

Single-cell sequencing analysis reveals two radioprotective cell subsets in hematopoietic stem and progenitor cells of naked mole rats

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Abstract

Bone marrow suppression is a common side effect of radiotherapy and a major cause of mortality following exposure to medium or high doses of total body irradiation (TBI). In this study, naked mole rats (NMRs) have been demonstrated to exhibit significantly greater tolerance to high-dose TBI than mice. Surviving NMRs maintain red blood cell (RBC) counts and restore the bone marrow microenvironment. A comprehensive single-cell RNA sequencing (scRNA-seq) analysis of NMR bone marrow cells in response identified two TBI-resistant subpopulations of HSPCs (C19 and C24) in NMRs, and C24 increased post-TBI. These subpopulations exhibit unique regulatory mechanisms related to oxidative stress damage, cell cycle regulation, DNA repair, and HSPC proliferation and differentiation. These mechanisms contribute to the enhanced survival of NMRs under high-dose TBI conditions. These findings suggest that NMRs serve as a valuable model for seeking radioprotection mechanisms and potential therapeutic and protective strategies, particularly in the context of long-term or high-dose irradiation therapies.

1 Introduction

Radiotherapy is a crucial treatment for more than half of all cancer patients ¹. However, the hematopoietic system is the body's most sensitive tissue to ionizing radiation. High-dose systemic radiation can cause severe biological injuries, including apoptosis, chromosomal aberrations, immunosuppression, hematopoietic dysfunction, infection, and even death ². Bone marrow (BM) suppression is a common and serious side effect of radiotherapy, contributing significantly to mortality following medium or high doses of total body irradiation (TBI) ³. Therefore, identifying potential radioprotective mechanisms from natural sources and developing strategies to safeguard the hematopoietic system from radiation-induced BM damage are essential for mitigating these adverse effects of radiotherapy.

Subterranean naked mole rats (NMRs) are known for their extraordinary longevity, remarkable resistance to cancer, and stable physiological and molecular states throughout aging ⁴. Notably, young blood and BM cell compositions in NMRs remain stable until middle age ⁵. Previous *in vivo* and *in vitro* studies have demonstrated that NMRs, as well as their isolated fibroblasts and induced pluripotent stem cells, exhibit resistance to a broad range of chemically induced oxidative stressors, heavy metals, and chemotherapeutic agents. These cells also decrease the incidence of cell death and attenuate inflammatory responses ⁶. These findings raise the question of whether NMRs are also resistant to the damaging effects of radiation injury.

NMR immune cells exhibit several unexpected functions. Hilton et al. demonstrated that NMRs exhibit a high myeloid cell-to-lymphocyte ratio. Though there is a novel subset of lipopolysaccharide-reactive granulocytes in NMRs, they lack NK cells. These characteristics, identified through single-cell RNA sequencing (scRNA-seq), enhance innate immune surveillance, contributing to the healthy longevity of the species ⁷. Additionally, NMRs have abundant macrophages that share NK cell markers and perform dual roles: killing senescent cells with NK cell-like activity and efficiently clearing dead cells via

macrophage function⁸. Emmrich et al. identified an increased quiescent HSPC compartment unique to NMRs, with HSPCs showing minimal age-related decline, likely contributing to their longevity⁵. Given that NMRs are resistant to various chemical stress conditions⁹, their response to physical damage, such as high doses of radiation, is intriguing.

Therefore, it is hypothesized that NMRs are resistant to TBI, and scRNA-seq was used to investigate potential mechanisms that may protect HSPCs from radiation-induced damage. Understanding how the BM cells of NMRs respond to such stressors, especially given their role as the primary site of blood cell production postnatally, could provide valuable insights into the hypoxic adaptation and longevity of NMRs. Moreover, this knowledge is crucial for developing strategies to protect BMs during radiotherapy for hematologic tumors.

2 Results

2.1 NMRs demonstrate greater tolerance to TBI than do C57BL/6J NMRs

LD50/30-day of NMRs post-TBI were evaluated as 10Gy (Figure s1). To investigate the response to radiation, both adult NMRs and C57BL/6J mice were exposed to 10 Gy of ⁶⁰Co γ-rays under the same conditions. Three days post-TBI, C57BL/6J mice experienced weight loss, decreased appetite, reduced activity, and unresponsiveness. Mortality began on day six, with all C57BL/6J mice dead by day 17, resulting in a 0% survival rate within 30 days. In contrast, NMRs started to die 17 days after TBI, and their 30-day survival rate was 60% (Fig. 1A). The NMRs experienced weight loss starting seven days post-TBI but began recovering by day 14 (Fig. 1B). In comparison, the body weights of C57BL/6J mice continued to decrease until 14 days after irradiation (Fig. 1C). Clinical scores, which assess body weight, temperature, physical appearance, posture, mobility, food consumption, and hydration^{1,11}, were used to evaluate animal status post-TBI. By day 18, the clinical scores of the C57BL/6J mice were significantly greater than those of the NMRs (Fig. 1D, Supplementary Table 1).

Peripheral blood cell counts were assessed on days 7 and 14 post-TBI. NMRs and C57BL/6J mice presented significant decreases in white blood cell (WBC) counts during this period (Fig. 1E). However, red blood cell (RBC) and platelet counts displayed species-specific differences (Fig. 1F and 1G). RBC counts in C57BL/6J mice decreased significantly at 7 and 14 days post-TBI (Fig. 1F), whereas they remained unchanged in NMRs. Compared with those in the controls, the platelet counts in the NMRs tended to decrease significantly by day 14 (Fig. 1G). Conversely, C57BL/6J mice had undetectable platelet counts on days 7 and 14 post-TBI (Fig. 1G).

BM cell apoptosis was also examined. In the NMRs, the percentage of apoptotic BM cells remained unchanged on day 7 but significantly increased to 11.34% by day 14, subsequently decreasing to near-normal levels (1.33%) by day 28 post-TBI (2.41%) (Figure s2A). In contrast, the percentage of apoptotic

BM cells in C57BL/6 mice increased from 13.15% on day 7 to 28.76% on day 14 (Figure s2B). No data were available for C57BL/6J mice on days 21 and 28, as they all died by day 17 post-TBI.

Further analysis of the BMs from both species revealed damage to the red BM and a decrease in cellularity (Fig. 1H, 1I), accompanied by an increase in the fat area within the BM cavity (Fig. 1J, 1K). Owing to the longer survival time of NMRs post-TBI, the fat area in their BM cavity peaked on day 14 and returned to near-normal levels by day 28. In contrast, the BM fat area in C57BL/6J mice significantly increased until day 14 post-TBI (Fig. 1J, 1K).

Considering the role of the spleen in hematopoiesis under stress, the spleens of C57BL/6J mice and NMRs post-TBI (Figure s3A-s3C) were also observed. Compared with those of mice, the red/white pulp ratios of NMR-generated spleens were higher, consistent with previous reports^{8,17}. The red pulp regions in the NMRs showed no significant changes post-TBI (Figures s3A and s3B). In contrast, the red pulp regions in C57BL/6J mice decreased significantly at 7 days and 28 days post-TBI (Figure s3A and s3B). NMRs and C57BL/6J mice showed no significant recovery in white pulp regions post-TBI (Figures s2A and s2C). The differing responses of the red pulp regions and peripheral RBC levels suggest that radiation resistance in NMRs may be closely related to their greater regenerative capacity following TBI.

Whole BM cells (excluding RBCs) from NMRs were transplanted into irradiated NOD-SCID mice. Compared with those receiving mouse BM cells, recipients of NMR BM cells presented significant increases in cellularity and decreases in fat area (Fig. 1L-1N). Compared with BM cells from C57BL/6J mice, BM cells from NMRs led to significant hematopoietic hyperplasia in NOD-SCID mice (Fig. 1L and 1O).

2.2 Single-cell atlas of NMR BM

Given that the hematopoietic system is one of the most radiation-sensitive tissues and that high-dose TBI can lead to hematopoietic dysfunction, infection, and even death¹⁸, the observed radiation survival of NMRs prompted us to conduct detailed research on their BM cells. BM samples from two adult NMRs were collected for scRNA-seq. Using the uniform manifold approximation and projection (UMAP) algorithm, eight cell types (Fig. 2A), namely, neutrophil progenitors, neutrophils, monocytes, macrophages, T cells, pre_pro B cells, B cells, and hematopoietic stem progenitor cells (HSPCs), were identified from 25 cell subclusters, and the differentially expressed genes and potential marker genes in each subcluster were analyzed (Fig. 2B). The proportions and compositions of the eight cell types are shown in Figs. 2C and 2D. Neutrophils were the most abundant, whereas hematopoietic stem progenitor cells (HSPCs) were the least abundant (Fig. 2d, Supplementary Table 2). A visualization of marker gene expression levels in 25 cell subclusters is shown in Fig. 2E and Supplementary Table 3. Eight cell types were identified based on the expression of known marker genes (Fig. 2F, Supplementary Table 3). Specific markers were used to identify each cell type: neutrophil progenitors (MMP9, CD38, IL8, and CFD), neutrophils (CD44, MMP8, ITGB2, and CXCR4), monocytes (CD14, MPO, and TOP2A), macrophages (CD14, TLR2, and TLR4), pre_pro B cells (CD19, KEL, GPR56, and PTRRC [CD45]), B cells (JCHAIN,

CD79A, MS4A1, and CD19), and T cells (CD3E). Notably, NMR-derived HSPCs express Thy1, a marker similar to that in primates ⁵, along with CD34, CXCL12, VCAM1, and BPGM.

2.3 Changes in HSPC subclusters after TBI in NMRs

HSPCs are highly sensitive to radiation. Understanding how their proportions and functions change post-TBI could provide insights into their high-dose radiation tolerance. ScRNA-seq was performed on BM cells from control NMRs and those irradiated at 7 and 28 days post-TBI. The cells were divided into 25 subclusters on the basis of the scRNA-seq data and were classified into 8 classes according to the gene annotation results (Fig. 3A).

Compared with that in the controls, the percentage of HSPCs in the NMR BM significantly decreased at 7 days post-TBI (Fig. 3A and 3B). Under normal conditions, HSPCs accounted for 2.15% of BM cells, which decreased to 1.35% on the 7th day and further decreased to 0.30% on the 28th day post-TBI (Fig. 3B).

According to the scRNA-seq results, pre_pro B cells decreased from 7.90% to 0.40% at 7 days post-TBI and increased to 7.60% by 28 days, approaching normal levels. The percentage of B cells increased sharply from 0.59% to 1.25% at 7 days post-TBI and then recovered to 1.9% by 28 days, although it was still below normal. T cells decreased from 3.50% to 2.35% at 7 days post-TBI but recovered to 5.90% by day 28. The number of macrophages was reduced between 7 and 28 days post-TBI. The percentage of monocytes increased from 19.60% to 25.36% at 7 days post-TBI and to 49.77% by 28 days. The number of neutrophils did not significantly decrease at 7 days post-TBI, but decreased to 21.80% by 28 days. By 28 days post-TBI, the sum of neutrophils and monocytes had returned to pre-irradiation levels.

Flow cytometry confirmed the proportions of HSPCs from C57BL/6J mice and NMRs before and post-TBI. While murine HSPCs are characterized by Lin⁻Sca-1⁺c-Kit⁺ expression, corresponding to hematopoietic stem cells (HSCs), multipotent progenitors (MPPs), and hematopoietic progenitors (HPCs), NMR HSPCs are characterized by Lin⁻Thy1⁺CD34⁺ expression (Fig. 3D). The sorting of NMR and mouse HSPCs was performed according to different strategies reported previously ⁵. HSPCs from BM were sorted via lineage depletion and specific markers according to previously published protocols. The mouse HSPC fraction was sorted via lineage (LIN = CD4/CD8a/CD45R/CD127/TER-119/Ly-6G) depletion and Sca-1 and c-kit staining ¹⁵. The NMR HSPC fraction was sorted by staining with lineage (LIN = CD11b/CD18/CD90/CD125) depletion and with Thy1.1 and CD34 ⁵. In C57BL/6J mice, the percentage of HSPCs decreased from 0.197% to 0.033% at 7 days post-TBI (Fig. 3C, 3D). In contrast, the proportion of HSPCs in the NMRs increased from 0.0367% to 0.083% at 7 days post-TBI and then returned to 0.04% by 28 days, nearly the same level as that in the naïve NMRs (Fig. 3E, 3F).

