Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

# The Flow Cytometry Marker for Early Detection of Irradiation Injury in NOG Mice Bone Marrow: A Short Report

**Maria Kavianpour**
Tehran University of Medical Sciences https://orcid.org/0000-0002-8047-8754
**Samad Muhammadnejad**
Tehran University of Medical Sciences
**Kobra Moradzadeh**
Tehran University of Medical Sciences
**Zahra Jabbarpour**
Tehran University of Medical Sciences
**Amir Arsalan Khorsand**
Tehran University of Medical Sciences
**Sajjad Aghayan**
Tehran University of Medical Sciences
**Mohammad Vasei**
Tehran University of Medical Sciences
**Javad Verdi** ( javad0verdi@gmail.com )
Tehran University of Medical Sciences

**Short report**

**Keywords:** Flow Cytometry, Radiation, NOG Mice, Annexin V, Propidium Iodide, Bone Marrow, Apoptosis, Reactive Oxygen Species

**Posted Date:** September 17th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-892573/v1

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

## Abstract

# Background

Radiation is used for myeloablation in mice's bone marrow (BM) for hematopoietic stem cells (HSC) engraftment experiments. To assess post-radiation damage to the BM, an accessible and convenient marker is needed. This study comparatively evaluated the flow cytometer's effectiveness in detecting the ROS and Annexin V-PI level.

# Methods

We divided 30 NOG mice between irradiated (n = 20) and control groups (n = 10) for each time point. After sacrificing BM samples were collected, the percentage of annexin V, PI, and ROS were investigated at two different time points (Day 2 and 14 after exposure).

# Results

At the first timepoint, the level of ROS was higher in the irradiated group than in the control group, and this difference was statistically significant ($P < 0.05$). Also, at the second timepoint, the mean differences of all markers in the irradiated group were significant compared to the control group ($P < 0.05$).

# Conclusions

Thus, in this mice strain to assess the BM irradiation-induced injury, measurement of ROS level is helpful.

## Introduction

The irradiation of rodents is a well-known useful tool in immunology and oncology studies (1). Radiation studies are used to understand biophysical and biological factors affecting animal models and improve experimental results (2). Biological targets, such as bone marrow (BM) and intestinal epithelial cells, are susceptible to irradiation (3). Rodents are irradiated to myeloablation BM either as immunosuppression or for donor graft regeneration (4). Sublethal systemic radiation is usually conducted before transplanting; this is useful for engrafting tumors or human hematopoietic stem cells (HSCs) to create humanized mice (5, 6). In immunodeficient rodents, such doses of radiation are often used to protect against innate immune rejection or to induce the development of chimeric animals expressing several types of HSC (7).

Irradiation leads to oxygen-derived free radicals and reactive nonradical molecules, so-called reactive oxidative species (ROS), and apoptosis resulting from DNA strand breaks (8). It is essential to wait for at least 4 to 12 weeks later to observe the levels of apoptosis using terminal deoxynucleotidyl transferase

dUTP nick end labeling (TUNEL) assay or fibrosis by Hemotoxylin and Eosin (H&E) to assess the level of radiation-induced injury (6, 9). However, flow cytometry is a valuable tool for the assessment analysis of necrosis and apoptosis quickly. Apoptosis can be detected by measuring the release of phosphatidylserine from the plasma membrane that Annexin V attaches to it using fluorescently-tagged annexin V and the DNA-binding dye propidium iodide (PI), necrosis can be detected (10, 11). Also, flow cytometry can be used to detect intracellular ROS (12). In this study, we aimed to comparatively evaluate the flow cytometer's effectiveness in detecting the ROS and Annexin V-PI level in BM samples following irradiation exposure in NOG mice to find the best way to measure the amount of radiation-induced injury quickly.

