

High-dose X-ray irradiation induces NETosis via the eCIRP/TREM-1 axis in mouse neutrophils

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Abstract. High-dose ionizing radiation induces multiple types of tissue injuries, including hematopoietic dysfunction characterized by neutropenia. Neutrophil extracellular traps (NETs) released during NETosis may contribute to the neutropenia, and subsequent infection and inflammation. Triggering receptor expressed on myeloid cells-1 (TREM-1) is one of receptors responsible for NET formation and extracellular cold-inducible RNA-binding protein (eCIRP) is a ligand for the TREM-1 receptor. The present study aimed to investigate NET formation after exposure to high-dose ionizing radiation and to explore the underlying role of the eCIRP/TREM-1 axis as its mechanism. Bone marrow-derived neutrophils (BMDNs) isolated from C57BL/6 mice were exposed to 5 to 15 Gy irradiation. C57BL/6 wild-type (WT), CIRP^{-/-} and TREM-1^{-/-} mice were exposed to 10 Gy total body irradiation (TBI). NET formation was analyzed 24 h after irradiation using flow cytometry and fluorescence microscopy, and also

after treatment with eCIRP. TREM-1 cell surface expression on neutrophils was assessed using flow cytometry. Peptidyl arginine deiminase 4 (PAD4) protein expression levels in BMDNs were evaluated using western blotting. TREM-1 and PAD4 mRNA expression levels in BMDNs were assessed using reverse transcription-quantitative PCR. *In vitro* irradiation of neutrophils resulted in a dose-dependent increase in NET formation, as assessed using flow cytometry and validated using fluorescence microscopy, which demonstrated the characteristic long extracellular DNA structures of NETs in irradiated neutrophils. The *in vivo* mouse model of TBI exhibited similar results. Furthermore, TREM-1 expression in BMDNs was significantly increased after irradiation. Protein and mRNA levels of PAD4 were significantly upregulated after irradiation. The addition of eCIRP to BMDNs further increased NET formation post-irradiation *in vitro*. Conversely, knockout of CIRP and TREM-1 *in vivo* significantly attenuated radiation-induced NET formation compared with that of WT mice. High-dose ionizing radiation induced NET formation through the eCIRP/TREM-1 pathway and may contribute to early neutropenia post-irradiation.

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Abbreviations: ARS, acute radiation syndrome; BMDNs, bone marrow-derived neutrophils; CIRP, cold-inducible RNA-binding protein; DAMP, damage-associated molecular pattern; eCIRP, extracellular CIRP; HMGB1, high mobility group box 1; IL, interleukin; I/R, ischemia/reperfusion; MFI, mean fluorescence intensity; MPO, myeloperoxidase; NE, neutrophil elastase; NETs, neutrophil extracellular traps; PAD4, peptidyl arginine deiminase 4; ROS, reactive oxygen species; Syk, spleen tyrosine kinase; TBI, total body irradiation; TLR, toll-like receptor; TREM-1, triggering receptor expressed on myeloid cells-1

Key words: neutrophil extracellular traps, neutrophil, ionizing radiation, extracellular cold-inducible RNA-binding protein, triggering receptor expressed on myeloid cells-1

Introduction

The threat of radiation exposure from nuclear terrorism and warfare, amplified by ongoing global conflicts and events such as the occupation of Ukraine's Zaporizhzhia nuclear power plant in 2022 (1), the Fukushima Daiichi nuclear disaster in Japan in 2011 (2,3) and the Chernobyl disaster in Ukraine in 1986 (4), has become a significant concern. Exposure to high-dose radiation causes acute radiation syndrome (ARS), characterized by various clinical complications, including hematopoietic, gastrointestinal and neurovascular syndrome (5). Damage to radiosensitive hematopoietic stem and progenitor cells leads to anemia, neutropenia and thrombocytopenia (6). Neutrophils are crucial to host defense against pathogens. Following radiation exposure, neutropenia increases susceptibility to life-threatening infections and risk of mortality (7). Furthermore, severe ARS typically includes multiple organ dysfunction (8,9). Radiation-induced inflammation has been shown to contribute to organ dysfunction in several systems, including hematopoiesis, the gastrointestinal tract and lungs, and is associated with an increased risk of mortality (5,10-13). Medical management of ARS focuses on the targeted treatment

of the subsyndromes. Treatment of hematopoietic syndrome involves the administration of granulocyte colony stimulating factor, granulocyte macrophage stimulating factor, erythropoiesis-stimulating factor and platelet-stimulating thrombopoietin mimetic (14). Hematopoietic stem cell transplantation may be considered in cases of persistent aplasia (8). Management of gastrointestinal syndrome is currently limited to symptomatic treatment, including antiemetics, antidiarrheals and antibiotics (5,8). Neurovascular syndrome is primarily addressed with supportive care (14).

Neutrophil extracellular traps (NETs) are critical for host defense against infection, trapping and eliminating pathogens such as bacteria, fungi and viruses (15). NET formation is initiated by peptidyl arginine deiminase 4 (PAD4)-mediated citrullination of histones, leading to decondensation of chromatin (16). Expanded chromatin is released into the cytoplasm, causing the rupture of the plasma membrane and the release of NETs, which form web-like structures (17). Released NETs include histones, myeloperoxidase (MPO) and other antimicrobial proteins such as neutrophil elastase (NE) (18). Conversely, excessive NETs can exacerbate inflammation and contribute to organ dysfunction in sepsis (19-21). Furthermore, NETosis is a form of regulated cell death of neutrophils, which can potentially contribute to neutropenia (22,23). NET formation is also implicated in sterile inflammatory conditions such as autoimmune disorders, vasculitis and thrombosis (19). However, the impact of high-dose ionizing radiation on NET formation in healthy tissue and the underlying mechanisms involved remains largely unexplored. Current research on radiation-induced NETs primarily focuses in the context of cancer radiotherapy (24).

Cold-inducible RNA-binding protein (CIRP) is an 18 kDa RNA chaperone that regulates the translation of stress response genes (25,26). Extracellular CIRP (eCIRP), which is released actively from stressed cells and passively from dying cells (27), acts as a damage-associated molecular pattern (DAMP). eCIRP exacerbates inflammation and worsens survival in conditions such as sepsis, hemorrhagic shock, ischemia/reperfusion (I/R) injury and radiation injury (28-30). Triggering receptor expressed on myeloid cells-1 (TREM-1) is an immune receptor expressed on the cell surface, primarily on neutrophils and macrophages, that initiates intracellular signaling cascades via phosphorylation of DNAX-activation protein 12 (DAP12) and spleen tyrosine kinase (Syk) (31). TREM-1 is known to induce pro-inflammatory responses and amplify the severity of inflammatory diseases (32,33). Our previous studies have identified eCIRP as a novel ligand for TREM-1 and eCIRP-mediated activation of TREM-1 as a key trigger for NET formation in sepsis (34,35). Notably, it was demonstrated that radiation-induced eCIRP upregulates TREM-1 surface expression on macrophages, and that TREM-1 deficiency improves survival after total body irradiation (TBI) (36). The present study aimed to investigate NET formation after exposure to high-dose ionizing radiation and the role of eCIRP and TREM-1 in radiation-induced NET formation.