BM counts revealed that hematopoietic hyperplasia in NMRs significantly decreased at 7 days post-TBI, increased significantly at 14 days, and returned to near-normal levels by 28 days. In contrast, myelodysplastic syndromes in C57BL/6J mice decreased significantly throughout the period post-TBI until 14 days (Fig. 3G, 3H).

The enhanced function of HSPCs, along with the maintenance of RBC counts, might contribute to the radiation protection observed in NMRs. We then focused on analyzing the HSPC fractions, which were further divided into two subclusters, C19 and C24. A comparison of marker gene expression revealed that C24 specifically expressed C-X-C chemokine ligand 12 (*Cxcl12*), platelet-derived growth factor receptor-like (*Pdgfrl*), c-KIT ligand (*Kitlg*), s subcomponent (*C1s*), olfactomedin-like 3 (*Olfml3*), vascular cell adhesion molecule 1 (*Vcam1*), Nidogen-1 (*Nid1*), syndecan-2 (*Sdc2*), and serpin family H member 1 (*Serpinh1*) (Fig. 3I), whereas C19 uniquely expressed *Hbz*, *Bpgm* (bisphosphoglycerate mutase), and *Gadd45a* (growth arrest and DNA damage-inducible gene a) (Fig. 3J). In normal NMRs, the cell count of C19 was significantly larger than that of C24 (Fig. 3K). However, 28 days post-TBI, the proportion of C19 cells significantly decreased, whereas that of C24 cells dramatically increased to levels comparable to those of C19 cells, suggesting that the C24 subcluster may play a critical role in maintaining hematopoiesis.

To confirm that C19 and C24 are functional HSPCs, the specific marker profiles of both subclusters were analyzed (Fig. 3L-3N). The markers for C24 were notably specific, with VCAM1 identified as a cell membrane surface molecule suitable for flow cytometry screening. NMR-generated HSPCs were sorted into VCAM1⁺ (C24) and VCAM1⁻ (C19) populations via flow cytometry, and *in vitro* colony formation assays were conducted. NMR and C57BL/6J HSPCs formed various types of colonies over a 10-day culture period. Consistent with findings by Emmrich et al.⁵, the self-renewal capacity of NMR HSPCs was slightly lower than that of C57BL/6J HSPCs under normal conditions. Crucially, following irradiation, the colony formation capacity of C57BL/6J HSPCs was significantly reduced, whereas the BFU-E colony formation levels of NMR HSPCs, comprising C19 and C24 clusters, were significantly increased, surpassing those of C57BL/6J HSPCs (Fig. 3O, 3P, and supplementary table 4).

2.4 Analysis of the trajectories of NMR BM cells and HSPC subclusters after TBI

HSPCs are the origin of all blood and immune cells, making their differentiation trajectories after TBI crucial for understanding the radiation tolerance of NMRs. To investigate the differentiation pathways of BM cells post-TBI, Monocle 2 was used to construct differentiation trajectories for the entire BM cell population, simulating their biological differentiation processes. The results revealed that HSPCs followed two distinct cell fates (Fig. 4A). Pseudotime analysis revealed an increase in the number of neutrophil progenitors that entered the differentiation process at 7 days post-TBI, with a significant influx of monocytes into the differentiation pathway at 28 days post-TBI (Fig. 4A). The expression peaks of the recognized marker genes at various pseudotime stages were consistent with these cell fates (Fig. 4B).

Furthermore, differential expression gene (DEG) analysis of the trajectory nodes revealed that the top DEGs included lineage-specific gene expression trends aligning with the observed cell fates (Fig. 4C, supplementary table 5). Using HSPCs as the initial cell population, Palantir was used to analyze the hematopoiesis data to identify all expected cell types (Fig. 4D). The trajectory identified by Palantir followed the anticipated progression from HSPCs to differentiated cell types (Fig. 4D). Early in the

trajectory, the cells exhibited the potential to reach any terminal state, gradually losing plasticity as they committed to specific lineages (Fig. 4E). To evaluate the trajectories, we calculated the dynamic expression of key markers across cell trajectories in the nodal heatmap (Fig. 4C). As expected, the expression of lineage-specific factors significantly changed: *Mmp9* was upregulated in neutrophil progenitor cells and downregulated in other cells, *Mpo* was markedly increased in monocytes, and *Itgb2* was significantly upregulated in neutrophils. *C1qa* was significantly upregulated in macrophages, *Kel* was significantly increased in pre_pro B cells but decreased in other lineages, *Cd3e* was significantly upregulated in T cells, and *Jchain* was significantly upregulated in B cells. *Bpgm* showed a downward trend across all lineages as the cells progressed through their trajectories (Fig. 4F).

2.5 GSEA reveals hallmark characteristics related to radiation protection of NMR HSPC subclusters

To determine the radiation resistance characteristics of the HSPC subclusters C19 and C24, we conducted gene set enrichment analysis (GSEA) via Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analyses. Our focus was on genes related to the cell cycle, metabolism, oxidative stress defense, HSPC proliferation and differentiation, and DNA damage repair. These analyses aimed to reveal differences in radiation response models between C57BL/6J mice and NMRs, providing insights into the unique radiation tolerance of NMRs.

Characteristics of the C19 subcluster

The signaling pathway enrichment of the C19 cluster has been explored under normal conditions (Fig. 5A). GO-GSEA revealed that, seven days post-TBI, erythrocyte differentiation pathways were significantly enriched, whereas at 28 days post-TBI, erythrocyte development, transcriptional regulation, and DNA-templated pathways were significantly enriched. GSEA based on the KEGG functional results revealed that 7 days post-TBI, pathways related to porphyrin metabolism, DNA repair, and protein recombination were significantly enriched, and by 28 days post-TBI, pathways such as aminoacyl-tRNA biosynthesis, mitochondrial biogenesis, FoxO signaling, and GnRH secretion (with MAP2K1 significantly enriched) were significantly enriched (Fig. 5A).

Volcano plots illustrate the expression patterns of enriched genes in C19 cells at 7 and 28 days post-TBI (Fig. 5B, 5C). Key genes involved in processes such as the ROS damage response, DNA repair, cell cycle regulation, metabolism, HSC proliferation and differentiation, and heme production, including nuclear factor erythroid 2-related factor 2 (*Nfe2l2*), casein kinase I isoform (*Csnk1d*), cyclin-dependent kinase inhibitor p21 (*Cdkn1a*), glutamyl-prolyl-tRNA synthetase (*Eprs*), DNA damage-induced transcript 4 (*Ddit4*), glucocorticoid receptor gene (*Nr3c1*), GATA binding protein 1 (*Gata1*), coproporphyrinogen oxidase (*Cpox*), and hydroxymethylbilane synthase (*Hmbs*), were validated. Notably, *Nfe2l2* expression continued to increase up to 28 days post-TBI. Other genes, such as *Csnk1d*, *Cdkn1a*, *Nr3c1*, *Ddit4*, and *Nfe2l2*, were significantly upregulated at 7 days post-TBI. *EPRS* was significantly increased at 28 days post-TBI. Conversely, *Hmbs*, *Gata1*, and *Cpox* decreased at 7 days but increased significantly at 28 days post-TBI (Fig. 5D). *Gata1*, a well-known transcription activator, drives genes responsible for erythroid cell

differentiation, including *Hmbs*^{19,20}. These gene expression changes in NMR BM cells after TBI were distinct from those observed in C57BL/6J mice (Fig. 5D).

To further explore the roles of these genes in radioprotection, lentiviral vectors containing NMR-specific genes were constructed and transfected into HSPCs from C57BL/6J mice before irradiation. Initial experiments revealed that 1 and 2 Gy of ⁶⁰Co significantly induced apoptosis in C57BL/6J HSPCs, but increasing the dose to 10 Gy did not lead to a further significant increase in apoptosis rates *in vitro* (Figure s4). Following transfection, the results indicated that *Nfe2l2*, *Nr3c1*, *Gata1*, *Csnk1d*, *Cdkn1a*, and *Eprs* significantly reduced the apoptosis rate in irradiated C57BL/6J HSPCs, whereas *Cpox*, *Gata1*, and *Hmbs* did not have the same effect (Fig. 5E, 5F, and s5).

Previous studies have reported an increase in the resting cell pool in NMR BMs⁵. To understand the cell cycle changes in various BM cells after irradiation, we conducted cell cycle analyses of NMR-generated HSPCs. The results revealed that the S phase of HSPCs remained almost unchanged at 7 days post-TBI and did not increase until 28 days (Fig. 5G). The S phase of neutrophil progenitors was significantly reduced at 7 days but returned to normal levels by 28 days. The S phase of pre_pro B cells decreased at 7 days post-TBI, whereas it increased at 28 days post-TBI. The G1 phase of pre_pro B cells also returned to normal levels by 28 days post-TBI. T cells and neutrophils exhibited relatively low proliferative capacities, whereas monocytes and macrophages strongly proliferated in response to radiation (Fig. 5G).

Further analysis of the two HSPC subclusters revealed that the percentage of S-phase C19 cells continued to increase by approximately twofold compared to that before TBI. The percentage of S-phase C24 cells decreased on Day 7 and returned to normal levels 28 days after TBI (Fig. 5H).

Characteristics of the C24 subcluster

The C24 subcluster was then analyzed. The GO-GSEA results revealed that seven days post-TBI, pathways involving the positive regulation of T-cell proliferation, neutrophil chemotaxis, and the transcription regulator complex were significantly enriched. At 28 days post-TBI, there was notable enrichment in the transmembrane receptor protein tyrosine kinase signaling pathway, positive regulation of type I interferon production, and T-cell proliferation pathways.

KEGG-based GSEA further revealed that seven days post-TBI, pyruvate metabolism, hematopoietic cell lineage, and oxytocin signaling pathways were enriched. By 28 days post-TBI, enrichment was observed in T-cell receptor signaling, neurotrophin signaling, beta-alanine metabolism, TNF signaling, CD molecules, and MAPK signaling pathways, with ALCAM being significantly enriched in CD molecules (Fig. 6A). Volcano plots illustrate the expression patterns of these enriched genes in C24 cells at 7 and 28 days post-TBI (Fig. 6B, 6C).

Several genes related to ROS damage, such as carnosine dipeptidase 2 (*Cndp2*), the synthesis of cytochrome C oxidase 1 (*Sco1*), and the CD44 molecule (*Cd44*), have been validated to be involved in

DNA repair, the cell cycle, metabolism, and HSPC proliferation and differentiation. Notably, *Cd44* expression significantly increased at 7 days post-TBI, whereas *Sco1* and *Cndp2* expression significantly increased at 28 days post-TBI (Fig. 6D-6H). These gene changes in the BM cells of C57BL/6J mice at 7 days post-TBI were distinct from those observed in NMRs (Fig. 6D-6H, and s6).

Further experiments demonstrated that overexpressing the control vector or *Cd44*, *Sco1*, or *Cndp2* in mouse HSPCs significantly reduced the apoptosis rate following irradiation (Fig. 6F and 6G).

2.6 Phenotypic and functional analysis identifies C19 and C24 as true HSPCs

To further investigate the effects of *Csnk1d*, *Cdkn1a*, *Nr3c1*, *Ddit4*, *Nfe2l2*, *Hmbs*, *Gata1*, *Eprs*, *Cpox*, *Cd44*, *Sco1*, and *Cndp2* on the function of HSPCs after irradiation, these genes were overexpressed in C57BL/6J HSPCs and processed for colony formation assays postirradiation. The results demonstrated that the overexpression of NMR *Gata1*, *Cpox*, and *Hmbs* significantly increased the rate of BFU-E cloning in mouse HSPCs after radiation (Fig. 7A, 7B). Furthermore, *Nr3c1*, *Nfe2l2*, *Eprs*, *Ddit4*, *Cd44*, *Cndp2*, and *Sco1* significantly increased the total number of HSPC colonies as well as the cloning rates of BFU-E and CFU-GM in C57BL/6J mouse HSPCs after irradiation (Fig. 7A, 7B, and supplementary table 8).