# Materials And Methods

A total of 30 female (12-week-old) *NOD.Cg-Prkdcscidll2rgtm1Sug/ShiJic* (NOG) mice with uniform body weight were obtained from the Digestive Disease Research Institute of Tehran University of Medical Sciences animal facility. The mice were housed in a positive, individually ventilated caging (IVC) system under controlled temperature and humidity, 12h light/12h dark cycles, and fed a sterilized pellet diet and water ad libitum. Animals were divided into two groups; Group 1 was exposure total body irradiation (TBI) at a sub-lethal dose of (3.5 Gy, 2.5 Gy/min, Elekta Precise accelerators), Group 2 was used as a control group. Bone marrow samples were collected on days 2 and 14. All procedures were conducted under a protocol approved by the Ethical Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1399.227).

The mean fluorescence intensity (MFI) of dye 2',7'-dichlorofluorescein diacetate (DCFH-DA) was analyzed using flow cytometry to evaluate ROS production in BM samples. DCFH-DA is a stable, non-fluorescent, cell-permeable compound, which penetrates the cell and converts to DCFH by intracellular esterase and then trapped within the cell and stable for a few hours. On oxidation, ROS de-esterified product is transformed to the highly fluorescent 2',7' dichlorofluorescein (DCF), which emits green fluorescence when excited at 488 nm, proportional to the intracellular level of ROS. DCFH-DA (10 µM) was added to the BM-mononuclear cells and incubated at 37°C for 30 min in the dark. Subsequently, cells were washed, resuspended in PBS, and detected by flow cytometry. Data were analyzed using the FlowJo 7.6.1 software (Tree Star, Ashland, OR, USA).

High-quality staining is achieved by combining Annexin V with PI; a DNA intercalating counterstain often used to distinguish between apoptotic and necrotic cells in a population. To carry out the Annexin V-PI staining procedure (BD, Cat. No. 556547). After washing cells and adding Annexin V and PI, the cells were stored in the dark, and then flow cytometry was used to evaluate them within one hour. Untreated cells were gated at the FSC-SSC (forward scatter-side scatter) plot, and percentages felt into four zones were computed.

The implantation rates were compared to determine the effect of exposure to radiation on BM injury. The Mann-Whitney U test was used to compare differences between groups. Results are reported as the mean

± SD and P < 0.05 were considered statistically significant. Data analysis was performed with GraphPad Prism 9 for Windows (GraphPad Software, Inc; CA). The irradiation group consisted of 20 animals (n = 20), and the nonirradiated control group consisted of 10 animals (n = 10) then divided into two timepoints.

# Results

After exposure to 3.5 Gy of radiation, irradiated mice (n = 20) and the control group (n = 10) were sacrificed at two different time points (2 and 14 days after exposure). Apoptosis, necrosis, and ROS in BM samples were measured via the flow cytometry method. In time point 1, data showed mean differences in the intracellular amount of ROS was significant compared to the control group (P < 0.05) (Fig. 1). However, there was no significant difference between the irradiated group and control for early and late apoptosis or necrosis at this time point. Also, the results demonstrated that in time point 2 (day 14), apoptosis, necrosis, and ROS significantly increased in the irradiated group compared to control (P < 0.05) (Fig. 2).

# Discussion

Radiation is one of the BM myeloablation methods for HSC engraftment study in animal models (13). To assess the level of injury after radiation exposure, we determined ROS, apoptosis, and necrosis by flow cytometry method at two different time points. Our goal was to identify a suitable flow cytometry marker among them within a few days of irradiation. At the first time point, the mean difference ROS level was higher than other markers like Annexin V and PI, that this marker increased before apoptosis or necrosis induction. According to Lorimore et al.'s study, activated macrophages and inflammatory-type reactions occurred in the hemopoietic system after exposure to ionizing radiation. Based on their results, increased Nitric oxide synthase (NOS) enzyme activity and neutrophil infiltration in the tissue, activation of macrophages provide a mechanism for damage production through a "bystander" effect, which may contribute to radiation-induced genomic instability and leukemogenesis (14). In Burr et al. study, phosphorylated histone H2AX, Caspase 3, and TUNEL by immunocytochemistry 24h after radiation were assayed. So, their findings demonstrated that radiation exposure led to the production of a damaging BM microenvironment 24 h post-irradiation that macrophages had the potential to contribute secondary damage after the initial radiation-induced injury. This delayed bystander-type effect indicated the importance of tissue responses (15). Their study results were not in line with our study because macrophages have a significant role in this mechanism, but we used the immunodeficient NOG mice that have reduced macrophage function. Thus, this mechanism might cause the delay in increased apoptosis and necrosis, and in NOG mice, a better marker is needed for early determination.