Materials and methods

Experimental animals. A total of 84 adult C57BL/6 wild-type (WT) male mice (age, 8-12 weeks; weight, 20-25 g)

were purchased from Jackson Laboratory. CIRP knockout mice (CIRP^{-/-}) were obtained from Professor Jun Fujita (Kyoto University; Kyoto, Japan). TREM-1 knockout mice (TREM-1^{-/-}; Trem1^{tm1(KOMP)Vlcg}) were generated by the trans-National Institutes of Health Knockout Mouse Project (KOMP) and obtained from the KOMP repository (University of California) (37). Six each of CIRP^{-/-} and TREM-1^{-/-} mice were used for the assessment of NET formation *in vivo* by flow cytometry. Mice were maintained in a temperature-controlled room ranging between 20 and 26°C, with a humidity level of 30-70%, under a 12-h light/dark cycle and were fed a standard rodent diet and water *ad libitum*. All animal experiments were performed in accordance with the guidelines for using experimental animals by the National Institutes of Health. All study procedures were approved by Institutional Animal Care and Use Committees of the Feinstein Institutes for Medical Research (approval no. 2023-007; Manhasset, NY, USA).

TBI and isolation of bone marrow cells. A total of 54 WT mice were randomly assigned to experimental and control groups, with 24 sham and 30 TBI mice. Mice were exposed to a single dose of 10 Gy TBI at a dose rate of 1 Gy/min (320 kV, 12.5 mA and 50 cm source-to-skin distance) using an X-Rad320 X-ray irradiation system (Precision X-Ray, Inc.). The 10 Gy model is considered to be high-dose irradiation in mice based on prior studies (38-40). Mice were restrained and placed in a fitted container without shielding during irradiation. The container was continuously rotated for even exposure. Mice were returned to their cages after completion. Sham (control) mice were not irradiated. After 24 h of TBI, mice were euthanized by exsanguination under isoflurane anesthesia (induction, 3%; maintenance, 2%). The femurs and tibias were then dissected for analysis. The bone marrow was flushed with PBS supplemented with 2% FBS (Gibco; Thermo Fisher Scientific, Inc), and suspensions of cells were filtered through a 70-µm cell strainer. Red blood cells in the bone marrow were lysed with RBC lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.). Bone marrow cells were collected by centrifugation at 300 x g and suspended with FACS buffer (PBS containing 2% FBS). The humane criteria established for the present study were as follows: minimal or no response to stimuli, hunched or recumbent posture, grimace score of =2, body condition score ≤2, weight loss of ≥20% or bleeding. All animals fulfilling ≥2 humane criteria were subjected to euthanasia.

Isolation and irradiation of bone marrow-derived neutrophils (BMDNs). A total of 30 naïve WT mice were euthanized by CO₂ asphyxiation at a fill rate of 50% displacement of the chamber volume per min followed by cervical dislocation, and the femurs and tibias were subsequently dissected. The bone marrow was flushed with ice-cold PBS supplemented with 2% FBS, and suspensions of cells were filtered through a 70-µm cell strainer and placed on ice. BMDNs were purified by negative selection using the EasySep mouse neutrophil enrichment kit (Stemcell Technologies, Inc.) according to the manufacturer's instructions and cultured in complete RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) on a cell culture plate at 37°C in 5% CO₂ for *in vitro* experiments. BMDNs were either exposed to radiation at a dose rate of 3 Gy/min using the X-ray Precision X-Rad320 (Precision X-Ray, Inc.) or not

irradiated (control). After irradiation, cells were incubated at 37°C in 5% CO₂. For the experiment of additional stimulation of eCIRP, BMDNs were treated with 1 µg/ml eCIRP, prepared in-house using a bacterial expression system (28), immediately after irradiation for 24 h at 37°C in 5% CO₂.

Assessment of NETs and TREM-1 expression on neutrophils by flow cytometry. The purity of the sorted neutrophils was assessed by labeling the cells with APC-rat anti-mouse Ly6G antibody (cat. no. 127614; BioLegend, Inc.) using a LSRFortessa™ flow cytometer (BD Biosciences). NETs were assessed using flow cytometry for both *in vivo* and *in vitro* experiments (35). Single-cell suspensions of neutrophils after irradiation were blocked with TruStain FcX™ PLUS (anti-mouse CD16/32) antibody (cat. no. 156604; BioLegend, Inc.) for 15 min at 4°C and surface-stained with APC-Ly6G, FITC anti-MPO (cat. no. ab90812; Abcam) and rabbit anti-histone H3 (citrulline R2+ R8+ R17) antibodies (cat. no. ab5103; Abcam) for 30 min at 4°C, followed by staining with PE-donkey anti-rabbit IgG antibody (cat. no. 406421, BioLegend, Inc.) as a secondary antibody for 30 min at 4°C. For the assessment of TREM-1 expression, neutrophils were also stained with BV421-rat anti-mouse TREM-1 antibody (cat. no. 747899; BD Biosciences) or BV 421-rat IgG2a κ isotype control (cat. no. 562602; BD Biosciences) for 30 min at 4°C. Unstained cells were used to establish control voltage setting and single-color compensation was performed using UltraComp eBeads™ (Invitrogen; Thermo Fisher Scientific, Inc.). Data were obtained using the LSRFortessa flow cytometer (BD Biosciences) and analyzed using the FlowJo software (version 10.10.0; BD Biosciences). The population of MPO and citrullinated H3 double-positive neutrophils were identified as NET-forming neutrophils. Mean fluorescence intensity (MFI) was used to evaluate the levels of TREM-1 expression.

Assessment of NETs via fluorescence microscopy. NET formation by BMDNs after radiation exposure was assessed by fluorescence microscopy *in vitro* (41). At 24 h after irradiation, BMDNs were incubated with 250 nM SYTOX Green (cat. no. S7020; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 37°C in 5% CO₂. The cells were visualized as live-cell images without fixation using fluorescence microscopy (EVOS FL Auto Imaging System; Thermo Fisher Scientific, Inc.).