ROS and antioxidant levels in NMRs

Given that radiation damage results primarily from the breakdown of water into reactive oxygen species (ROS) ²¹, ROS levels were measured via NMR. Compared with C57BL/6J mice, NMRs presented higher MDA levels before irradiation (Figure s7A). Fourteen days post-TBI, the GSH level in the NMRs significantly increased to 10 IU, surpassing that in the C57BL/6J mice at the same time point (Figure s7B). SOD levels did not significantly differ between the two species (Figure s7C). However, T-AOC levels in NMRs were considerably higher than those in C57BL/6J mice before irradiation and remained elevated post-TBI (Figure s7D).

3 Methods and Materials

3.1 Animals

To compare the NMR response with that of short-lived rodents following TBI, C57BL/6J mice of comparable age were selected. Both species were maintained at similar body weights to ensure unbiased TBI dosing. Adult male NMRs (mean age: 1.5 years) from different colonies and male C57BL/6J mice (mean age: 8 weeks) were provided by the Laboratory Animal Department of Naval Medical University (Shanghai, China). All the animals were cared for and used in accordance with Chinese laws for animal experimentation and regulations. All animal procedures were performed in accordance with experimental protocols approved by the Naval Medical University Animal Care and Use Committee of China (No. 2021CSF0611).

NMRs were bred and housed in cages connected by tunnels of varying lengths, as previously reported¹⁰. The room was maintained at 30°C with controlled humidity, and red lighting was used daily between 08:00 and 16:00¹⁰. The mice were kept on a standard 12-h light/dark cycle with food and water available ad libitum. A total of 197 NMRs, 171 C57BL/6J mice, and 120 NODSCID mice were used in this study. For sample collection, animals were anesthetized using 4–5% isoflurane and then euthanized by cervical dislocation.

3.2 Irradiation treatments

3.2.1 Total body irradiation (TBI)

For dose response relationship (DRR) studies, unanesthetized NMRs were placed in ventilated plastic pie cages and exposed to 0, 2.0, 3.0, 4.0, 5.5, 6.0, 7.0, 8.0, 10.0, 12.0, 20.0, 30.0, 40.0, and 50.0 ⁶⁰Co γ-rays (n = 20 / group). ⁶⁰Co γ-rays (10Gy, dose rate: 1.16 Gy/min) were then used for TBI in the following studies on mice and NMRs. This procedure was conducted at the Naval Medical University in Shanghai, China.

3.2.2 Cell irradiation

The cells were exposed to ⁶⁰Co γ-rays at doses of 1, 2, 4, 6, 8, and 10 Gy to determine the appropriate irradiation dose for *in vitro* experiments. A dose of 2 Gy was selected for *in vitro* experiments, which was consistent with previous reports.

3.3 Survivability monitoring

Unanesthetized C57BL/6J mice (control group, n = 30; irradiation group, n = 30) and NMRs (control group, n = 30; irradiation group, n = 30) with similar body weights were used for this study. The animals were placed in ventilated plastic pie cages and exposed to 10 Gy of ⁶⁰Co γ-rays to analyze survival rates without additional treatments. After TBI, the animals were housed with standard chow and water provided ad libitum unless otherwise noted. The rats were monitored for changes in body weight and other parameters for 30 days post-TBI.

The clinical score was determined via a cumulative scoring system (supplementary table 1) based on previously reported methods, with some modifications^{1,11}. This scoring system is used to evaluate factors such as body weight loss, temperature changes, physical appearance, posture, mobility, food consumption, and hydration¹¹. Body temperatures were detected by infrared thermometers, and body weights were recorded with an electronic scale (Yuyan Instruments, Shanghai, China) at fixed times to avoid disturbing the animals' biorhythm. The animals used for survival monitoring were distinct from those used for blood, tissue, and cell collection.

3.4 Blood sample collection and analysis

Animals processed for blood sample collection were also used for tissue collection, but were not included in the survivability monitoring cohort. Blood samples were collected from 10 animals per group, including those in the sham group and those at 7 and 14 days post-TBI (NMRs and C57BL/6J mice) and

at 21 and 28 days post-TBI (NMRs). For mice, blood was collected from the orbital venous plexus under anesthesia, while cardiac blood was collected from NMRs after anesthesia. Following euthanasia, other organs were simultaneously fixed and collected.

For blood cell count analyses, blood was placed into lavender-top collection tubes containing EDTA and kept at ambient temperature (n = 10/group). The samples were immediately analyzed via a small animal blood cell analyzer (HEMAVET950, USA).

For oxidative stress-related substance detection, animals that were similar to those used for blood cell count analysis were used (n = 4/group). Fresh blood samples were allowed to clot at 4°C for at least 30 min. Serum was then collected by centrifugation at 3000 × g for 15 min at 4°C, aliquoted into 50 µL/tube, and stored at -80°C. The concentrations of malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC) were measured via a mouse GSH kit (S0053, Beyotime, China), a mouse MDA kit (S0131, Beyotime, China), a mouse T-AOC kit (S0119, Beyotime, China), and a mouse SOD kit (S0109, Beyotime, China) according to the manufacturer's instructions (n = 4/group).

3.5 Histopathology analysis and BM cell counts

The animals used for blood sample collection were also utilized for tissue fixation. BM and spleen tissues were collected for histopathological analysis. Femurs were surgically removed, fixed in 10% buffered formalin for 48 h, decalcified, and then embedded in paraffin. Spleens were also fixed in 10% buffered formalin. Sections of 4 µm thickness were subjected to hematoxylin and eosin (H&E) (Servicebio, Wuhan, China) (n = 10/group) or oil red O staining kit (Beyotime Biotechnology, Shanghai, China) (n = 3/group) using standard procedures. The spleen (n = 5/group) scoring system was based on the extent and pattern of extramedullary hematopoiesis (EMH), as previously reported.

BM cells from the metaphysis end were directly coated onto a slide, air-dried at room temperature, fixed, and stained via a Giemsa staining solution kit (Jingkang Biotechnology, Shanghai, China). Hematopoietic hyperplasia was evaluated by calculating the percentage of nucleated cells among the total cell count. Three animals per group were used for analysis, with nine NMRs and eighteen mice in the irradiated groups.

3.6 RNA extraction and qPCR

NMR and mouse HSPCs were harvested for qPCR analysis. RNA extraction with TRIzol (Takara, Japan) and reverse transcription with a Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher, USA) were performed according to the manufacturer's instructions. The primers used for the qPCR analysis are listed in the supplemental data (Supplementary Table 7). Three animals per group were used for qPCR analysis, with eight NMRs being irradiated.

3.7 Single-cell RNA sequencing (scRNA-seq)

BM cells from NMRs after TBI were analyzed via scRNA-seq to obtain unbiased molecular profiles of myeloid cells in NMRs. Two samples from each group, the control group and the groups 7 days and 28 days after TBI, were prepared for scRNA-seq, accounting for six samples in total. To ensure representation from two animals, approximately 6000 high-quality cells per sample were processed for further analysis. RBCs were removed from BM cells with RBC lysis buffer (Beyotime Biotechnology, Shanghai, China), which does not affect premature red blood cells or other cells containing a nucleus. The cells were subsequently collected for further analysis. BM cells with viability greater than 95% were used for scRNA-seq. Briefly, scRNA-seq was performed via a BD Rhapsody system. Single-cell capture was achieved by the random distribution of a single-cell suspension across > 200,000 microwells through a limited dilution approach. The cells were lysed in the microwell to hybridize mRNA molecules to barcoded capture oligos on beads. The beads were collected into a single tube for reverse transcription and Exol digestion. Whole-transcriptome libraries were prepared via the BD Rhapsody single-cell whole-transcriptome amplification (WTA) workflow, which included random priming and extension (RPE), RPE amplification PCR, and WTA index PCR. The libraries were quantified via a high-sensitivity DNA chip (Agilent) on a Bioanalyzer 2200 and a Qubit high-sensitivity DNA assay (Thermo Fisher Scientific). Sequencing was performed via an Illumina sequencer (Illumina, San Diego, CA) on a 150 bp paired-end run. In the process of dimensionality reduction, the mutual nearest neighbors (MNN) method was used to correct the batch effect.

3.8 Data analysis of scRNA-seq data

All BM cells without mature red blood cells were collected for scRNA-seq. Saturation curve analysis revealed that the sequencing depth was sufficient to detect genes in each sample, and the median number of genes detected per cell was comparable across samples. The percentage of UMI counts of mitochondrial genes, the number of genes, and the summation of UMI counts per cell were detected to assess the quality of the data. The generalized linear model was fitted by filtering the delocalized cells from the two samples. To rule out potential low-quality data that may result from damaged cells, non-single cells, and other technical issues, a strict threshold was set for the quality indicators of the samples. The high-quality data from 6,000 cells were ultimately preserved. In summary, high-quality, large-scale scRNA-seq datasets from BM specimens were generated.

Data analysis of the scRNA-seq data was performed by NovelBio Bio-Pharm Technology Co., Ltd., with the NovelBrain Cloud Analysis Platform. Fastp with default parameter filtering of the adaptor sequence was applied, and the low-quality reads were removed to obtain clean data. UMI tools were applied for scRNA-seq analysis to identify a cell barcode whitelist. To obtain the UMI counts of each sample, the UMI-based clean data were mapped to the NMR genome (HetGla_female_1.0 Ensembl100) via STAR via the UMI-tools standard pipeline. Cells containing more than 200 expressed genes and with a mitochondrial UMI rate less than 20% passed the cell quality filter, and mitochondrial genes were removed from the expression table. The Seurat package (version 3.1.4, <https://satijalab.org/seurat/>) was used for normalization and regression on the basis of the expression table according to the UMI count of each sample and percentage of mitochondria to obtain scaled data. PCA was constructed on the basis

of scaled data with the top 2000 highly variable genes, and the top 10 principal components were used for UMAP construction. The mutual nearest neighbors (MNN) method was used to correct the batch effect. Cell clusters expressed both marker genes from two different cell types were defined as doublets and removed from the analysis.

3.9 Inference of differentiation trajectories

Pseudotime analysis was conducted via Monocle2 to trace cell differentiation across the entire dataset and identify branching points. Specifically, the trajectories of the C19 and C24 subclusters were analyzed on the basis of the results from the pseudotime analysis of the total cell population.

For Palantir pseudotime analysis, the raw data from the Seurat object were imported into Palantir (v.0.2.2). After principal component analysis (PCA), diffusion mapping, and MAGIC imputation were completed, pseudotime analysis was performed. The start cell was specified during the initiation process of Palantir on the basis of prior validated information and its appropriate coordinate position in UMAP. The terminal cell states were automatically determined by Palantir. Each cell was assigned a pseudotime value and branch probabilities to terminal states. Gene expression trends along Palantir pseudotime or across different lineages were modeled via generalized additive models provided by Palantir.

3.10 GO, KEGG pathway analysis, and GSEA

Cells in the C19 and C24 subclusters were processed for GO analysis and pathway analysis. Gene Ontology (GO) analysis¹² was performed to elucidate the biological implications of the identified marker genes and differentially expressed genes. GO annotations were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/>), UniProt (<http://www.uniprot.org/>), and Gene Ontology (<http://www.geneontology.org/>). Fisher's exact test was applied to identify the significant GO categories, and FDR was used to correct the *P* values.

Pathway analysis was used to determine the significant pathways associated with the marker genes and DEGs according to the KEGG database. Fisher's exact test¹³ was used to select the significant pathway, and the threshold of significance was defined by the *P* value and FDR.

GSEA was performed based on GO and KEGG pathway analyses of the C19 and C24 subclusters.

3.11 Cell cycle analysis

Cell cycle analysis was conducted for eight cell types, including the C19 and C24 subclusters, via Seurat (v3.1).

