

## Immunomodulatory effects of ionizing radiation on peripheral blood mononuclear cells

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### ABSTRACT

#### ► Original article

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**Background:** Avoiding exposure to ionizing radiation due to environmental factors is almost inevitable in daily life. Here, we aimed to investigate the possible immunomodulatory effects of ionizing radiation on NK and T cell activation using Peripheral Blood Mononuclear Cells (PBMC). We measured the pro-inflammatory cytokines INF $\gamma$ , IL-2 and TNF $\alpha$ , as well as Granzyme B. In addition, we determined the expression levels of CD28, NKG2D (CD314) receptors, which play a key role in the activation of T and NK cells, respectively. **Materials and Methods:** 20 ml peripheral blood samples were taken from healthy volunteer donors and exposed to radiation doses of 0, 1, 3 and 5 Gy. ELISA analysis was used to measure Granzyme B, INF $\gamma$ , TNF $\alpha$  and IL2. Expression of CD28, NKG2D (CD314) receptors was measured by qRT-PCR analysis. Apoptosis and necrosis were measured by AnnexinV/7AAD analysis. Catalase activity was measured using hemolysates from irradiated blood samples. **Results:** Here we show that IR exposure induces necrotic cell death in PBMCs as the main response. IR exposure significantly induced secretions of Granzyme B, TNF $\alpha$ , IL2, and INF $\gamma$  in a dose-dependent manner. In addition, mRNA levels of CD28 and NKG2D expressions were increased by 3 Gy IR exposure, but decreased by 5 Gy, while catalase activity increases with 1 Gy IR treatment, 3 and 5 Gy decreases. **Conclusions:** Our results suggest that not only high doses but even low doses of radiation can modulate the immune response through cytokine secretion and activation of T and NK cell receptors.

### INTRODUCTION

Ionizing radiation (IR) is the energy emitted in the form of particles or electromagnetic waves in order to become stable as a result of the deterioration of its stable structure by itself or by an external effect <sup>(1)</sup>. Currently, IR is applied in diverse fields including medicine, industry, agriculture and research. Thus, the human body may be exposed to environmental IR on a daily basis <sup>(2)</sup>. Environmental IR sources include the foods contaminated with radio-nucleotides, accidents in nuclear power plants or radioactive wastes <sup>(3)</sup>. Additionally, health workers, doctors, hospital workers of radiation technicians or many occupational groups may be exposed to IR in hospitals and/or workplaces such as radiology or radiotherapy, where nuclear medicine techniques are used for diagnosis and treatment, respectively <sup>(4)</sup>. Therefore, it is almost inevitable to avoid IR exposure due to environmental factors <sup>(5)</sup>. Furthermore, exposure to IR can have dangerous adverse effects such as permanent DNA damage and cancer development <sup>(6)</sup>. One of the most important consequences of IR exposure is that it can modulate biological processes in living organisms at the

molecular and cellular levels. IR-induced changes can be short-term or long-term, depending on the type, amount, and duration of ionizing radiation. According to previous studies, the most affected cells exposed to IR are immune system cells <sup>(7)</sup>. Peripheral blood mononuclear cells (PBMC) constitute important portion of the immune system cells in adaptation against pathogens and infections <sup>(8)</sup>. PBMCs can be easily isolated from peripheral blood and represents any type of blood cell with a nucleus including lymphocytes (T cells, B cells, and NK cells), monocytes, and dendritic cells <sup>(9)</sup>. The highest proportion of peripheral blood cells consists of lymphocytes with 70-90%. Lymphocytes consist of 70-85% CD3+ T cells, 5-10% B cells and 5-15% NK cells. Monocytes are found in the peripheral blood at a rate of 10-20%, while dendritic cells are found only at a rate of 1-2% <sup>(9)</sup>. Recent studies suggest that exposure to IR can not only have deleterious effects on immune system cells, but also modulate the activation of the immune response. For example, Sologuren *et al.* (2014) showed that high-dose radiation applications have immunosuppressive effects on immune system cells and low-dose immunomodulatory effects <sup>(10)</sup>. IR was also shown to

affect the cytokine secretion levels of PBMCs which may activate or inhibit stress and co-stimulatory receptors of immune cells including T cells, NK cells and dendritic cells in a dose dependent manner<sup>(11)</sup>. A recent study also suggests that IR may modulate the expression of immune system-related genes<sup>(12)</sup>. Therefore, there is increasing interest in investigating the effects of IR on immune cells, but it is still not fully defined and needs further investigation.