Western blotting. PAD4 protein expression levels were assessed by western blotting. BMDNs were collected by centrifugation at 300 x g at 20 h after irradiation and lysed in RIPA lysis buffer [10 mM Tris-buffered saline (TBS; pH 7.5), 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate and 0.2 mM phenylmethylsulfonyl fluoride] containing protease inhibitors (Pierce; Thermo Fisher Scientific, Inc.). Protein concentrations in the cell lysates were measured using DC protein assay kit (Bio-Rad Laboratories, Inc.). The cell lysates of BMDNs (20 µg/lane) were dissolved in 4X SDS sample buffer and resolved on NuPAGE 4-12% Bis-Tris gels (Invitrogen; Thermo Fisher Scientific, Inc.), after which, they were transferred to nitrocellulose membranes (Invitrogen; Thermo Fisher Scientific, Inc.). After blocking with 0.1%

casein in TBS for 1 h at room temperature, each membrane was incubated overnight at 4°C with primary antibodies of rabbit anti-PAD4 antibody (1:1,000; cat. no. 17373-1-AP; Proteintech Group, Inc.) and mouse anti-β-actin antibody (1:5,000; cat. no. A5441; MilliporeSigma). The membranes were washed with TBS and then incubated with the corresponding fluorescent secondary antibodies for 1 h at room temperature. The secondary antibodies IRDye 800CW goat anti-rabbit IgG (1:5,000; cat. no. 926-32211; LI-COR Biosciences) and IRDye 680RD goat anti-mouse IgG (1:5,000; cat. no. 926-68070; LI-COR Biosciences) were used. Detection was performed using the Odyssey Clx (LI-COR Biosciences) imaging system and quantification with the Image Studio software (version 5.2; LI-COR Biosciences).

Reverse transcription-quantitative PCR. BMDNs were collected by centrifugation at 300 x g at 4°C for 10 min a total of 4 h after irradiation. Illustra™ RNAspin Mini RNA Isolation kit (Cytiva) was utilized to extract total RNA according to the manufacturer's instructions. Subsequently, 1 µg RNA was reverse-transcribed into complementary DNA (cDNA). Briefly, first-strand cDNA was synthesized using oligo(dT) primers (Invitrogen; Thermo Fisher Scientific, Inc.) via incubation at 70°C for 10 min, followed by cooling to 4°C. RT was performed by adding a reaction mixture containing PCR buffer with MgCl₂ (Applied Biosystems; Thermo Fisher Scientific, Inc.), dNTP mix (Invitrogen; Thermo Fisher Scientific, Inc.), RNase inhibitor (Applied Biosystems; Thermo Fisher Scientific, Inc.) and reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) and by incubation at 42°C for 60 min, heating to 95°C for 5 min and cooling to 4°C. PCR was performed using a final volume of 20 µl containing forward and reverse primers (final concentration, 0.06 mM each), cDNA and SYBR Green master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a Step One Plus real-time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following program was run: 95°C for 10 min as an initial denaturation step, followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 1 min. Mouse β-actin mRNA served as a reference gene for normalization. Relative gene expression levels were calculated using the comparative 2^{-ΔΔC_q} method (42). Relative expression levels of mRNA were represented as fold-change relative to the control group. The primer sequences used were as follows: PAD4 forward (F), 5'-GCAGGACATGTCTCCAATGA-3' and reverse (R), 5'-AGCTCCAGGCAATACGAGAA-3'; TREM-1 F, 5'-CTACAACCCGATCCCTACCC-3' and R, 5'-AAACCAGGCTCTTGCTGAGA-3'; and β-actin F, 5'-CGTGAAAAGATGACCCAGATCA-3' and R, 5'-TGGTACGACCAGAGGCATACAG-3'.

Statistical analysis. Data represented in the figures are expressed as mean and SEM. An unpaired two-tailed Student's t-test was used for comparisons of two groups and one-way analysis of variance followed by Tukey's multiple comparisons post hoc test were used for comparisons of multiple groups. Data were analyzed using the GraphPad Prism software (version 10.3.1; Dotmatics). P<0.05 was considered to indicate a statistically significant difference.