In another study, irradiation at ranges of 2 to 20 Gy was used that did not cause direct cellular apoptosis or necrosis but induced mitochondrial damage in cells; thus, an easier and faster method is needed (16). In another study, the level of apoptosis in two mice strains (C57 and CBA) in different radiation doses was assessed. The results demonstrated that apoptosis levels differed between the strains with CBA that

apoptosis levels were higher at 24 h than C57, but C57 showed a higher level of apoptosis at the delayed time point (17). However, in our study, NOG mice had a higher level of apoptosis on day 14 than two days after irradiation; this may be due to differences in the strains of the study mice from other studies. On the other hand, these mice have low irradiation tolerance, and basically, doses higher than 4 Gy cannot be used for this strain of mice (18).

Mukherjee et al. investigated the relationships between apoptotic responses of cells exposed and tissue cytotoxicity in irradiated (0.25-2 Gy) murine BM. Up to 24 h post-irradiation, BM cellularity showed reductions, but *in vivo* apoptosis measurements were not detected. There was ongoing cell death up to 24 h post-irradiation, while the level of apoptosis is not elevated and cytokines are produced in response to the initial tumor protein 53 (p53)-induced apoptosis. In the presence of low levels of measured apoptosis, intramedullary cell death and apoptotic processes associated with pro-inflammatory mechanisms contribute to additional ongoing cell death (19). In our study, due to the absence of immune cells (B-cells and T-cells, macrophages, and natural killer (NK) cells) and pro-inflammatory mechanisms, BM damage has been delayed in NOG mice (20, 21). So, determining intracellular ROS in BM samples could be a helpful marker to measure injury of irradiation for establishment HSC engraftment in NOG mice models.

# Abbreviations

**BM**
Bone Marrow
**Gy**
Gray
**NOG**
NOD/Shi-scid/IL-2Rγnull
**ROS**
Reactive Oxidative Species

# Declarations

### Ethics approval and consent to participate

This study was conducted under a protocol approved by the Ethical Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1399.227).

### Consent for publication

All the authors have approved the manuscript and agree with submission to your journal.

### Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

### Competing interests

The authors declare that there is no conflict of interest.


### Funding

This study was funded by a grant from the Tehran University of Medical Sciences (98-02-87-43338).


### Authors' contributions

Conceptualization: [Maria Kavianpour] and [Javad Verdi]; Methodology: [Kobra Moradzadeh], [Sajjad Aghayan], [Amir Arsalan Khorsand] and [Maria Kavianpour]; Formal analysis and investigation: [Maria Kavianpour] and [Zahra Jabbarpour]; Writing-original draft preparation: [Maria Kavianpour]; Writing-review and editing: [Samad Muhammadnejad]; Funding acquisition: [Javad verdi]; Resources: [Mohammad Vasei], [Javad Verdi]; Supervision: [Javad verdi]. The authors read and approved the final manuscript.


