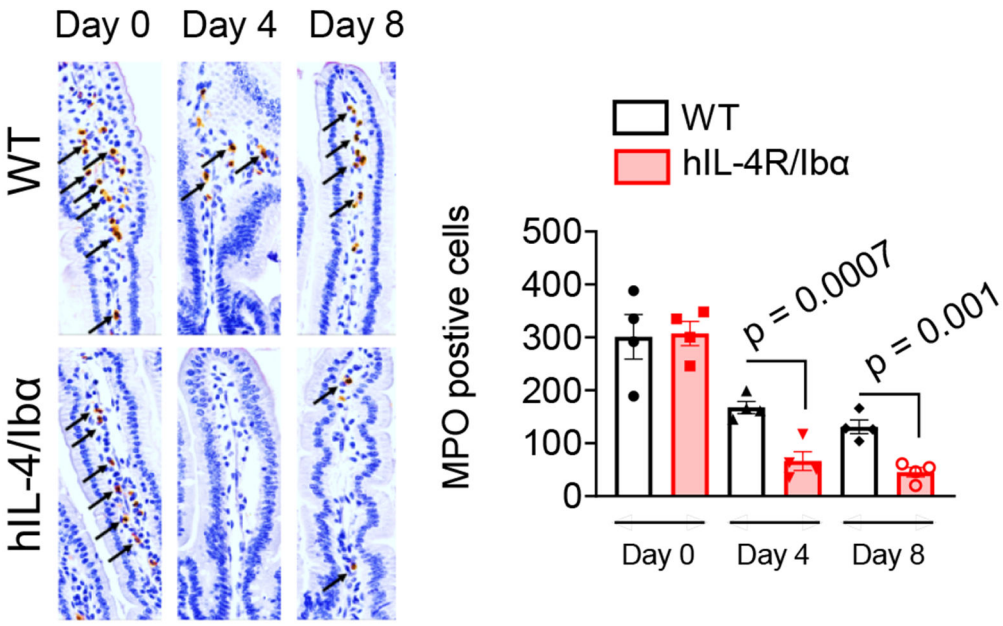

Figure 6. Neutrophil infiltration in the intestine following TBI.

Representative photomicrographs (40× magnification) of MPO immunostaining in samples from sham-irradiated (day 0), 4, and 8 days post-irradiation, and the number of MPO-positive cells in immunostained tissue at different time points in WT (n = 4) and hIL-4/Ibα mice (n = 4). To compare the differences in MPO positive cells between the WT and hIL-4R/Ibα mice before and after TBI, a Student's t-test was performed on mean values.