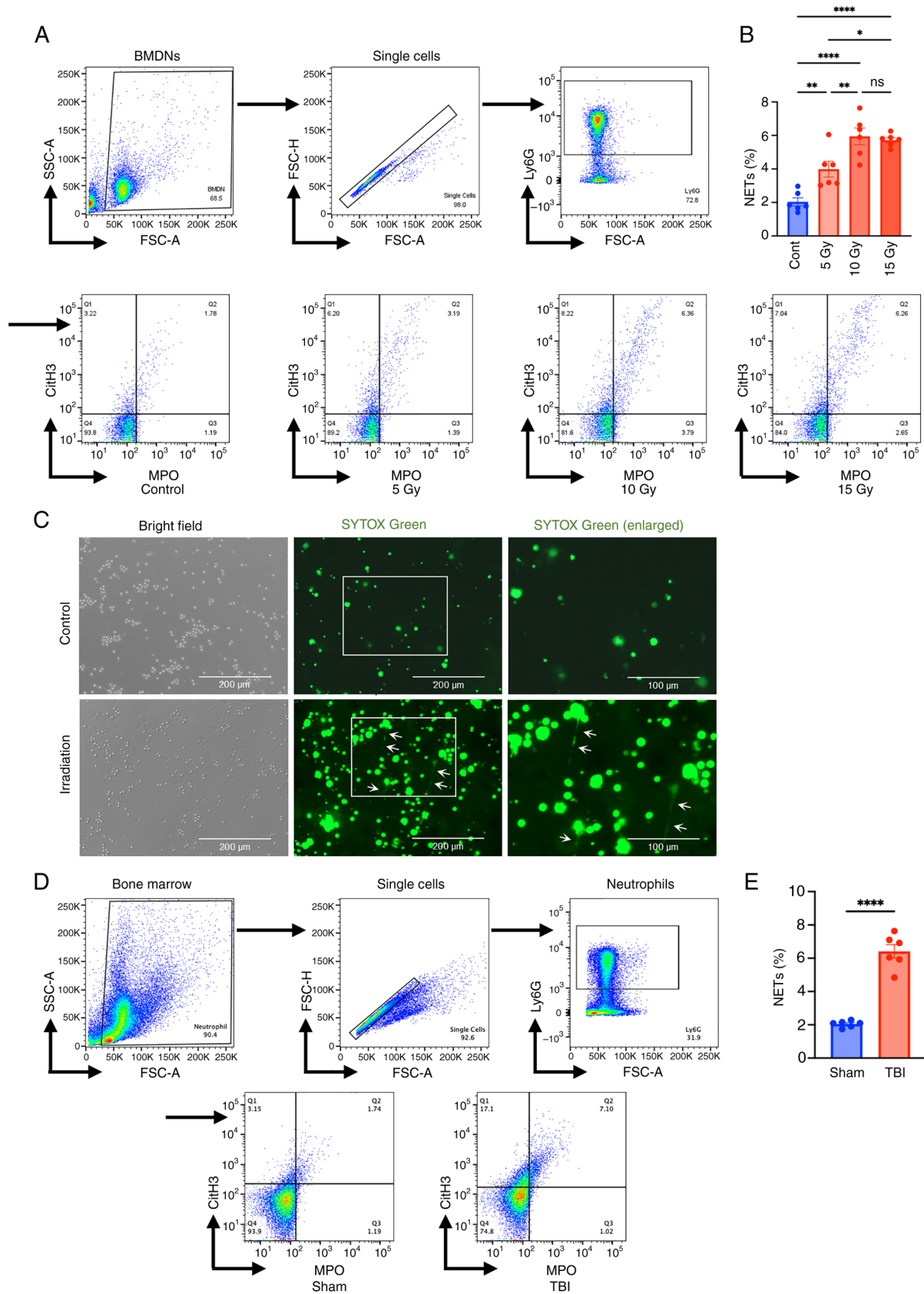


Figure 1. Exposure to ionizing radiation induces NETs. (A) BMDNs were exposed to 5, 10 and 15 Gy radiation. NETs were assessed via flow cytometry 24 h after irradiation. (B) The frequencies of NET positive neutrophils are shown. Groups were compared using a one-way ANOVA and Tukey's multiple comparison test. (C) Representative images of BMDNs that were exposed to 10 Gy radiation. NET formation was assessed by fluorescence microscopy using SYTOX Green 24 h after irradiation. White arrows indicate the NET structures. Scale bar, 200 μ m (Bright field and SYTOX Green); scale bar, 100 μ m [SYTOX Green (enlarged)]. (D) Wild-type adult mice were exposed to 10 Gy TBI. Bone marrow cells were isolated 24 h after TBI and NETs were assessed using flow cytometry. (E) The frequencies of NET positive neutrophils. Groups were compared using an unpaired two-tailed Student's t-test. Data are expressed as the mean $\pm$ SEM (n=6/group). * P <0.05, ** P <0.01 and **** P <0.0001. ns, not significant; cont, control; NET, neutrophil extracellular traps; BMDNs, bone marrow-derived neutrophils; TBI, total body irradiation.

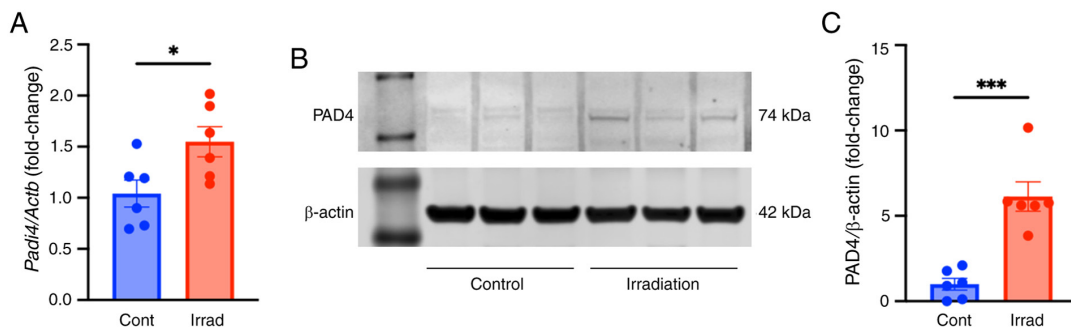


Figure 2. Radiation upregulates PAD4 mRNA and protein expression in neutrophils. BMDNs were exposed to 10 Gy radiation. (A) PAD4 mRNA expression levels were assessed 4 h after irradiation via reverse transcription-quantitative PCR. PAD4 protein expression levels were evaluated by western blotting 20 h after irradiation. (B) Representative PAD4 western blot and (C) quantitative bar diagrams are shown. Groups were compared using an unpaired two-tailed Student's t-test. Data are expressed as the mean $\pm$ SEM (n=6/group). *P<0.05 and ***P<0.001. Cont, control; Irrad, irradiation; NET, neutrophil extracellular traps; BMDNs, bone marrow-derived neutrophils; PAD4, peptidyl arginine deiminase 4.

Results

Exposure to ionizing radiation induces NET formation. To determine whether exposure to ionizing radiation induces NETs, BMDNs were exposed to 5 to 15 Gy radiation and NET formation was assessed 24 h after irradiation via flow cytometry. Citrullinated histone H3 and MPO double-positive cells were considered as neutrophils with NETs. The number of NET-forming neutrophils were significantly increased following 5 to 10 Gy irradiation in a dose-dependent manner and reached a plateau at 10 Gy (Fig. 1A and B). NET formation was further confirmed as extracellular DNA using microscopy with SYTOX Green labelling, a nucleic acid stain that fluoresces green when bound to DNA. Notably, SYTOX Green does not cross intact membranes but penetrates compromised membranes. The number of neutrophils with expanded nuclear area stained by SYTOX Green were increased after irradiation compared with that of the control cells, and a number of irradiated neutrophils showed long stretches of extracellular DNA, which indicated NET formation (Fig. 1C). NET formation was also evaluated *in vivo*. Bone marrow from irradiated WT mice 24 h after 10 Gy TBI were isolated and NETs evaluated using flow cytometry. Consistent with the *in vitro* experiments, NET-forming neutrophils were significantly increased after TBI compared with that of sham mice (Fig. 1D and E). Thus, these results demonstrated that exposure to ionizing radiation induced NET formation.

Radiation upregulates PAD4 expression levels in neutrophils. PAD4 catalyzes histone citrullination, leading to chromatin decondensation and NETosis (15). PAD4 mRNA and protein expression levels in BMDNs after irradiation were investigated. BMDNs were exposed to 10 Gy radiation and mRNA and protein expression levels of PAD4 were evaluated 4 or 20 h after irradiation, respectively. The mRNA levels of PAD4 were significantly upregulated after irradiation compared with that of control cells (Fig. 2A). Similarly, PAD4 protein expression levels were also significantly upregulated after irradiation compared with that of control cells (Fig. 2B and C). These findings further evidenced the induction of NET formation after irradiation.

eCIRP induces NET formation after irradiation. To determine the role of eCIRP on radiation-induced NET formation, NET

formation in bone marrow isolated from WT and CIRP^{-/-} mice 24 h after 10 Gy TBI was assessed. NET-forming neutrophils were significantly increased in WT TBI mice compared with that in sham mice. CIRP knockout ameliorated NET formation compared with that of WT TBI mice (Fig. 3A and B). Additional effects of eCIRP on radiation-induced NETs were assessed as a gain-of-function experiment. The addition of eCIRP to BMDNs in culture significantly increased NET formation after irradiation (Fig. 3C and D). These loss- and gain-of-functional experiments indicated that eCIRP promotes radiation-induced NET formation.

Radiation upregulates neutrophil TREM-1. TREM-1 has been identified as a key receptor of eCIRP and has been shown to initiate NETosis (35). Therefore, the effect of radiation on TREM-1 expression on BMDNs after irradiation was investigated. BMDNs isolated from WT mice were exposed to 10 Gy irradiation and TREM-1 cell surface protein expression levels were evaluated 24 h after irradiation using flow cytometry. Exposure to ionizing radiation significantly upregulated TREM-1 expression levels in BMDNs compared with that in control cells (Fig. 4A and B). A total of 4 h after irradiation, the mRNA level of TREM-1 was significantly upregulated compared with that of control cells (Fig. 4C). TREM-1 expression levels in WT mice were also significantly upregulated after TBI compared with those in the sham group (Fig. 4D and E). Thus, radiation exposure increases mRNA and surface protein expression of TREM-1 in neutrophils.