### Acknowledgements

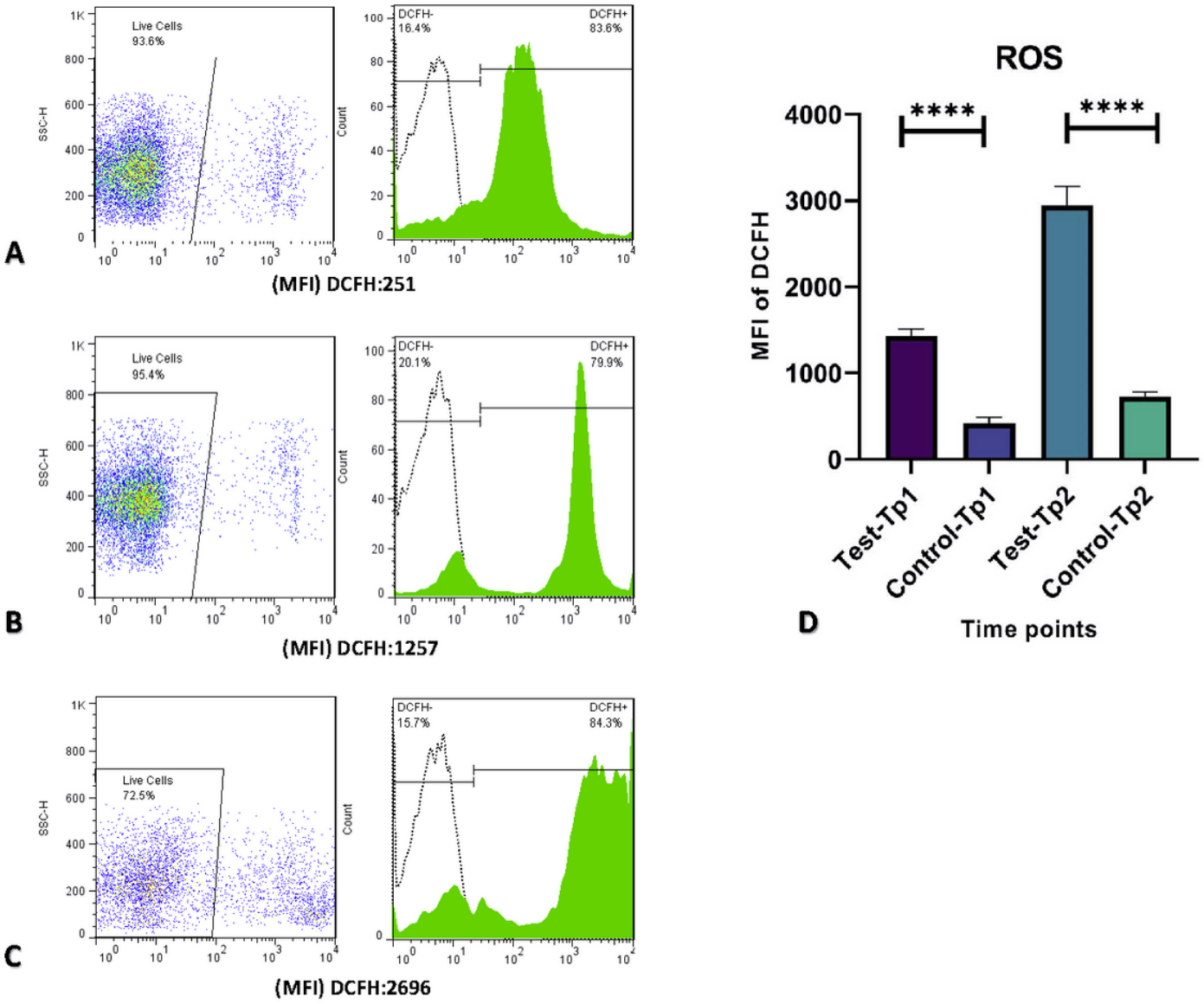
Not applicable.


## References


1. Williams JP, Brown SL, Georges GE, Hauer-Jensen M, Hill RP, Huser AK, et al. Animal models for medical countermeasures to radiation exposure. 2010;173(4):557–78.
2. Tatum JLJljorb. Hypoxia: importance in tumor biology, noninvasive measurement by imaging, and value of its measurement in the management of cancer therapy. 2006;82(10):699–757.
3. Li T, Cao Y, Li B, Dai RJPiB, Biology M. The biological effects of radiation-induced liver damage and its natural protective medicine. 2021.
4. Huang CA, Fuchimoto Y, Scheier-Dolberg R, Murphy MC, Neville DM. Sachs DHJTJoci. Stable mixed chimerism and tolerance using a nonmyeloablative preparative regimen in a large-animal model. 2000;105(2):173–81.
5. Rajan V, Melong N, Wong WH, King B, Tong RS, Mahajan N, et al. Humanized zebrafish enhance human hematopoietic stem cell survival and promote acute myeloid leukemia clonal diversity. 2020;105(10).
6. Cheng W, Xiao L, Ainiwaer A, Wang Y, Wu G, Mao R, et al. Molecular responses of radiation-induced liver damage in rats. 2015;11(4):2592–600.
7. Zitvogel L, Pitt JM, Daillère R, Smyth MJ, Kroemer GJNRC. Mouse models in oncoimmunology. 2016;16(12):759–73.