In this study, we aimed to investigate the possible immunomodulatory effects of IR, especially on NK and T cell activation, by measuring the proinflammatory cytokines INF $\gamma$ , IL-2 and TNF $\alpha$ , and Granzyme B. Expression levels of the CD28 receptor, which allows T cells to interact with antigen presenting cells (ASH), and the NKG2D (CD314) receptor, which plays a key role in the recognition of stressed, infected, or malignant transformed cells by NK cells. The effect of ionizing radiation on the secretion of immunomodulatory cytokines as well as on the costimulatory receptor (CD28) and cytotoxic receptor (NKG2D) expressions were demonstrated for the first time in this study.

## MATERIALS AND METHODS

### Ethics statement

Peripheral blood samples were obtained from healthy male (7) and female (3) volunteers (10) of Caucasian origin (25-55 years, median mean 44), and sample size was determined by G power analysis as described in Das et al. (N=10) (table 1)<sup>(13)</sup>, this study complies with all the provisions of the Declaration of Helsinki and was approved by the non-interventional clinical research ethics committee of Aydin Adnan Menderes University Faculty of Medicine, Aydin, Turkey (Ethics Committee April No 2020/ 14-decision 18, date: 01/ 23/2020).

Table 1. G power analysis.

Input Parameters		Output Parameters	
Effect size f	1.0000000	Noncentrality parameter $\lambda$	48.0000000
A err prob	0.05	Critical F	4.4589701
Power (1- $\beta$ err prob)	0.80	Numerator df	2.0000000
Number of groups	4	Denominator df	8.0000000
Number of measures	3	Total sample size	8
Corr among rep measures	0.5	Actual power	0.9994012
Nonsphericity correction $\epsilon$	1		

### Peripheral blood irradiation

All peripheral blood samples (100 ml) collected in the blood bag were divided into 20 ml 50 ml tubes. Each tube containing 20 ml of peripheral blood was exposed to radiation doses of 0, 1, 3 and 5 Gy (Best Theratronic GC 3000, Radiation Source: Cesium-137).

### Peripheral blood mononuclear cell isolation

20 ml of irradiated peripheral blood samples were

isolated with Ficoll Histopaque-1077 (Sigma Aldrich, USA) and centrifuged at 400 g for 30 min. PBMCs were collected at the buffy coat and washed with phosphate buffered saline (PBS) (Gibco, Thermo Fisher Scientific, USA)<sup>(14)</sup>.

### Cell death analysis

Analysis of the apoptosis and necrosis profiles of mononuclear cells collected from irradiated peripheral blood was obtained using Annexin V/7-Amino Actinomycin D [7AAD] assay using the Muse cell analyzer according to the manufacturer's instructions. Quantification of apoptotic and necrotic cells death was also performed with the Muse Cell Analyzer (Millipore, Austin, TX, USA).

After irradiation, blood samples were diluted 1:1 in 50 ml centrifuge tubes with RPMI 1640 (Capricorn Scientific, Germany) medium supplemented with 10% FBS (Capricorn Scientific, Germany) and incubated for 24 hours at 37 °C and 5% CO<sub>2</sub>. Next, blood samples were used for buffy coat collection. Buffy coats were collected by Ficoll Histopaque separation for RNA isolation. Plasma was collected for ELISA analysis, hemolysate was prepared for catalase assay and all samples were stored at -80°C.

### RNA extraction, reverse transcription and gene expression analysis by qRT-PCR

Total RNA was isolated with the "GeneJet RNA Purification Kit" (Thermoscientific, Rockford, IL, USA) according to the manufacturer's instructions. cDNA was synthesized by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). Gene expression levels were measured with Taqman probes for CD28, KLRC4 (CD314) (Applied Biosystems, CA, USA) and for the housekeeping gene control  $\beta$ -actin (Applied Biosystems, CA, USA). Relative mRNA levels were calculated by StepOne Software (Applied Biosystems, CA, USA) based on the 2- $\Delta\Delta CT$  value<sup>(15)</sup>. Relative mRNA expression levels were plotted as arbitrary units in fold change normalized to internal control using the Origin Graph® program.