TREM-1 positively regulates radiation-induced NETs. Next, the association between radiation-induced NETs and the intensity of TREM-1 expression was investigated in BMDNs *in vitro*. Irradiated BMDNs were classified into two populations, neutrophils with high and low TREM-1 expression based on the median TREM-1 expression as the cut-off value, and NET formation was evaluated (Fig. 5A). There was a positive association between the intensity of TREM-1 expression and the number of NET-forming neutrophils (Fig. 5B). The association in the bone marrow neutrophils isolated from WT TBI mice was determined; neutrophils with high TREM-1 expression demonstrated a significant increase in NETs after irradiation compared with that in the low TREM-1 expression group (Fig. 5C and D). The effect of TREM-1 knockout on

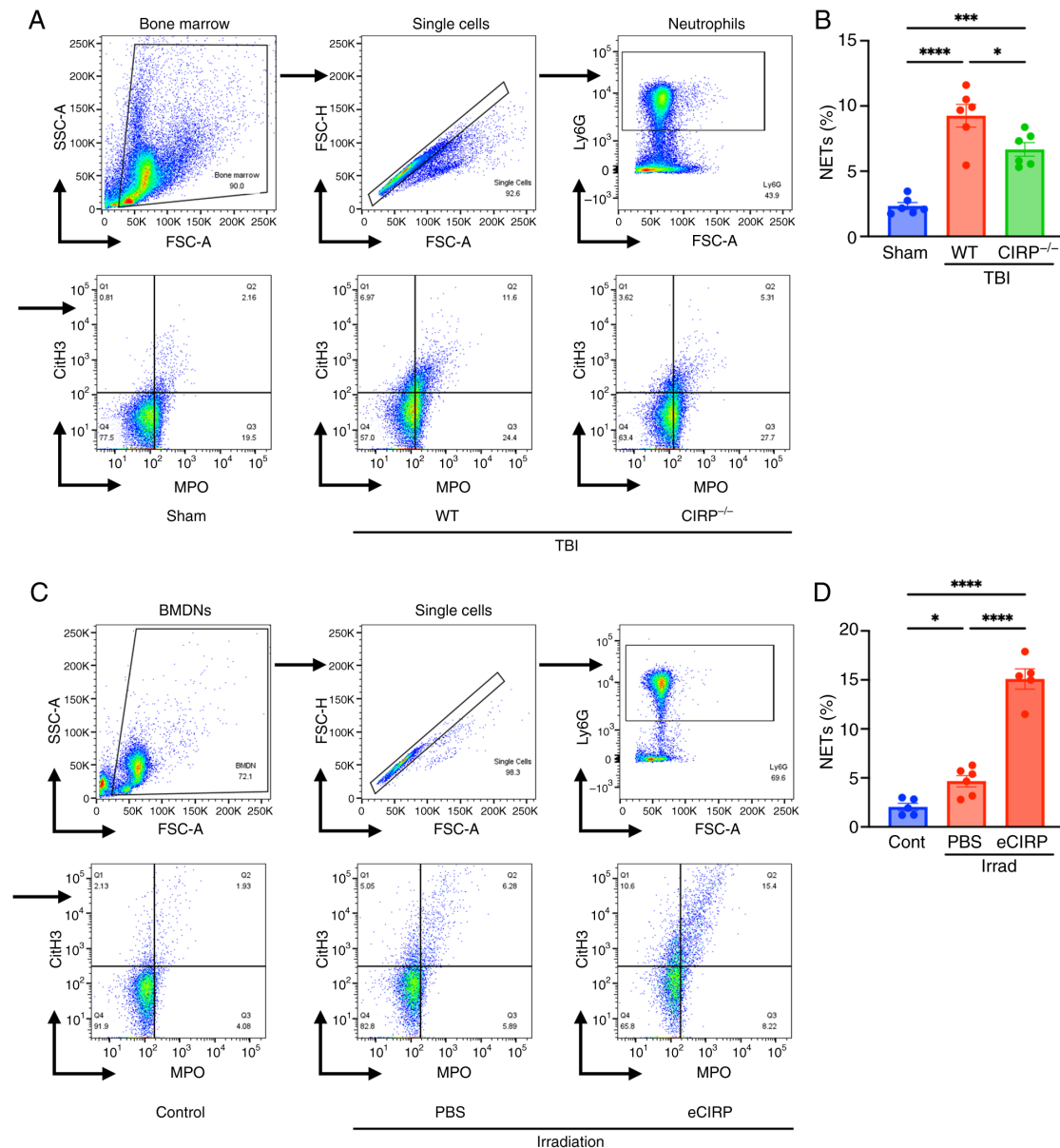


Figure 3. eCIRP induces NET formation after irradiation. (A) Adult WT and CIRP^{-/-} mice were exposed to 10 Gy TBI. Bone marrow cells were isolated 24 h after TBI and NETs were assessed by flow cytometry. (B) Frequencies of NET positive neutrophils. Data are expressed as the mean $\pm$ SEM (n=6/group). Groups were compared using a one-way ANOVA and Tukey's multiple comparison test. (C) BMDNs were exposed to 10 Gy radiation and treated with 1 μ g/ml eCIRP immediately after irradiation. NETs were assessed via flow cytometry 24 h after irradiation. (D) Frequencies of NET positive neutrophils. Groups were compared using a one-way ANOVA and Tukey's multiple comparison test. Data are expressed as the mean $\pm$ SEM (n=5-6/group). *P<0.05, ***P<0.001 and ****P<0.0001. Cont, control; Irrad, irradiation; NET, neutrophil extracellular traps; BMDNs, bone marrow-derived neutrophils; CIRP, cold-inducible RNA-binding protein; eCIRP, extracellular CIRP; WT, wild-type.

NET formation after irradiation was evaluated. Bone marrow cells were isolated from irradiated WT and TREM-1^{-/-} mice 24 h after 10 Gy TBI and NET formation assessed via flow cytometry. NET-forming neutrophils were significantly increased in WT TBI mice compared with that in sham mice, whereas NET-forming neutrophils were significantly decreased in TREM-1^{-/-} TBI mice compared with that in WT TBI mice (Fig. 5E and F). These data indicated that TREM-1 was associated with NET formation after exposure to ionizing radiation.

Discussion

Clinical experiences from nuclear accidents and war have shown that ARS exhibits a variety of clinical manifestations

with lethal consequences (8). The initial treatment of patients exposed to high doses of radiation focuses on hematopoietic dysfunction. Bacterial translocation due to gastrointestinal injury, combined with neutropenia, can lead to severe infections and sepsis (43). In addition, uncontrolled inflammatory responses worsen multi-organ dysfunction in severe ARS (8,9). NETosis is a form of regulated cell death that could contribute to the initiation and duration of neutropenia (23). Furthermore, excessive NET formation has been shown to induce pro-inflammatory responses and exacerbate organ damage (19). The present study demonstrated that exposure to 5-15 Gy of ionizing radiation induces NET formation, and identified the eCIRP/TREM-1 pathway as the mechanism that mediates NET formation after radiation exposure (Fig. 6).