3.12 Flow cytometry analysis (FCA)

3.12.1 Cell death assay

Nineteen NMRs and twenty-eight mice were irradiated for this study, with four animals per group analyzed for cell death. Cell death was assessed via Annexin V-APC/7-AAD double staining or an Annexin V-FITC/PI Apoptosis Detection Kit according to the manufacturer's instructions (BioLegend, USA). Briefly, 1.0×10^6 cells/ml were washed in ice-cold PBS, centrifuged at 1000 rpm for 5 min, resuspended in Annexin V binding buffer, and incubated with APC-conjugated Annexin V and 7-AAD or FITC-conjugated Annexin V and PI. After 15 min of incubation in the dark, the cells were diluted in 300 μ l of binding buffer and analyzed by flow cytometry (Becton Dickinson, USA). For each test, at least 10,000 events from 1.0×10^6 cells were obtained via Cell Quest software (Becton Dickinson, USA).

3.12.2 HPSC apoptosis after lentivirus transfection

HSPCs were irradiated with 1, 2, 4, 6, 8, or 10 Gy of ^{60}Co to determine a moderate irradiation dose for further experiments. The coding sequences (CDSs) for NMR NR3C1, NFE2L2, CDKN1A, GATA1, CSNK1D, EPRS, CPOX, HMBS, SCO1, CNDP2, and CD44 were inserted separately into pLVX-puro vectors (Hanhang Biology). These constructs were used to produce lentivirus particles. PLVX-puro vectors without the target gene sequence were used as controls. QPCR was used to verify the overexpression levels of these genes. Mouse HSPCs transfected with either the control or the aforementioned genes were exposed to 2 Gy ^{60}Co irradiation prior to further analysis. Experiments have been repeated three times. Flow cytometry analysis was performed 6 h after irradiation, as previously reported ¹⁴.

3.12.3 HSPC identification via FCA

HSPC identification was performed via CytoFLEX S (Beckman, Germany), and data analysis was conducted via CytExpert software (Beckman, Germany). Red blood cells were lysed following the manufacturer's instructions. After resuspension in FACS buffer, the BM cells were incubated with antibodies according to the manufacturer's instructions. 7-AAD (1 μ M, Biolegend, USA) was used to assess cell viability. Three animals per group were used for HSPC identification, with three mice and five NMRs irradiated. The antibodies used for the characterization of NMRs and mouse HSPCs are listed in Supplemental Table 7, as reported previously ¹⁵.

3.13 Mouse HSPC purification

Mice (8–10 weeks old, male and female) were euthanized by cervical dislocation, and the tibias and femurs were aseptically removed. The BM cavity was flushed with PBS containing 2% FBS via a 5 mL syringe. The BM cell suspension was repeatedly aspirated with a 1 mL syringe fitted with a No. 4 needle. The supernatant was then filtered through a 70 μ m filter, centrifuged at $600 \times g$ for 5 min, and treated with 2 mL of red blood cell lysate. After 10 min of incubation at room temperature, the suspension was centrifuged again at $600 \times g$ for 5 min. The supernatant was discarded, and the pellet was washed once with PBS. HSPCs were then isolated via a mouse hematopoietic progenitor cell enrichment kit (19865, Stem Cell, USA).

3.14 NMR-based HSPC sorting

The collection of BM cells from NMRs (1.5–2 years old, male and female) was performed similarly to the procedure used for mouse BM cells. NMR-generated BM HSPC populations were sorted as previously reported⁵ via a lineage cocktail of antibodies, including CDD11b FITC, CD18 FITC, CD90 FITC, and CD125 FITC (NMR LIN), along with Thy1.1 PE and CD34 APC. For sorting C19 and C24 cells from the NMR HSPC populations, the panel included VCAM1 PE-Cy7 (Clone 429 (MVCAM. A) (BioLegend, USA). The C19 and C24 subpopulations were sorted as VCAM1⁻ and VCAM1⁺ cells, respectively. A total of thirty animals were used for this analysis.

3.15 HSPC characterization

Mouse HSPCs were cultured *in vitro* in StemSpan SFEM (09650, Stem Cell, USA). NMR HSPCs, including the C19 and C24 subclusters, were cultured in StemSpan SFEM supplemented with 1% GlutaMax (Thermo Fisher, USA), 1% nonessential amino acids (Thermo Fisher, USA), 0.25% CDLC, 25 ng/ml hSCF, 25 ng/ml hFLT3L, and 20 ng/ml hIL-6 (Pepro Tech, USA) as previously reported⁵. All cultures were supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), and gentamicin solution (50 µg/ml) (Solarbio, Beijing, China). Mouse HSPCs were cultured at 37°C with 5% CO₂, while NMR HSPCs were cultured at 32°C with 5% CO₂. The media were replaced every three days, and after three weeks, hIL-6 was replaced with 20 ng/ml hIL-7.

3.16 Colony formation assay

NMR cells were sorted into methylcellulose-enriched media H4034 Optimum (Stem Cell, USA), while mouse HSPCs were sorted into MethoCult™ GF M3434 (Stem Cell, USA) following the manufacturer's instructions. The sorted cells were plated onto 35-mm dishes and incubated at 32°C (NMR) or 37°C (mouse) in a 5% CO₂ humidified incubator. Colonies were scored on day 10 as burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte/macrophage (CFU-GM), CFU-granulocyte, erythrocytes, megakaryocytes, or macrophages (CFU-GEMM). Phase-contrast photomicrographs of the colonies were taken on day 10 via an Olympus phase-contrast inverted microscope¹⁶.

3.17 BM cell transplantation

Eight-week-old male NOD-SCID mice (Slake Company, Shanghai, China) were randomly grouped into Ctrl (without irradiation and transplantation), PBS (irradiated and transplanted with PBS), NMR BMC (irradiated and transplanted with NMR BMCs), and C57BL/6J BMC (irradiated and transplanted with C57BL/6J BMCs) groups. NOD-SCID mice were irradiated with 10 Gy ⁶⁰Co γ-rays (dose rate of 1.16 Gy/min) 12 hours before BM cell transplantation. Red blood cells were removed from isolated NMR BM via Red Blood Cell Lysate (Beyotime, Shanghai, China), and the remaining cells were labeled with the cell membrane dye Dil (10 µM/L, Beyotime, Shanghai, China) for 20 min at room temperature. After 3 rinses with PBS, the labeled cells were subjected to bilateral femoral injection into NOD-SCID mice. Each femur was injected with 1.0×10^7 cells in a volume of 30 µL.

3.18 Statistical analysis

All values are presented in the figures as means $\pm$ SEMs, with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. The number of animals (n) used in the experiments is indicated in the figures. Statistical analyses were performed via GraphPad Prism 8 software. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test or Tukey's multiple comparisons test, as recommended by the software. The Kruskal–Wallis test followed by Dunn's multiple comparisons test was used when one-way ANOVA could not be performed because the data did not pass the normality test. Radiation DRRs were analyzed using a logistic regression model for 30-day mortality with the natural log of radiation dose to estimate LD50/30 by GraphPad Prism 8 software. The log-rank (Mantel–Cox) test was used to analyze the survival rates, and the significance between the two groups was determined via one-way ANOVA in GraphPad Prism with the default parameters. The Mann–Whitney test for the area under the curve (AUC) was used to analyze the clinical scores.

4 Discussion

This study highlights the remarkable radiation tolerance of NMRs compared with that of C57BL/6J mice following high-dose TBI with 10 Gy of ^{60}Co γ -rays. The surviving NMRs could maintain RBC counts and restore the BM microenvironment. After high-dose TBI, the percentage of HSPCs in the NMR BM increased, whereas the percentage of HSPCs in the NMR BM significantly decreased in C57BL/6J mice. ScRNA-seq was utilized to analyze BM cells comprehensively from NMRs, revealing two subpopulations (C19 and C24) of BM HSPCs from NMRs that exhibited resistance to TBI. Radiotherapy is one of the key factors that induce aging in the body, especially in the bone marrow hematopoietic system. Previous studies have shown that ionizing radiation and various cancer treatments induce a pro-inflammatory senescent phenotype in bone marrow cells ²², especially with a myeloid bias ²³. Revealing the characteristics and mechanisms by which NMR bone marrow tolerates radiation damage may help in finding effective strategies to protect humans from the side effects of radiotherapy.

NMRs (*Heterocephalus glaber*) are known for their extraordinary lifespan among rodents ²⁴. Despite being similar in size to mice, these hairless, highly social animals can live nearly ten times longer, with lifespans of up to 40 years in captivity and approximately 17 years in the wild ^{24,25}. Interestingly, BM cell abnormalities have not been observed in NMRs throughout their lifespan. In contrast, previous studies have shown high mortality rates in C57BL/6J mice following high-dose TBI (9.2 Gy), with only a few surviving. In those cases, tryptophan metabolites produced by *Lachnospiraceae* and *Enterococcaceae* are critical for mitigating BM and gastrointestinal tract injuries caused by TBI ¹¹. In this study, the mortality rate of C57BL/6J mice was approximately 2.5 times greater than that of NMRs, underscoring the intrinsic ability of NMRs to protect themselves from radiation-induced mortality.

Following TBI, BM cells in NMRs begin to recover by 14 days post-TBI, indicating the restoration of hematopoietic function. Notably, from the peripheral blood data, the number of peripheral blood red blood cells in NMRs after TBI remained constant. Bone marrow cell apoptosis levels gradually declined after peaking on 14 days post-TBI. In contrast, the mouse bone marrow cell apoptosis levels exceeded

those of NMRs by more than twice at both 7 and 14 days. A dose of 10 Gy did not trigger extramedullary hematopoiesis in NMRs, but it did induce extramedullary hematopoiesis in mice. However, due to severe bone marrow damage, extramedullary hematopoiesis was still unable to produce enough blood cells to meet normal blood demands. This likely supports oxygen delivery to tissues affected by radiation injury²⁶ and aids in repair processes. Radiation exposure often triggers oxidative stress in multiple organs, leading to an inflammatory response and the depletion of neutrophils and macrophages, which are involved in both the inflammatory response and the clearance of damaged cells²⁷. Consequently, the recovery of peripheral WBCs and splenic white pulp regions was slower, likely reflecting the prolonged nature of the body's overall repair process.

Consistent with previous studies showing that NMRs lack natural killer (NK) cells^{7,28}, NK cells were not detected in these BM scRNA-seq data. However, NMRs that express the NK1.1 antigen in the steady state may function dually as both macrophages and NK cells⁸. Our scRNA-seq results revealed an increase in macrophages from 7–28 days post-TBI, suggesting that these cells play a key role in clearing senescent and injured cells, thereby reducing the inflammatory response. Peripheral blood counts in NMRs further suggest that these animals may selectively allocate energy following radiation exposure²⁹, initially focusing on survival, then on repairing radiation-induced damage, and finally on mounting an inflammatory response to ensure long-term survival.

According to the scRNA-seq data, pre_proB cells decreased significantly by 7 days post-TBI but then sharply increased by 28 days, whereas B cells began to recover 7 days post-TBI and showed a substantial increase by 28 days. This pattern suggests that the increase in pre_proB cells contributes to the gradual recovery of B cells over time. In healthy individuals, 24 hours after receiving 2 Gy of radiation, the number of B cells in the body also significantly decreases, leading to subsequent aging phenotypes of the immune system. After TBI, the significant increase of pre_proB cells in NMRs quickly replenishes the missing B cells in the body, thereby preventing immune system aging. T cells decreased by one-third at 7 days post-TBI but also recovered by 28 days. Since both T cells and B cells originate from lymphoid hematopoietic progenitor cells, these findings indicate that the radiation tolerance characteristics of NMR HSPCs are crucial in replenishing WBCs, including T cells and B cells.

Seven days post-TBI, a decrease in macrophages was observed in the BM, accompanied by a slight increase in monocytes, suggesting that monocytes may replenish macrophages in tissues. Both monocytes and neutrophils differentiate from myeloid hematopoietic progenitor cells. However, while neutrophil levels were slightly reduced at 7 days post-TBI, they decreased significantly by 28 days. However, by 28 days post-TBI, the combined levels of neutrophils and monocytes had returned to normal levels. Given the strong phagocytic functions of both neutrophils and macrophages, playing synergistic roles in response to stress. The significant reduction in neutrophils at 28 days post-TBI may not have had a major effect on the overall recovery of bodily functions in NMRs.

The shifts in the cellular landscape within the BM suggest that as these cells enter the bloodstream, they contribute to the steady improvement in the physical function of NMRs and the restoration of various

blood markers, including WBCs, following radiation exposure.