Primers:

CD28 Forward Primers:

5'-GAGAAGAGCAATGGAACCATTATC-3'

CD28 Revers Primers:

5'-TAGCAAGCCAGGACTCCACCAA-3'

KLRC4 (CD314) Forward Primers:

5'-CAAATGGGCGGTAGGCGTG-3'

KLRC4 (CD314) Revers Primers:

5'-CCAGGTTCCGGCCCTCAC-3'

$\beta$ -actin Forward Primers:

5'-CACCATGGCAATGAGCGGTTC-3'

$\beta$ -actin Revers Primers:

5'-AGGTCTTGCAGATGTCCACGT-3'

### Analysis of cytokines by ELISA (Enzyme linked immunoabsorbent assay)

Blood plasma levels of Granzyme B, INF $\gamma$ , TNF $\alpha$

and IL2 were measured using commercial ELISA kits (Boster Bio, ELISA Kits, Antibodies, Antibody Company, China) according to the manufacturer's instructions. The blood plasma levels of Granzyme B, INF $\gamma$ , TNF $\alpha$  and IL2 were determined by measuring absorbance at 450 nm in a microplate reader. A standard curve is constructed according to the manufacturer's instructions and determined by appropriate accurate regression analysis.

#### Catalase measurement

This measurement was achieved according to the previously published protocol. Briefly, erythrocytes were washed three times with cold 0.9% NaCl solution. Red cells were then resuspended in 0.9% NaCl solution and used for catalase assay. The sample was added to phosphate buffer solution containing 30 mM H<sub>2</sub>O<sub>2</sub> and the absorbance drop at 240 nm was recorded for 2 minutes. Results are given as U/gHb enzyme activity<sup>(16)</sup>. Formula was used to calculate it. Formula:  $2,3/\Delta t \times \log \text{absorbance}_0/\text{absorbance last} \times k$   
Absorbance 0: first absorbance  
Absorbance last: last absorbance  
K: rate constant.

#### Statistical analysis

Statistical analysis was performed using Origin 8.5 Graph software. ANOVA and Tukey analysis were used where indicated (\*p<0.05). Data are expressed as means of three independent experiments  $\pm$  SD (standard deviation).

## RESULTS

#### Radiation induces necrotic cell death in PBMCs

In an attempt to evaluate the effects of IR on NK and T cell activation, peripheral blood samples were initially treated with IR at doses of 1, 3 and 5 Gy, and PBMCs were isolated and analyzed with the Annexin V/7AAD dead cell assay 24 hours after exposure. As shown in figure 1A-B, IR treatment primarily induced necrotic cell death in PBMCs. 1, 3, and 5 Gy IR treatments induced 21.30%, 23.35% and 24% of necrotic cell death, respectively (figure 1A-B) A mild

induction of apoptosis was detected as well as necrosis. Increasing radiation doses caused a gradual increase in necrotic cell death of PBMCs; however, it did not cause a gradual increase in apoptosis.

#### Effect of radiation exposure on cytokines secretion

As next, in order to examine whether increased IR doses influences on cytokine secretion of mononuclear cells, we measured the levels of Granzyme B, INF $\gamma$ , TNF $\alpha$  and IL2 by ELISA utilizing the PBMCs isolated from 1, 3 and 5 Gy IR treated blood samples. As shown in figure 2A-D, in PBMCs Granzyme B, TNF $\alpha$  and IL2 secretions were significantly increased in a dose dependent manner. Likewise a slight increase in INF $\gamma$  secretion of PBMCs in response to 1, 3 and 5 Gy of IR exposure was also detected (figure 2B).