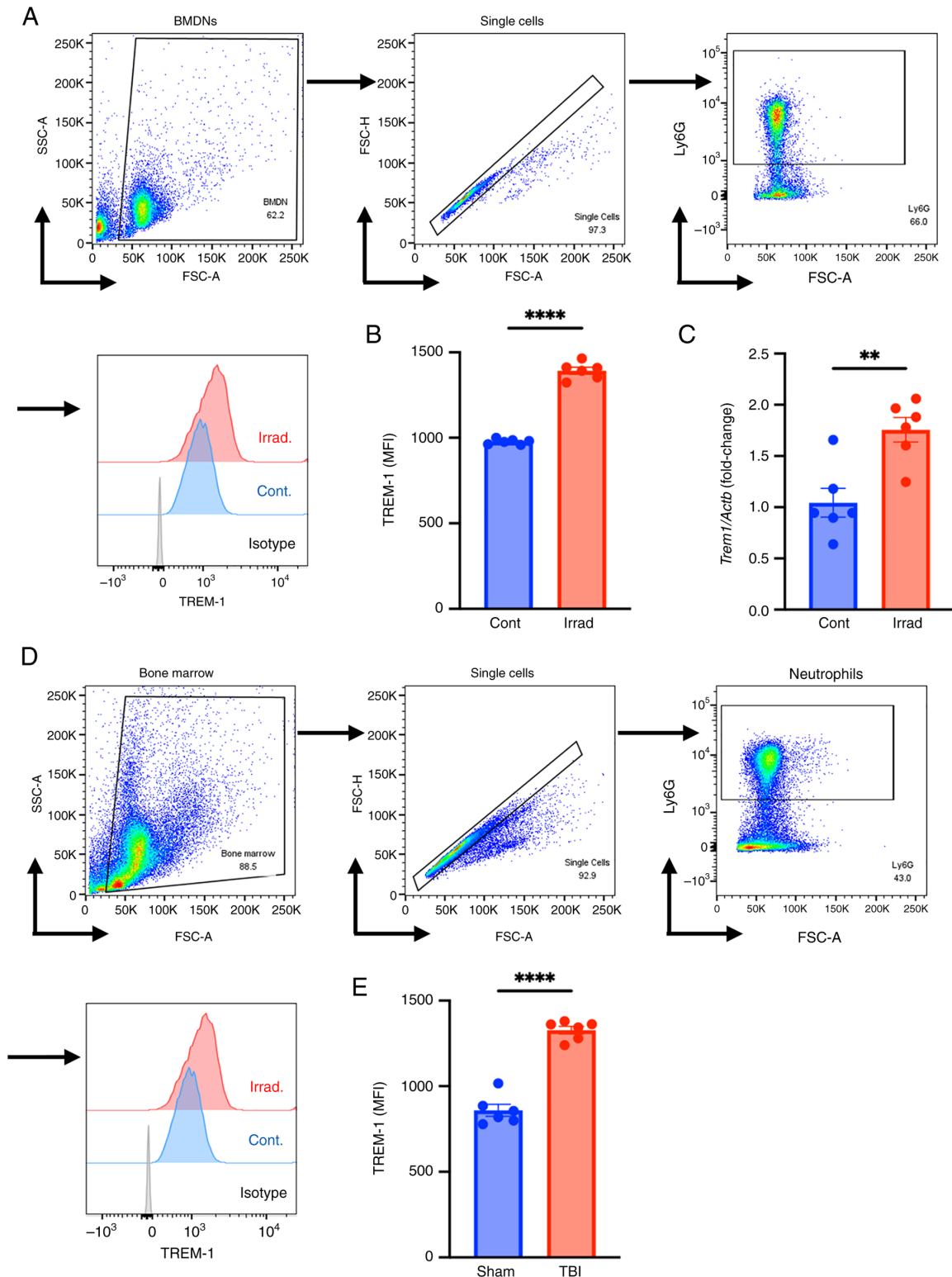


Figure 4. Radiation upregulates neutrophil TREM-1. BMDNs were exposed to 10 Gy radiation. (A) TREM-1 cell surface expression was assessed via flow cytometry 24 h after irradiation. (B) MFI values of TREM-1. (C) TREM-1 mRNA expression levels were assessed 4 h after irradiation via using reverse transcription-quantitative PCR. Groups were compared using an unpaired two-tailed Student's t-test. (D) WT adult mice were exposed to 10 Gy TBI. Bone marrow cells were isolated 24 h after TBI and TREM-1 cell surface expression were assessed via flow cytometry. (E) MFI values were used to evaluate TREM-1 expression levels. Groups were compared by unpaired two-tailed Student's t-test. Data are expressed as the mean $\pm$ SEM (n=6/group). **P<0.01 and ****P<0.0001. Cont, control; Irrad, irradiation; NET, neutrophil extracellular traps; BMDNs, bone marrow-derived neutrophils; TREM-1, triggering receptor expressed on myeloid cells-1; TBI, total body irradiation; MFI, mean fluorescence intensity; WT, wild-type.

Therefore, focusing on radiation-induced NET formation by targeting eCIRP/TREM-1 axis can provide novel therapeutic strategies for acute neutropenia in the future.

To the best of our knowledge, only two studies of ionizing radiation-induced NETosis and NET formation have been conducted, and they were confined to the