The hematopoietic system is highly susceptible to the cytotoxic effects of radiation, leading to immunosuppression, including neutropenia, thrombocytopenia, myelosuppression, and anemia³⁰. Myelosuppression, a common side effect of radiotherapy, is a significant cause of mortality following exposure to moderate- or high-dose TBI³¹. Unfortunately, the effects of myelosuppression cannot be thoroughly studied in clinical settings due to ethical constraints. BM hematopoietic stem cells, the primary source of all blood cells, play crucial roles in maintaining blood cell renewal and immune function. In this study, peripheral blood tests revealed that NMRs maintained stable RBC counts for 28 days post-TBI, in stark contrast to the significant declines typically observed in C57BL/6J mice. Additionally, the NMR results revealed no significant changes in the red pulp regions after TBI, further distinguishing these regions from those in C57BL/6J mice. These marked differences suggest that NMRs possess unique mechanisms of radiation resistance. Previous research by Oka et al. has shown that NMR skin tissues exhibit a strong anti-inflammatory response to chemical carcinogens². It is hypothesized that the delayed recovery of lymphocytes in NMRs after TBI may help mitigate radiation-induced inflammation, providing an extended window for tissue recovery and contributing to their extended lifespans.

BM histological analysis further highlighted the differences between NMRs and C57BL/6J mice post-TBI, emphasizing the intrinsic features of the NMR hematopoietic system. The area of BM fat is considered a useful indicator for assessing the degree of BM injury after irradiation³². H&E staining revealed that the NMR BM hematopoietic system began to recover from TBI damage by 14 days post-TBI. Moreover, only HSCs from NMRs, but not those from mice, reduced TBI-induced injuries in NOD-SCID mice, strongly indicating the intrinsic differences between NMRs and C57BL/6J mice.

To further explore the characteristics of NMR-generated HSPCs, scRNA-seq was performed to analyze these cells. Postirradiation, the number of NMR HSPCs rapidly increased and gradually returned to normal levels, which coincided with the rapid replenishment of RBCs—a process distinct from that observed in C57BL/6J mice. While RBC recovery was swift, the recovery of WBCs and PLTs appeared slower. ScRNA-seq also revealed two novel subclusters of HSPCs, C19 and C24, in NMRs. The C24 subcluster population gradually rose and maintained a sustained HSPC pool, whereas C19 decreased after radiation. It could be speculated that the reduction in C19 may be partially involved in differentiation and in stabilizing RBC counts in peripheral blood to support essential life functions.

Unlike in C57BL/6J mice, where high doses of TBI induced premature senescence in HSPCs, leading to long-term myelosuppression and even death³¹, flow cytometry results revealed recovery of HSPCs after irradiation in NMRs but not in C57BL/6J mice. ScRNA-seq data confirmed that the two HSPC subclusters in NMRs, C19 and C24, exhibited unique resistance to irradiation and recovered 28 days post-TBI.

The C24 subcluster predominantly expressed genes such as C1S, CXCL12, SDC2, PDGFRL, KITLG, VCAM1, OLFML3, and NID1. Among these, CXCL12 is a pleiotropic chemokine that serves as a ligand for

CXCR4, which is expressed in both hematopoietic and nonhematopoietic cells. CXCL12 plays a critical role in the homing of CXCR4-expressing HSCs within the BM microenvironment by attracting these cells³³. Additionally, KITLG is significantly upregulated in the C24 subcluster of NMR HSPCs. The KIT proto-oncogene ligand (KITLG) is expressed in endothelial and leptin receptor (LepR)-expressing perivascular stromal cells and is a key niche component for the maintenance of HSCs and HSPCs^{6,34}. Currently, it remains unclear whether KITLG/KIT signaling inhibits HSC differentiation to maintain their stem cell state and what regulates KITLG and KIT in coordinating HSC self-renewal and differentiation. Vascular cell adhesion molecule 1 (VCAM1), another gene expressed in this subcluster, is typically present in healthy HSCs, is upregulated in leukemic stem cells (LSCs), and is associated with poor prognosis. In our study, it could be speculated that CXCL12, KITL, and KITLG together contributed to the expansion of the C24 subcluster following irradiation, particularly from 7–28 days post-TBI.

The C19 subcluster specifically expressed three notable genes: HBZ encodes hemoglobin subunit zeta. There are four transcripts, three of which are specific to NMRs (ENSHGLT00000003834.2, ENSHGLT00000080339.1, ENSHGLT00000049673.1). One transcript (ENSHGLT00000003833.2), also known as G5BXY3, is mainly involved in oxygen transport and heme. The role of the BPGM in hematological physiology and pathology has not been extensively reported. However, recent studies suggest that BPGM may cooperate with FNDC3B under conditions of HPV-mediated premalignant transformation of the normal epithelium³⁵. This finding implies that BPGM plays important roles in promoting HSPC proliferation and maintaining the HSPC pool³⁶. GADD45A is regulated by Hesperadin and has been shown to play a crucial role in promoting cellular oxidative stress damage and accelerating apoptosis³⁷.

GSEA further elucidated the radiation resistance characteristics of the C19 and C24 subclusters, revealing their roles in synergistic metabolic regulation, cell cycle control, and DNA repair mechanisms in NMRs. These processes collectively contribute to combating radiation-induced BM injury and sustaining hematopoietic homeostasis.

The NMR subclusters presented increased expression of genes associated with antioxidant defense, DNA repair, cell cycle regulation, and cell differentiation. These findings combined with the results of the qPCR and apoptosis analyses revealed that the expression of oxidative stress-related genes, particularly *Nfe2l2*, which continued to increase until 28 days post-TBI, was elevated under normal conditions. The *Nfe2l2* gene encodes the transcription factor Nrf2, which is known for regulating the expression of antioxidant and detoxification genes³⁸. The overexpression of NMR *Nfe2l2* reduced the degree of apoptosis in mouse HSPCs after irradiation *in vitro*. Notably, NMRs exhibit constitutively high Nrf2 signaling activity³⁹, which prepares them for TBI injuries and protects against further damage, as evidenced by reduced splenic damage after TBI.

In terms of DNA repair, *Csnk1d* levels increased significantly up to 28 days post-TBI. CSNK1D is crucial for regulating cellular DNA repair processes, including cell growth and survival, following radiation exposure⁴⁰. Additionally, both CDKN1A and CNDP2 were significantly upregulated after irradiation.

CDKN1A and CNDP2 play roles in regulating the cell cycle after radiation. CDKN1A promotes cell cycle arrest by inhibiting CDK2 activity and works synergistically with breast cancer susceptibility gene 2 (Brca2) to enhance DNA repair ⁴¹, which may help regulate cell cycle arrest and reduce apoptosis ⁴². A tightly controlled cell cycle could provide the necessary preparations for DNA repair in NMR BM HSPCs following radiation. EPRS has been reported to regulate DNA repair functions by interacting with nuclear BRCA1 in chickens ⁴³.

With respect to metabolic regulation, the sustained increase in Ddit4 expression until 28 days post-TBI is important for radiation tolerance. Recent studies have indicated that DDIT4 is vital for regulating cell proliferation during injury-induced periods ⁴⁴. It is speculated that high levels of DDIT4 are crucial for energy metabolism and stress response adaptation in NMR cells, possibly facilitating rapid activation of emergency response states. Studies have shown that NMRs possess a larger pool of quiescent HSPCs than do mice ⁵. Interestingly, despite radiation exposure, changes in the cell cycle of NMR-HSPCs were minimal, likely due to the heterogeneity of cell cycle changes in C19 and C24. This ensures HSPC stability and enables an energy-efficient response to radiation damage. Only a small fraction of C19 cells re-entered the cell cycle at 7 days post-TBI to replenish blood cells, with levels normalizing by 28 days post-TBI. In contrast, C24 cells maintain a stable cell cycle post-TBI, avoiding excessive proliferation and conserving energy.

NMR monocytes and macrophages also exhibit strong proliferative capabilities. Recent studies suggest that monocytes proliferate and differentiate into various macrophage subtypes ⁴⁵. The robust proliferation of monocytes and macrophages in NMRs likely reduces the consumption of HSPCs differentiated into erythrocytes and may accelerate the clearance of inflammation. With respect to cell differentiation, GATA1 binds to a DNA locus with a consensus sequence in the regulatory region of globin genes and other erythrocyte-expressed genes ¹⁹. HMBS, which is involved in the heme biosynthesis pathway ⁴⁶. In K562 erythroleukemia cells, *Hmbs* is a critical target gene for erythroid differentiation controlled by GATA1. Additionally, the expression of *Cpox*, which catalyzes aerobic oxidative decarboxylation in the heme biosynthesis pathway ⁴⁷, also increased 28 days post-TBI. Overall, the upregulation of *Gata1*, *Hmbs*, and *Cpox* promoted erythroid cell differentiation following lethal irradiation, a finding supported by our apoptosis and colony formation analyses.

With respect to hematopoietic recovery, the significant upregulation of *Nr3c1*, *Sco1*, *Cpox*, and *Cd44* suggested their positive roles in HSPC proliferation and differentiation. NR3C1 influences inflammatory responses, cell proliferation, and differentiation processes in target tissues ⁴⁸. SCO1, an important regulator of copper homeostasis ⁴⁹, may play a critical role in modulating HSC proliferation and differentiation after irradiation. CD44 is involved in various cellular functions, including T lymphocyte recycling and homing, hematopoiesis, inflammation, and the response to bacterial infection ⁵⁰. A recent study indicated that CD44 in the endoplasmic reticulum contributes to tissue cell longevity in NMRs by regulating protein homeostasis independent of hyaluronic acid ⁵⁰.

At 28 days post-TBI, significant increases in *Gata1* and *Sco1* were observed, both of which are essential for the recovery of hematopoietic function. Additionally, activated leukocyte cell adhesion molecule (ALCAM), which is crucial for the engraftment of normal hematopoietic stem cells in the BM, was significantly enriched at 28 days post-TBI. ALCAM also interacts with CD6 to promote T-cell proliferation and activation⁵¹. MAP2K1, which is enriched in C19 cells 28 days post-TBI, plays a key role in cell growth, adhesion, survival, and differentiation by regulating transcription, metabolism, and skeletal rearrangement⁵². Therefore, ALCAM and MAP2K1 may facilitate the colonization of transplanted NMR BM cells in the BM of NOD-SCID mice, contributing to their protective effects. This may also explain why the NMR RBC counts remained stable while other blood cells were replenished at a later stage.

Thus, the hematopoietic progenitor cells of NMRs, represented by C19 and C24, exhibit a unique regulatory mode for managing oxidative stress damage, cell cycle progression, DNA repair, and the proliferation and differentiation potential of HSPCs. This not only promotes survival after high-dose TBI but also plays a protective role in recipient NOD-SCID mice. Notably, most genes mentioned in NMRs have well-established roles in humans and mice, facilitating future translational research for clinical applications. It would be intriguing to explore why these critical genes are highly expressed in NMRs and how they initiate repair mechanisms.

Despite these findings, some limitations remained. First of all, the sample size for single-cell sequencing was small, which may pose some inconvenience in deeply mining biological data and could easily lead to the omission of rare cells. In addition, there is nearly a 20-fold difference in maximum lifespan between NMRs and mice. Comparing adult mice around 8 weeks old with adult naked mole rats around 1.5 years old introduces some differences in age matching. And this study claims that the differences in radio-resistance are mainly due to species, but not age-related stem cell biology. Finally, functional validation of the C19 and C24 genes and BM cell transplantation into immunodeficient mice needs further in vivo investigations.

Conclusions

This study highlights the exceptional radiation tolerance of NMRs compared with C57BL/6J mice. We identified two novel subtypes of NMR HSPCs that exhibit remarkable resistance to high-dose TBI and elucidated their underlying regulatory mechanisms. TBI induced the expression of specific genes related to metabolic regulation, cell cycle control, and DNA repair within these HSPC subtypes, enabling them to combat radiation-induced BM injury. This, in turn, initiated radiation protection mechanisms that preserved the HSPCs pool in the BM and maintained RBC counts in peripheral blood. These findings underscore the significance of NMRs as a model for studying myelosuppression and radiation protection, offering valuable insights for therapeutic development and radiation protection strategies in clinical settings.