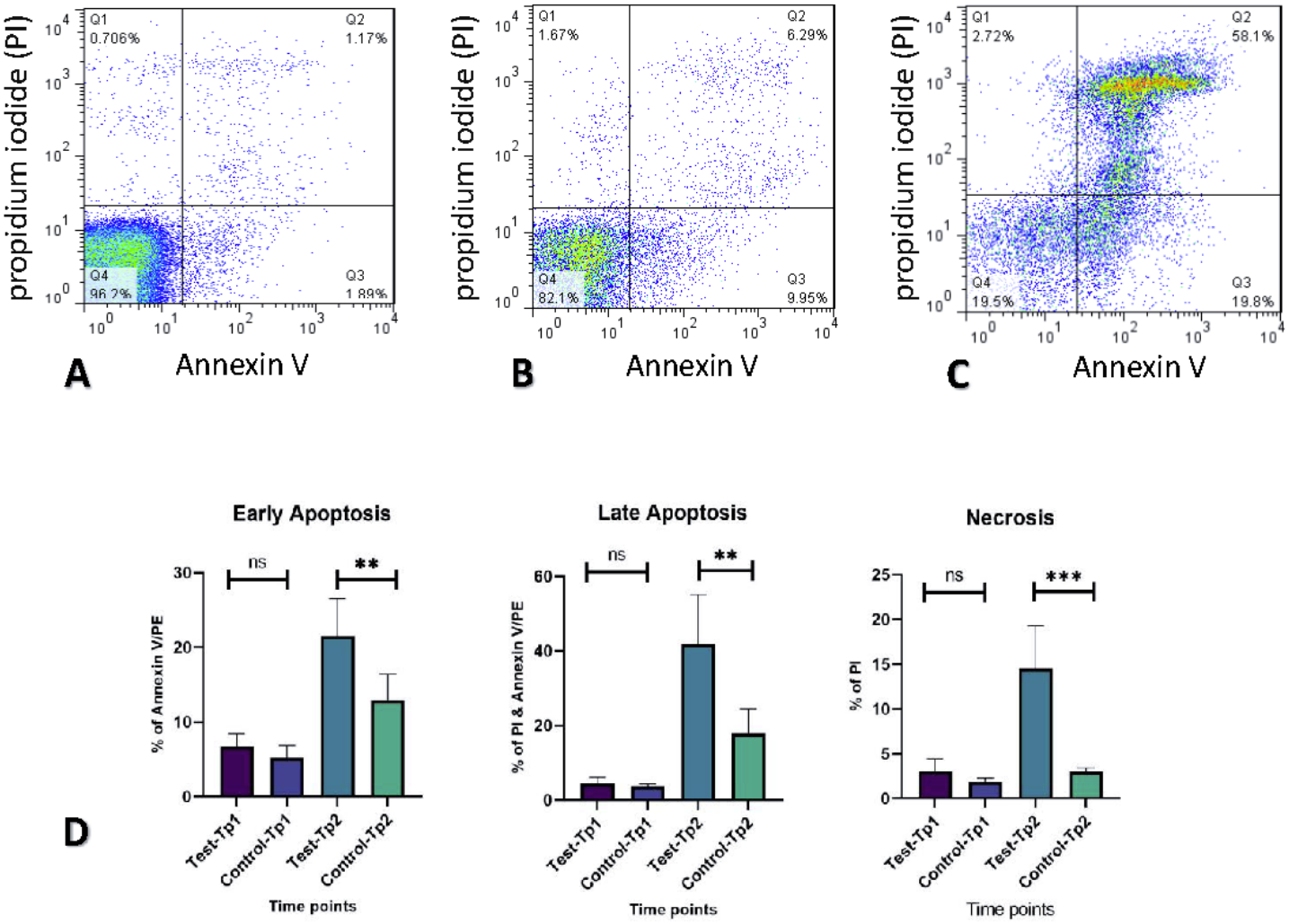
8. Bergamini CM, Gambetti S, Dondi A, Cervellati CJCpd. Oxygen, reactive oxygen species and tissue damage. 2004;10(14):1611-26.
9. Kezic JM, McMenamin PGJPO. The effects of CX3CR1 deficiency and irradiation on the homing of monocyte-derived cell populations in the mouse eye. 2013;8(7):e68570.
10. Moumaris M, Rajoelya B, Abuafa NJOBSJ. Fluorescein isothiocyanate-dextran can track apoptosis and necrosis induced by heat shock of peripheral blood mononuclear cells and HeLa cells. 2015;1(1).
11. Andrade R, Crisol L, Prado R, Boyano MD, Arluzea J, Aréchaga JJBotC. Plasma membrane and nuclear envelope integrity during the blebbing stage of apoptosis: a time-lapse study. 2010;102(1):25–35.
12. Eruslanov E, Kusmartsev S. Identification of ROS using oxidized DCFDA and flow-cytometry. Advanced protocols in oxidative stress II. Springer; 2010. pp. 57–72.
13. Boieri M, Shah P, Dressel R, Inngjerdingen, MJFii. The role of animal models in the study of hematopoietic stem cell transplantation and GvHD: a historical overview. 2016;7:333.
14. Lorimore SA, Coates PJ, Scobie GE, Milne G, Wright EGJO. Inflammatory-type responses after exposure to ionizing radiation in vivo: a mechanism for radiation-induced bystander effects? 2001;20(48):7085–95.
15. Burr KL, Robinson JI, Rastogi S, Boylan MT, Coates PJ, Lorimore SA, et al. Radiation-induced delayed bystander-type effects mediated by hemopoietic cells. 2010;173(6):760–8.
16. Zhou Z, Zhao J, Hu K, Hou X, Sun X, Pan X, et al. Single High-Dose Radiation Enhances Dendritic Cell Homing and T Cell Priming by Promoting Reactive Oxygen Species-Induced. Cytoskeletal Reorganization. 2021;109(1):95–108.
17. Irons SL, Serra V, Bowler D, Chapman K, MIliti S, Lyng F, et al. The effect of genetic background and dose on non-targeted effects of radiation. 2012;88(10):735–42.
18. Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2Rγnull mice engrafted with mobilized human hemopoietic stem cells. 2005;174(10):6477–89.
19. Mukherjee D, Coates PJ, Rastogi S, Lorimore SA, Wright EGJIjorb. Radiation-induced bone marrow apoptosis, inflammatory bystander-type signaling and tissue cytotoxicity. 2013;89(3):139–46.
20. Liou J-T, Liu F-C, Mao C-C, Lai Y-S, Day Y-JJTJotASoA. Inflammation confers dual effects on nociceptive processing in chronic neuropathic pain model. 2011;114(3):660–72.
21. Shultz LD, Lang PA, Christianson SW, Gott B, Lyons B, Umeda S, et al. NOD/LtSz-Rag1null mice: an immunodeficient and radioresistant model for engraftment of human hematolymphoid cells, HIV infection, and adoptive transfer of NOD mouse diabetogenic T cells. 2000;164(5):2496–507.

# Figures




## Figure 1

Flow cytometry with DCFH-DA staining for measuring ROS production in BM samples after radiation exposure. Flow cytometry plots were shown in A, B, and C for Control, Timepoints 1, and 2, respectively. The ROS production was assessed according to changes in the fluorescence intensity of DCF, the oxidation product of DCFH-DA. The data presented are means ± SD. (* p-value < 0.05, ** p-value < 0.01 and *** p-value < 0.001).




## Figure 2

Flow cytometry analysis of annexin-V and propidium iodide (PI) staining of apoptosis and necrosis, A) Control sample, B) Timepoint 1 (day 2), C) Timepoint 2 (day 14); D) Graphs showing % of apoptotic cells (annexin-V positive for early apoptosis, PI positive + annexin V double-positive cells for late apoptosis, and PI-positive for the percentage of necrosis). The data presented are means ± SD. (ns: not significant, * p-value < 0.05, ** p-value < 0.01 and *** p-value < 0.001).