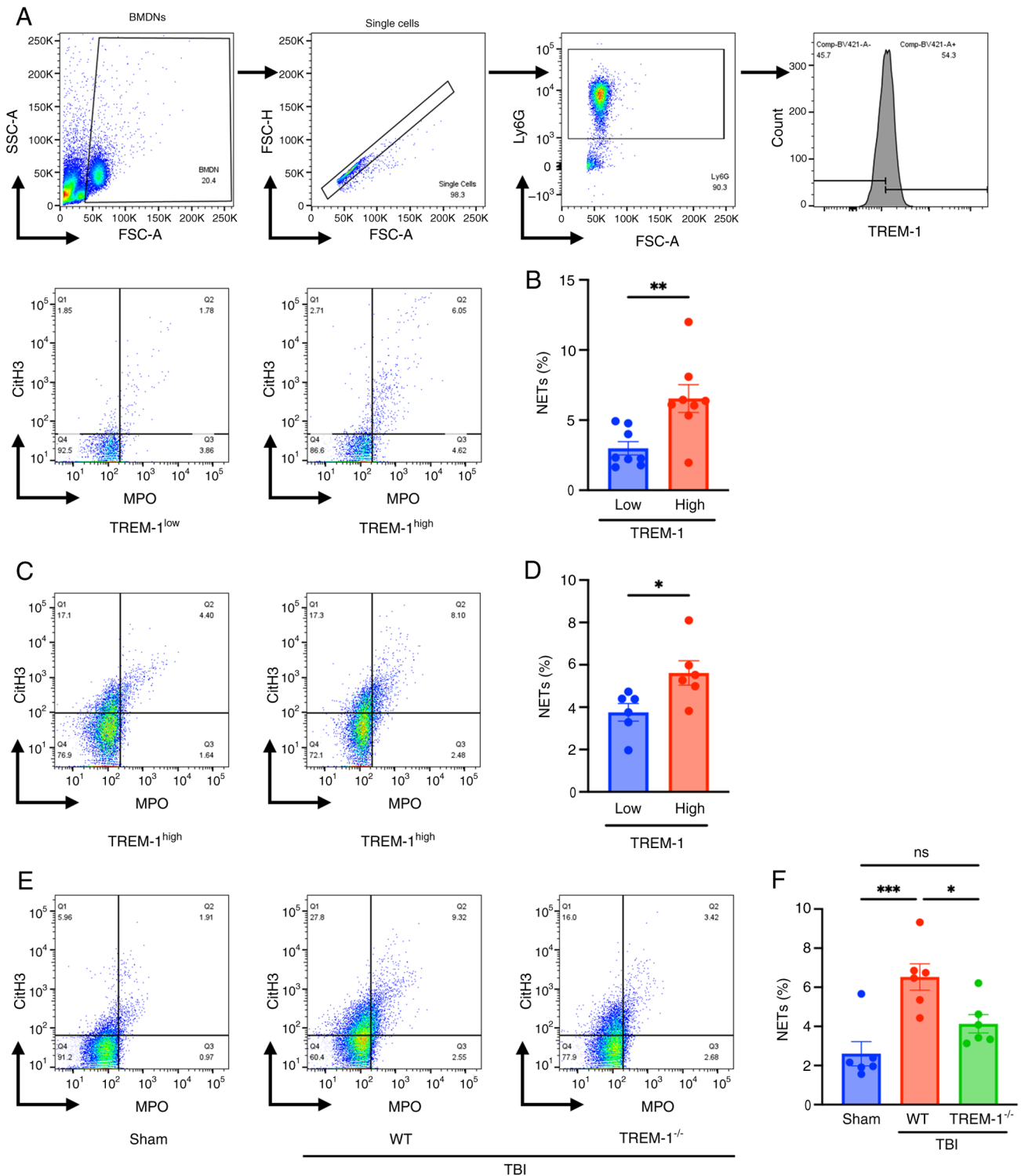


Figure 5. TREM-1 positively regulates radiation-induced NETs. (A) BMDNs were exposed to 10 Gy radiation. BMDNs were classified into two populations with high and low TREM-1 expression and NETs were assessed via flow cytometry 24 h after irradiation. (B) Frequencies of NET positive neutrophils. Groups were compared using an unpaired two-tailed Student's t-test. (C) WT adult mice were exposed to 10 Gy TBI. Bone marrow cells were isolated 24 h after TBI and classified into two populations with high and low TREM-1 expression. NETs were assessed via flow cytometry. (D) Frequencies of NET positive neutrophils. Groups were compared by unpaired two-tailed Student's t-test. (E) Adult WT and TREM-1^{-/-} mice were exposed to 10 Gy TBI. Bone marrow cells were isolated 24 h after TBI and NETs were assessed via flow cytometry. (F) Frequencies of NET positive neutrophils. Groups were compared using a one-way ANOVA and Tukey's multiple comparison test. Data are expressed as the mean $\pm$ SEM (n=6/group). *P<0.05, **P<0.01 and ***P<0.001. ns, not significant; NET, neutrophil extracellular traps; BMDNs, bone marrow-derived neutrophils; TREM-1, triggering receptor expressed on myeloid cells-1; TBI, total body irradiation; MFI, mean fluorescence intensity; WT, wild-type.

context of tumor microenvironment (24,44). Radiation therapy targeting cancer tissues has been shown to activate peritumoral neutrophils and induce NET formation, which

promotes angiogenesis, enhances tumor cell adhesion and increases the risk of radiation resistance and metastasis (19). Teixeira *et al* (44) recently showed that exposure of

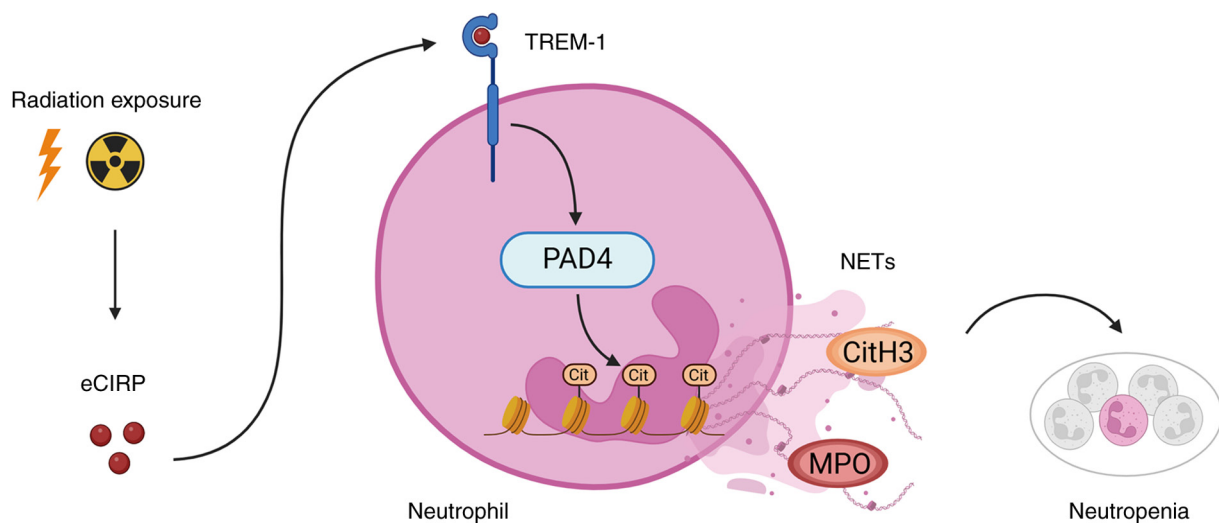


Figure 6. Exposure to ionizing radiation induces NETs via eCIRP/TREM-1 axis. Radiation-induced eCIRP upregulates and activates TREM-1 on neutrophils, resulting in increased expression of PAD4 and NET formation, causing neutropenia. NETs, neutrophil extracellular traps; PAD4, peptidyl arginine deiminase 4; eCIRP, extracellular cold-inducible RNA-binding protein; TREM-1, triggering receptor expressed on myeloid cells-1; MPO, myeloperoxidase; cit, citrulline; CitH3, citrullinated H3. The schema was created in BioRender. Murao, A. (2025) <https://BioRender.com/qhaiby0>