Declarations

CRediT authorship contribution statement

Wenjing Yang: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, and Writing - review & editing. **Xiaolong Jiang:** Investigation, Methodology. **Jingyuan Zhang:** Methodology. **Junyang Wang:** Investigation, Methodology, Software, Visualization. **Qianqian Zhang:** Investigation, Methodology, Software, Visualization. **Jinjia Liu:** Investigation, Methodology. **Chengcai Zhang:** Investigation. **Qian Zhou:** Investigation, Methodology. **Yan-Ru Lou:** Project administration, Supervision, review & editing. **Shufang Cui:** Funding acquisition, Project administration, Resources, Software, Supervision, Validation, Visualization, review & editing.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval and consent to participate

All animal experiments involved in this project are subject to the approval of the Animal Welfare Ethics Committee of the Naval Medical University (No. 2021CSF0611).

Author Contribution

Wenjing Yang: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, and Writing - review & editing. Xiaolong Jiang: Investigation, Methodology. Jingyuan Zhang: Methodology. Junyang Wang: Investigation, Methodology, Software, Visualization. Qianqian Zhang: Investigation, Methodology, Software, Visualization. Jinjia Liu: Investigation, Methodology. Chengcai Zhang: Investigation. Qian Zhou: Investigation, Methodology. Yan-Ru Lou: Project administration, Supervision, review & editing. Shufang Cui: Funding acquisition, Project administration, Resources, Software, Supervision, Validation, Visualization, review & editing.

Data Availability

The data can be obtained in GEO under the accession number: stqxmciubvwhpcj (GEO accession GSE254429: stqxmciubvwhpcj. <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE254429>).

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Figures

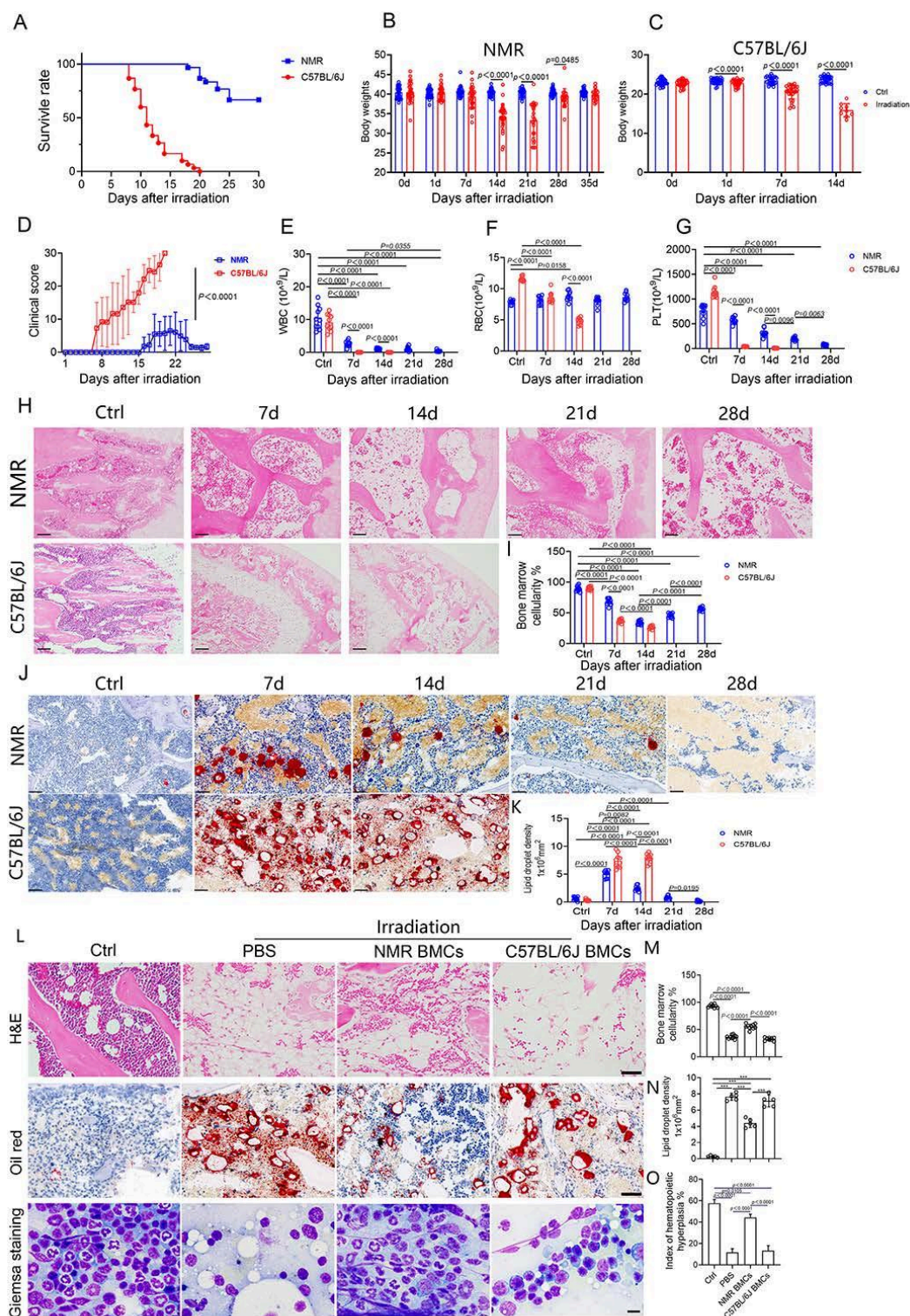


Figure 1

NMRs exhibit greater tolerance to total body irradiation (TBI) than do C57BL/6J mice.

A, Survival rates of C57BL/6J mice (n = 30) and NMRs (n = 30) after receiving 10 Gy of ^{60}Co γ -ray TBI. Survival was monitored for 30 days. The data represent two independent experiments (n = 30/group). The log-rank (Mantel–Cox) test was used to analyze the survival rates. **B–C**, Body weights of C57BL/6J

mice and NMRs after irradiation (NMRs: n = 30 for the Ctrl, 1 d, 7 d, and 14 d groups; n=20 for the 28 d and 35 d groups). C57BL/6J mice: n = 30 for the Ctrl, 1 d, and 7 d groups; n=9 for the 14 d group). **D**, Clinical scores of C57BL/6J mice and NMRs postirradiation (n = 6/group). **E-G**, White blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts in anticoagulant-treated peripheral blood (n = 10/group). The data are presented as the means $\pm$ SEMs. **H**, Histological changes in the bone marrow (BM) of C57BL/6J mice (0–14 days) and NMRs (0–28 days) after TBI, as detected by H&E staining (n = 10/group). **I**, Quantification of cellularity area proportions in BM (n = 10/group). **J**, Oil red O staining of BM. **K**, Quantification of fat area proportions in BM (n = 3/group). **L**, H&E staining and Oil Red O staining of BM from NOD-SCID mice 7 days after BM transplantation. Ctrl: NOD-SCID mice without irradiation and transplantation. PBS: NOD-SCID mice were irradiated and transplanted with PBS. NMR BMCs: NOD-SCID mice were irradiated and transplanted with NMR BM cells. C57BL/6J BMCs: NOD-SCID mice were irradiated and transplanted with C57BL/6J BM cells. **M**, Quantification of cellularity area proportions in BM (n = 10/group). **N**, Quantification of fat area proportions in BM (n = 5/group). **O**, Evaluation of myelohyperplasia in whole BM cells (n = 3/group). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test.

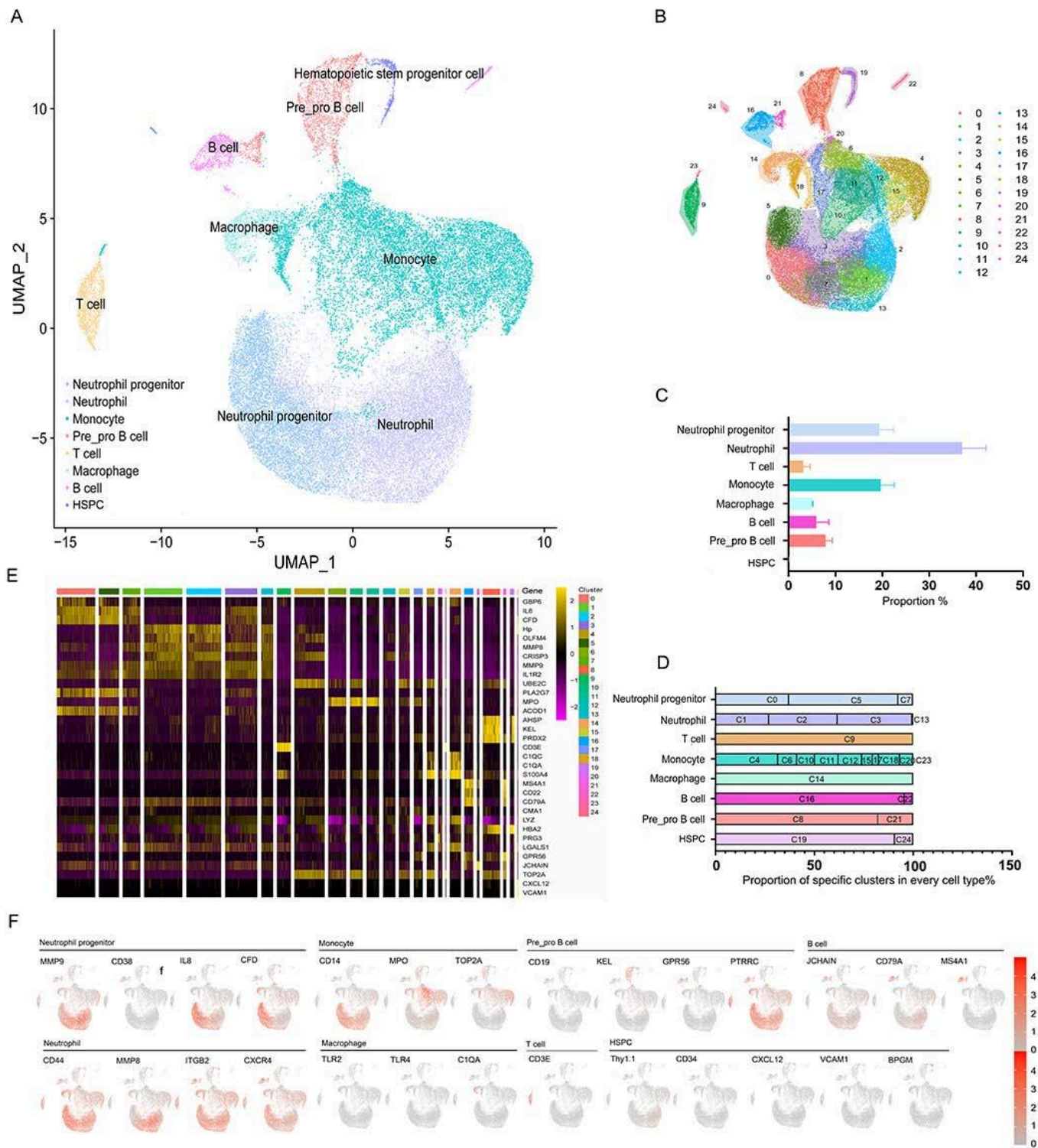


Figure 2

scRNA-seq analysis of NMR BM cells.

A, Uniform manifold approximation and projection (UMAP) analysis of NMR bone marrow (BM) cells (excluding erythrocytes) combined from all samples (n = 6000 cells/group). Each dot represents a cell, color-coded by its subcluster allocation and annotated cell type (see Materials and Methods).

Annotation of the indicated cell types. **B**, Twenty-five distinct cell subclusters were identified and numbered from 0–24. **C**, Proportions of the 25 cell subclusters. Heatmap showing marker gene expression levels across the 25 cell subclusters. **D**, Proportions of the 8 identified cell types. **E**, Annotation and relative proportions of subclusters C0–C24 within the 8 categories of identified cell types. **F**, Expression patterns of selected known marker genes in NMR BM cells, displayed on UMAP plots.