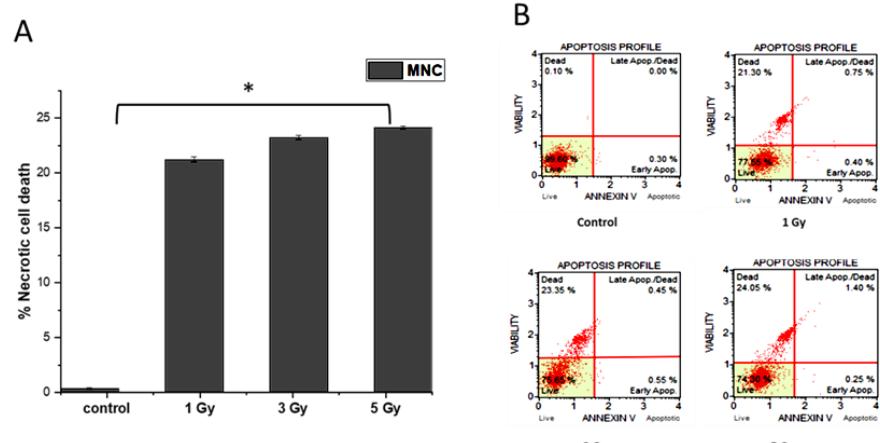
#### Effect of radiation on catalase activity

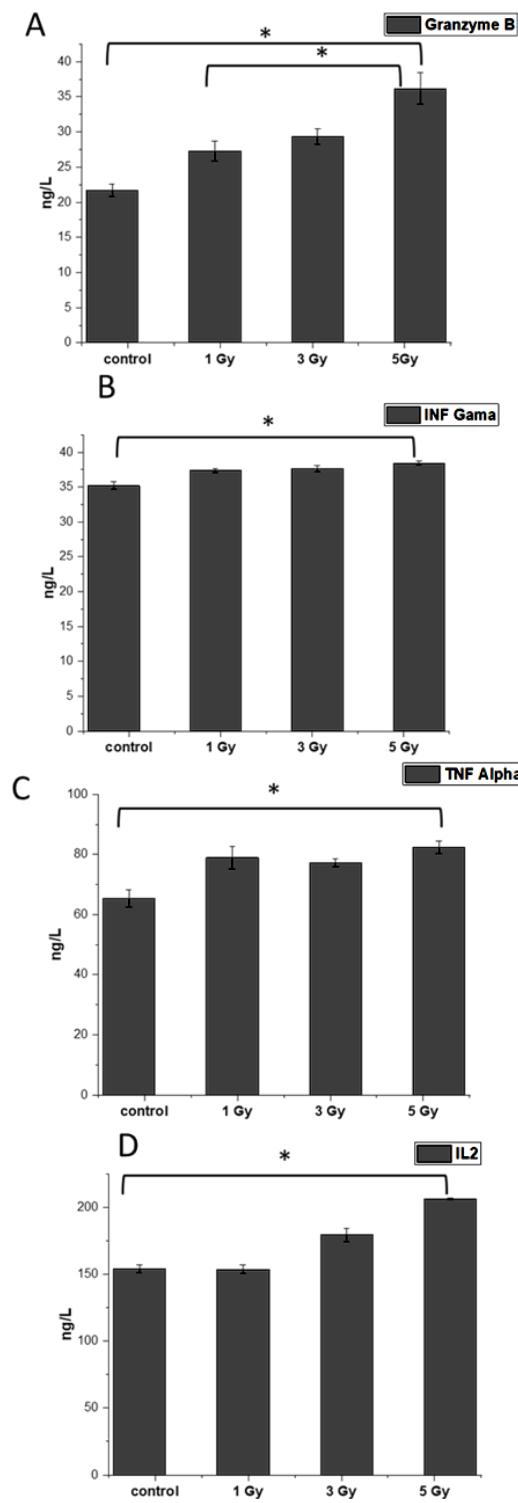
To examine whether increased IR exposure induced a systemic toxic effect, we measured the enzymatic activity of catalase in PBMCs isolated from blood samples treated with 1, 3 and 5 Gy IR. As shown in figure 3, catalase activity was increased in PMBCs treated with 1 Gy IR compared to untreated control samples. Interestingly, catalase activity decreases as increasing doses of IR treatment (3 and 5 Gy) decrease (figure 3).

#### Effect of radiation exposure on mRNA levels of CD314 and CD28 receptors

Next, we measured mRNA expression levels of the cytotoxic receptor NKG2D of NK cells and CD28, the co-stimulatory receptor of T cells, to gain further insight into the effects of increased IR exposure on immune cell activation, specifically T and NK cell activations. PBMCs isolated from 1, 3 and 5 Gy IR-treated blood samples were used for mRNA isolation followed by qRT-PCR analysis.