neutrophils isolated from healthy human blood to low-dose γ -radiation (0.5-1 Gy) induces NET formation. It was also shown that oxidative stress, NADPH oxidase activity and IL-8 triggers NET formation (44). Additionally, ultraviolet irradiation has been reported to induce NET formation via oxidative stress (45,46). However, the formation of NETs induced by high-dose ionizing radiation and the underlying mechanisms have not yet been studied. The present study first demonstrated radiation-induced NET formation by the quantitative evaluation of NETs using flow cytometry, along with direct observation through fluorescence microscopy. The increased mRNA and protein expression levels of PAD4 upon exposure to ionizing radiation further supports the radiation-induced NET formation. Subsequently, the mechanisms that regulate radiation-induced NET formation were investigated. Several types of receptors, such as Toll-like receptor (TLR) 2, TLR4, G protein-coupled receptor and Fc receptors, have been reported to be involved in NET formation (47-50). Neutrophils can be activated via these receptors by pathogens, lipopolysaccharide, cytokines and DAMPs to induce NET formation (15). Our previous studies reported TREM-1 as a key receptor responsible for NET formation in a sepsis model (35) and demonstrated the strong binding affinity between eCIRP and TREM-1 using surface plasmon resonance (37). The interaction of eCIRP with TREM-1 on mouse macrophages using a fluorescence resonance energy transfer assay was also demonstrated (37). In addition, our previous studies have shown that exposure to high-dose radiation induces release of eCIRP in mice (36,51). The present study demonstrated that the eCIRP/TREM-1 axis serves a critical role in NET induction after radiation exposure. Currently, it has not yet been established how TREM-1 activation promotes NET formation. However, in a sepsis model, eCIRP-mediated activation of TREM-1 induces the surface expression of ICAM-1, leading to NET formation via Rho activation (35). Furthermore, eCIRP activates the downstream signaling pathway of TREM-1, as evaluated by DAP12

and Syk phosphorylation (35). Syk activation mediates the phosphorylation of phospholipase C γ (PLC γ) (52). Activated PLC γ degrades phosphatidylinositol 4,5-bisphosphate in the plasma membrane to inositol 1,4,5-trisphosphate, triggering the release of calcium from endoplasmic reticulum (52). Elevated intracellular calcium levels induce PAD4 activation and extracellular calcium chelation has been shown to inhibit NETosis induced by IL-8 and phorbol-12-myristate-13-acetate (53,54). Furthermore, activation of Syk following TREM-1 stimulation has been shown to induce reactive oxygen species (ROS) production via the PIK3/AKT pathway (55). ROS are also critical in NETosis signaling, as demonstrated by the inhibition of NETosis upon treatment of neutrophils with ROS scavengers (53,54). A previous study has reported that low-dose γ -radiation induces NET formation depending on oxidative stress and ROS inhibition significantly attenuates radiation-induced NETosis (44). Since TREM-1 is associated with ROS production, the aforementioned findings with low-dose irradiation could potentially also be mediated, at least in part, via the TREM-1 pathway. Nevertheless, whether the induction of NETs triggered by the eCIRP/TREM-1 axis is concurrent after low-dose irradiation may require further investigation.

Bone marrow damage from radiation exposure leads to less recruitment of polymorphonuclear leukocytes into the systemic circulation (56). Furthermore, the half-life of circulating neutrophils is short, ranging from 18 to 19 h. (23). Consequently, induction of neutrophil cell death after radiation exposure inevitably exacerbates neutropenia, resulting in high susceptibility to infection (7). Furthermore, organ dysfunction caused by NETs has been demonstrated in a variety of diseases, including sepsis, I/R injury, and renal and hepatic injuries (57-65). In sepsis and I/R injury, NETs have been shown to cause systemic inflammation and epithelial barrier disruption, resulting in intestinal and lung injury (57-63). Inflammation and organ damage by NETs have also been demonstrated in liver and renal injuries (64,65). Histones, a

key component of NETs, have been shown to induce inflammatory responses via the TLR2/TLR4-MyD88 signaling or the NLRP3 inflammasome pathway (66). Histones also bind to phospholipids in the plasma membrane of epithelial and endothelial tissues, altering their permeability and causing cell death and tissue damage (67). NET components such as MPO and NE impair junctional integrity and promote vascular permeability (68,69). MPO has been shown to bind to the endothelial glycocalyx via heparan sulfate side chains, disrupting the endothelial glycocalyx structure and causing endothelial injury (68). Considering that excessive inflammatory responses accompanied by disruption of vascular homeostasis contribute to multi-organ dysfunction after severe ARS, radiation-induced NET formation may potentially be an exacerbating factor in radiation injury.

The detrimental role of DAMPs has been addressed in a number of inflammatory conditions such as sepsis, hemorrhage and I/R injury (27). Although evidence for DAMPs in radiation injury is limited, targeting DAMPs presents a promising potential strategy for mitigating radiation-induced organ damage (30). High mobility group box 1 (HMGB1) is a nuclear protein, which can be translocated to cytoplasm in response to cellular stress and act as a DAMP (70). Glycyrrhizin, an HMGB1 inhibitor, suppresses the HMGB1/TLR4 signaling, including NF- κ B, JNK and ERJ1/2, and reduces the production of inflammatory cytokines, resulting in mitigating radiation-induced lung injury (71). Our previous study has shown that pharmacological inhibition of eCIRP/TREM-1 binding using an eCIRP-derived decoy peptide improves systemic inflammation, tissue injury and survival in sepsis and intestinal I/R injury (37,72). Additionally, the TREM-1 inhibitor LP17 was shown to reduce eCIRP-induced NET formation (35). Furthermore, another TREM-1 inhibitor LR12 is currently in clinical trials for sepsis (73,74). In radiation injury, knockout of TREM-1 improves survival after TBI, and that treatment with a monoclonal neutralizing antibody against eCIRP improves macrophage phagocytic function following high-dose radiation exposure (36,51). The present study provides novel insights into the role of eCIRP and TREM-1 in radiation-induced immune dysfunction.

Several limitations of the present study existed. First, the impact of radiation-induced NETosis on neutropenia was not assessed. Studies evaluating the impact of NETosis inhibition on neutropenia and susceptibility to infection may provide valuable insights into the role of NETosis in radiation injury. Second, whether the formation of NETs contributes to the progression of organ injuries after radiation exposure was not investigated; elucidating the possible involvement of NETs in radiation-induced organ injuries may be important for the development of therapeutic strategies. Third, NET formation in the tissues by histopathological evaluation was not directly observed. Detection of NETs in the tissues including bone marrow and intestines may clarify the relevance of radiation-induced NET formation and organ injuries.

In conclusion, the present study demonstrated that exposure to ionizing radiation induces NET formation. NETosis resulted in neutrophil death to exacerbate neutropenia, which contributed to increased susceptibility to infection. The eCIRP/TREM-1 axis served a key role in radiation-induced NET formation. Therefore, targeting the eCIRP/TREM-1 axis

to regulate NETs may potentially offer a novel therapeutic strategy for alleviating neutropenia and preventing infection after radiation exposure in the future.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MA, MB and PW conceived the study. SY performed the experiments. SY and AM analyzed the data and drafted the manuscript. MA, MB and PW reviewed and revised the manuscript. MB and PW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All study procedures were approved by Institutional Animal Care and Use Committees of the Feinstein Institutes for Medical Research (approval no. 2023-007; Manhasset, NY, USA).

Patient consent for publication

Not applicable.

Competing interests

The authors declared that they have no competing interests.

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