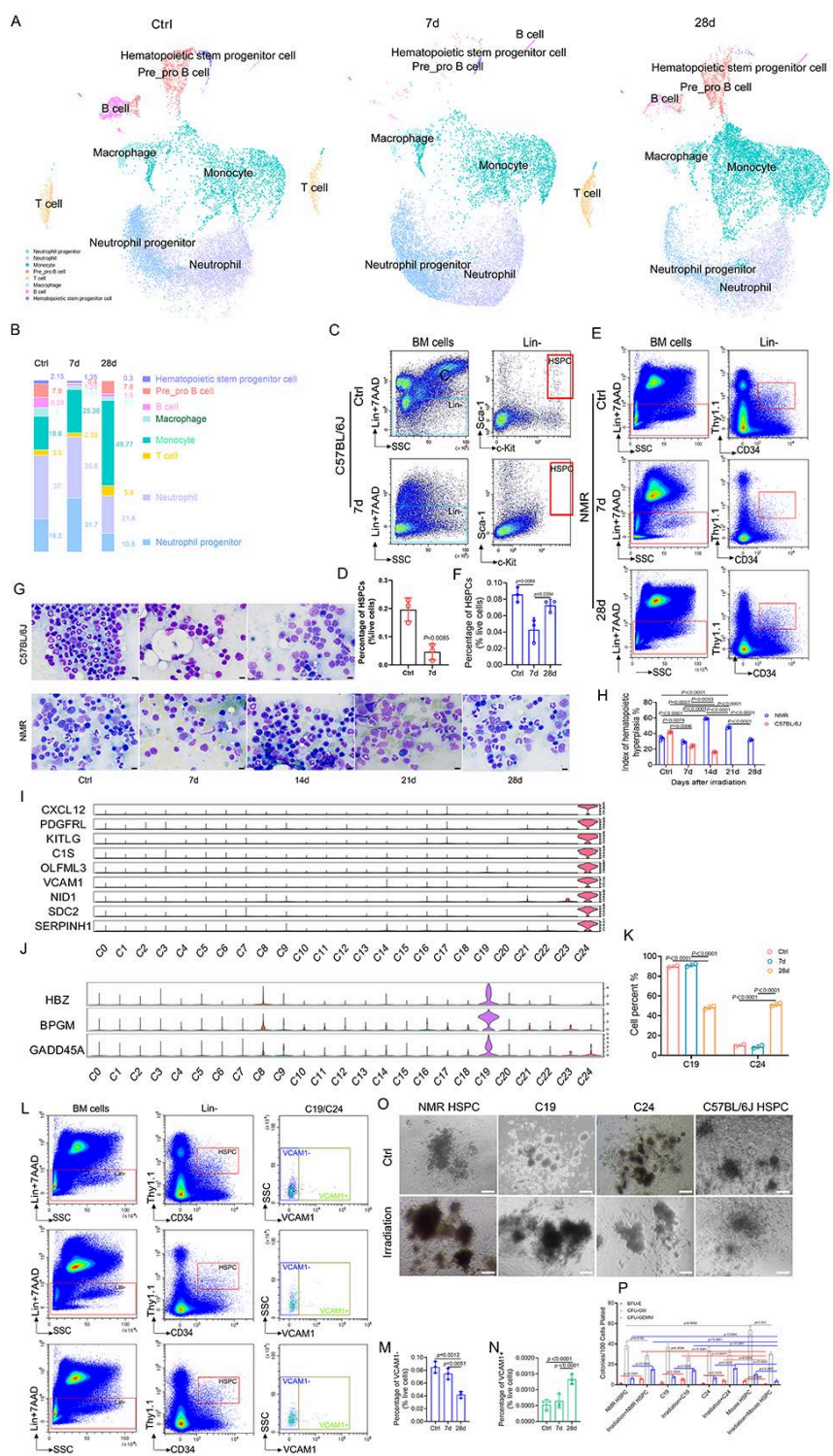


Figure 3

Changes in NMR BM cells at different time points after TBI and characteristics of HSPC subclusters (C19 and C24).

A, UMAP visualization of NMR bone marrow (BM) cells before total body irradiation (TBI, ctrl) and at 7 days (7 d) and 28 days (28 d) post-TBI. The color legend is consistent throughout the dataset. **B**, Quantification of the proportions of various cell types after TBI. The percentage of each cell type (%) is labeled in the corresponding color adjacent to each bar. **C**, Mouse hematopoietic stem and progenitor cells (HSPCs) from BM were sorted via lineage markers (LIN = CD4/CD8a/CD45R/CD127/TER-119/Ly-6G) for depletion and stained with Sca-1 and c-kit. The sorting gates for the HSPCs are shown. **D**, Quantification of flow cytometry results for mouse BM HSPC fractions (n=3). **E**, NMR-generated HSPCs from BM were sorted using lineage markers (LIN = CD11b/CD18/CD90/CD125) for depletion and stained with Thy1.1 and CD34. The sorting gates for the HSPCs are shown. **F**, Quantification of flow cytometry analysis (FCA) results for NMR BM-derived HSPCs (n=3). Statistical significance was determined via Student's t test (**c**) and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test (**e**). **G**, Wright–Giemsa staining of whole BM from NMRs and C57BL/6J mice on different days postirradiation. **H**, Evaluation of myelohyperplasia in whole BM cells, as shown in panel (g). **I**, Violin plots depicting the expression of C24 cell-specific markers. **J**, Violin plots depicting the expression of C19 cell-specific markers. **K**, Percentages of C19 and C24 cells before irradiation (ctrl) and at different days postirradiation. **L**, C19 and C24 clusters were identified within HSPCs by FCA as VCAM1⁻ and VCAM1⁺, respectively. **M–N**, Quantification of the C19 and C24 clusters shown in panel (I). **O**, Colony formation analysis of NMR-generated HSPCs, C57BL/6J HSPCs, and C19 and C24 clusters. Scale bars = 100 μ m. **P**, Quantification of the colonies shown in panel (o), including burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte/macrophage (CFU-GM), CFU-granulocyte, erythrocytes, megakaryocytes, and macrophages (CFU-GEMM). The data are expressed as the means $\pm$ SEMs. n = 3/group. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test.

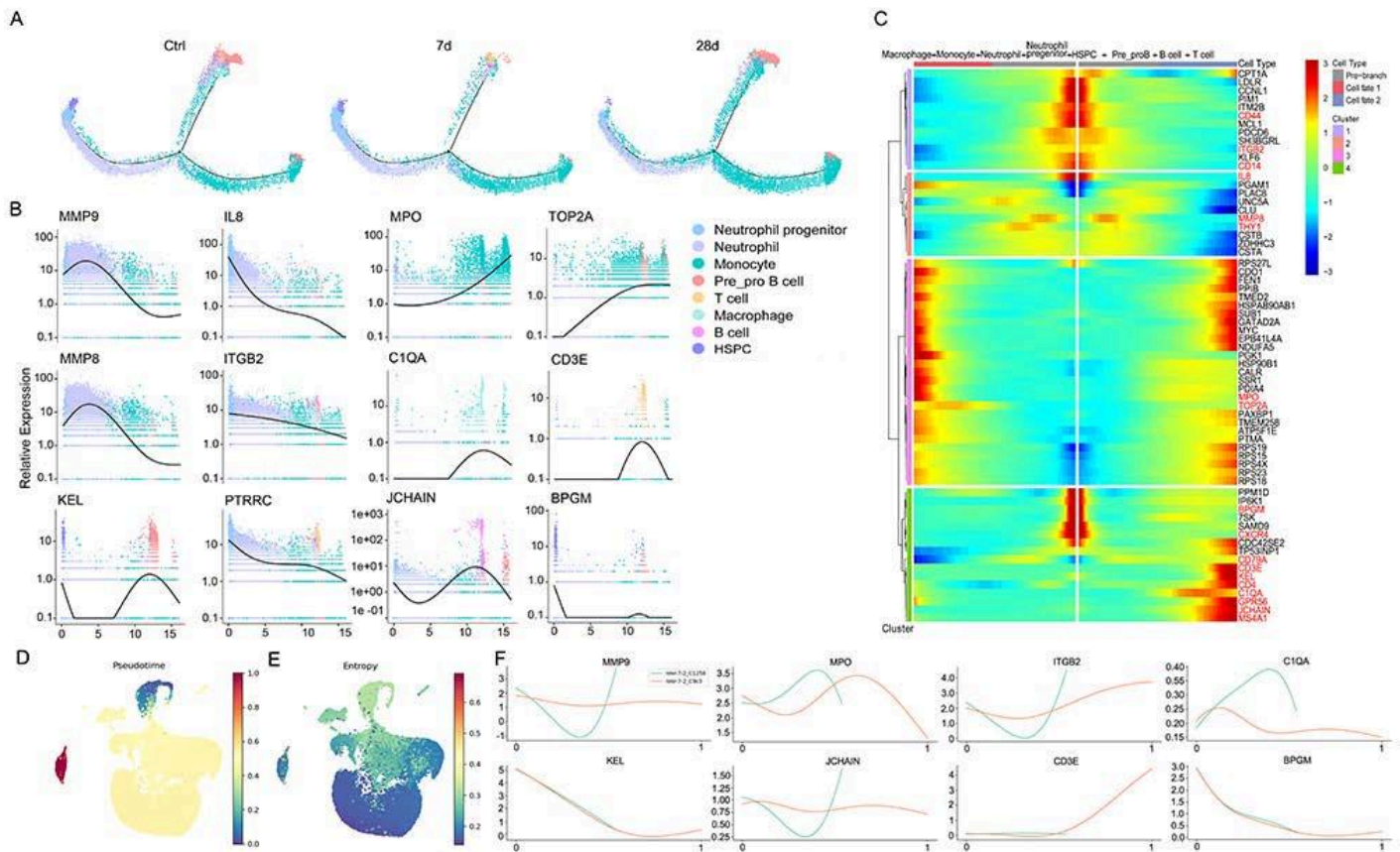


Figure 4

Single-cell population and trajectory analysis of NMR BM cells before and after irradiation.

A, Unsupervised differentiation trajectories of all cell populations before total body irradiation (TBI, Ctrl), 7 days after TBI (7d), and 28 days after TBI (28d), constructed with M3drop and Monocle v2.10.1. **B**, Lineage-specific gene expression changes over pseudotime. **C**, Heatmap analysis of branch-specific genes near branch point 1 in (A). **D**, Cells colored by Palantir pseudotime. **E**, Cells are colored according to their Palantir differentiation potential. **F**, Palantir analysis of the top genes shown in panel (B).

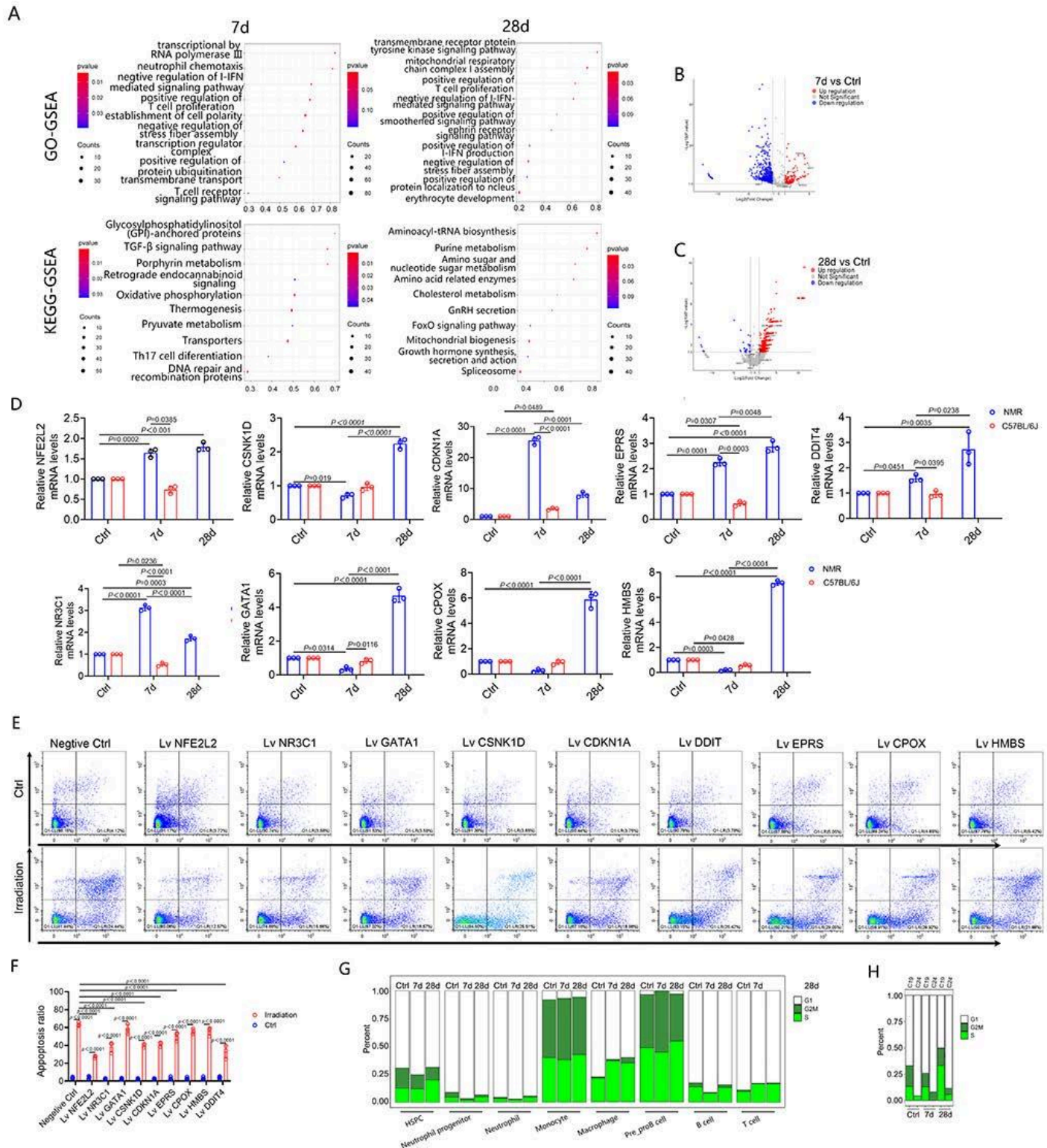


Figure 5

GSEA of the C19 subcluster.

A, Gene set enrichment analysis (GSEA) of the C19 subcluster based on gene ontology (GO) and KEGG analyses at different time points: Ctrl, 7 days, and 28 days **postirradiation**. **B–C** Volcano plots showing gene changes enriched by GSEA (b, 7 days vs Ctrl; c, 28 days vs Ctrl). **D**, Representative genes detected

by qPCR, including NFE2L2, CSNK1D, CDKN1A, EPRS, DDIT4, NR3C1, GATA1, CPOX, and HMBS (n=3). **E**, Roles of CDKN1A, CSNK1D, NR3C1, GATA1, and HMBS in regulating mouse HSPC apoptosis after 2 Gy of ⁶⁰Co irradiation. **F**, Quantification of the levels of apoptosis shown in panel (e). n=3. **G**, Seurat cell cycle analysis of NMR bone marrow cells in the Ctrl, 7 d, and 28 d groups. **H**, Seurat cell cycle analysis of the C19 and C24 clusters. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test.

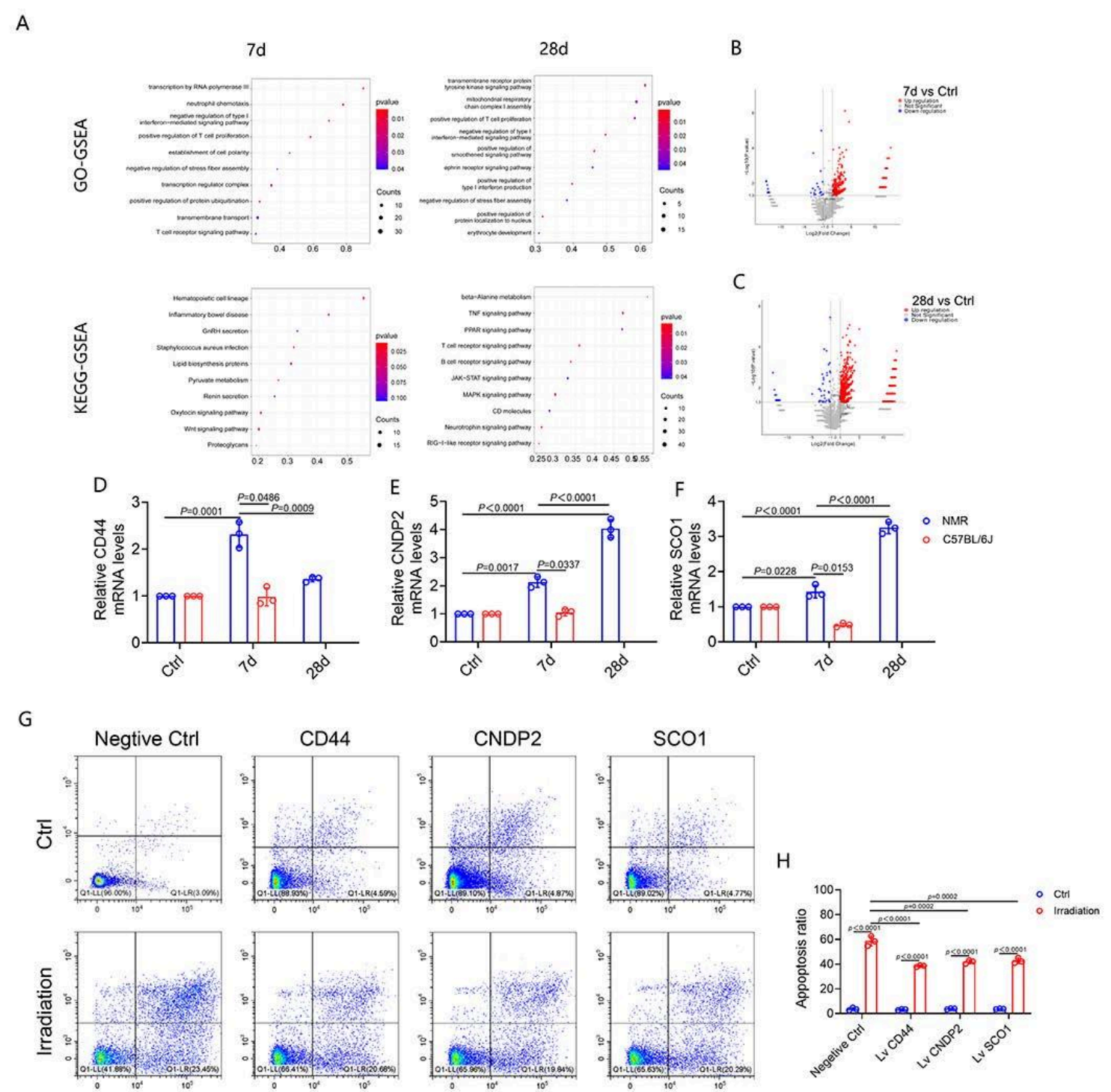


Figure 6

GSEA of the C24 subcluster.

A, Gene set enrichment analysis (GSEA) of the C24 subcluster cells based on gene ontology (GO) and KEGG analyses at different time points: Ctrl, 7 days, and 28 days postirradiation. **B-C**, Volcano plots showing gene changes enriched by GSEA (b, 7 days vs Ctrl; c, 28 days vs Ctrl). **D-F**, Representative genes detected by qPCR, including CD44, CNDP2, and SCO1 (n=3). **G**, Roles of CD44, CNDP2, and SCO1 in regulating mouse HSPC apoptosis after 2 Gy of ⁶⁰Co irradiation. **H**, Quantification of the levels of apoptosis shown in panel (g) (n=3). The data are expressed as the means ± SEMs. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test.

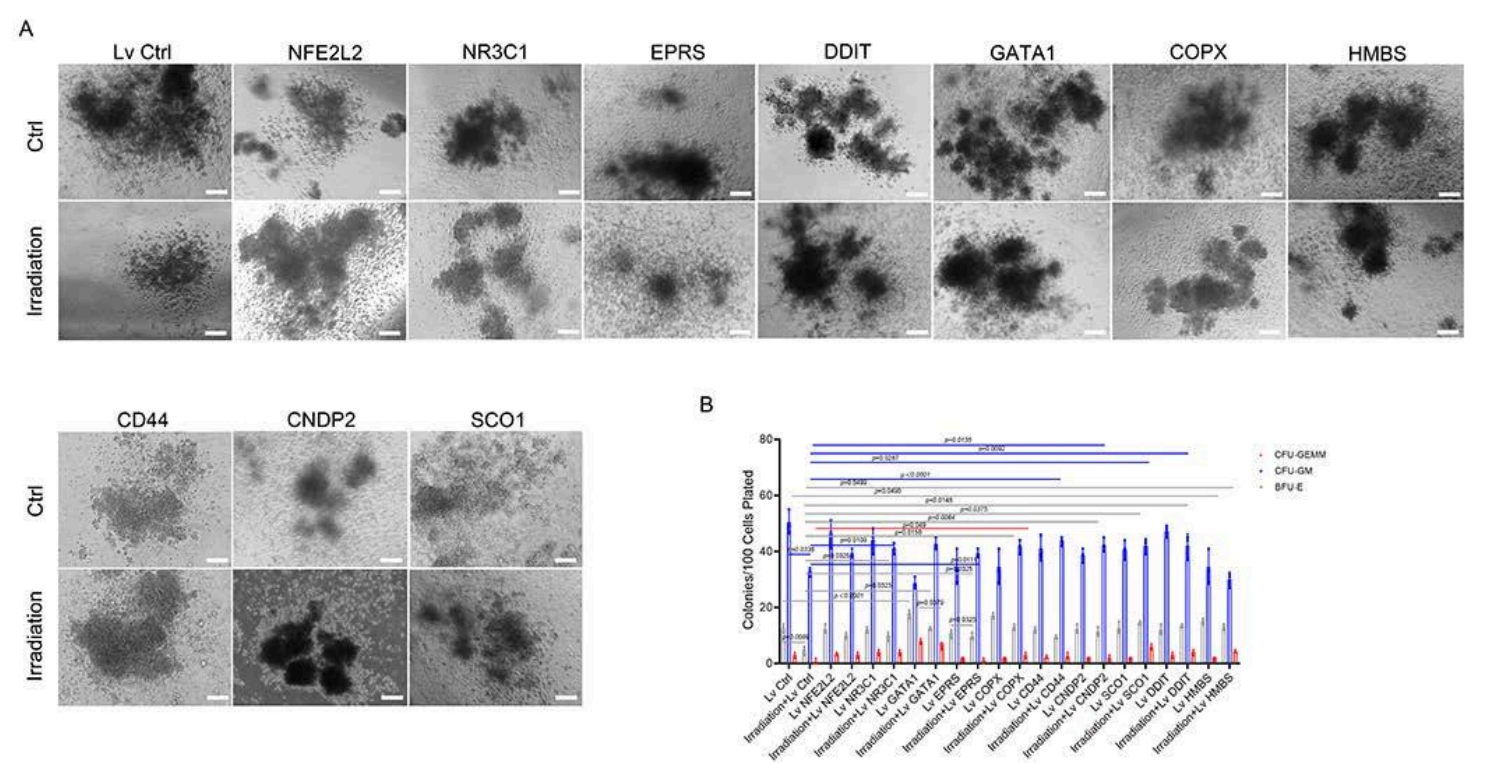


Figure 7

Colony formation analysis of mouse HSPCs transfected with NMR genes.

A, Colony formation analysis of C57BL/6J HSPCs transfected with NMR genes, including NFE2L2, NR3C1, EPRS, DDIT, GATA1, COPX, HMBS, CD44, CNDP2, and SCO1. **B**, Quantification of the colonies of BFU-E, CFU-GM, and CFU-GEMM is shown in panel (A). The data are expressed as the means ± SEMs. n =3/group. The data from every gene overexpression group was compared with the data in the Ctrl group under radiation or non-radiation conditions. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test.

Supplementary Files

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