Our results showed that 1 and 3 Gy IR treatment increased CD314 and CD28 mRNA levels in PBMCs, while 5 Gy decreased (figure 4A-B). As shown in figure 4A-B, CD314 and CD28 mRNA levels increased 2-fold with 3 Gy IR treatment compared to control (p ≤ 0.05).

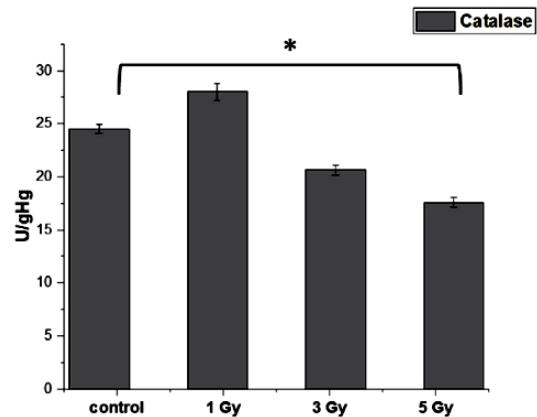




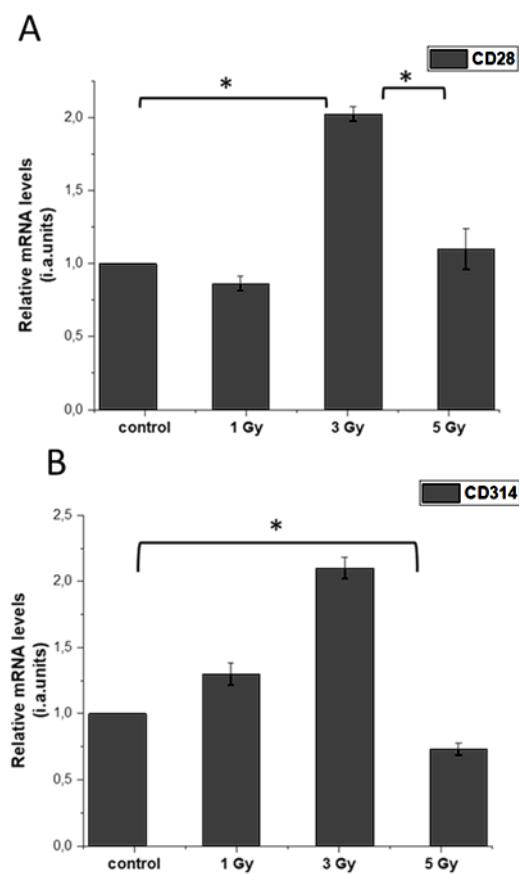
**Figure 2. A, D.** Granzyme B, INFy, TNF $\alpha$  and IL2 levels in PBMCs. ELISA was used to determine Granzyme B, Interferon gamma (INFy), Tumor necrosis faktör alpha and Interleukin 2 (IL2) levels in PBMCs isolated from healthy peripheral blood samples 24 h after exposure to 0 (control) 1,3,5 Gy of IR. ng/L (\*p<0.05)

## DISCUSSION

Daily IR exposure is almost inevitable due to both working conditions and natural radiation. Exposure to IR can induce permanent DNA damage, which can trigger a variety of cellular responses, including cell



**Figure 3.** Catalase activity in PBMCs. Catalase activity was measured in hemolysate samples of PBMCs isolated from healthy peripheral blood samples 24 h after exposure to 0 (control) 1,3,5 Gy of IR. Activity express as per gram hemoglobin (\*p<0.05).



**Figure 4. A, B.** mRNA expressions of CD28 and NKG2D receptors in PBMCs. qRT-PCR analysis was achieved to assess receptors expressions in PBMCs isolated from healthy peripheral blood samples 24 h after exposure to 0 (control) 1,3,5 Gy of IR. mRNA levels were expressed as fold increase in arbitrary units normalized to control. (\*p<0.05).

death (10). Available data suggest that the effects of low or high dose IR exposure may vary depending on cell types. Of these cell types, immune system cells are the cells most affected by IR treatment (10,17). In this study in order to better understand the effects of varying doses of IR exposure on immune cell

activation, mononuclear cells were isolated from peripheral blood samples and exposed to 1, 3, and 5 Gy doses of IR followed by cell death analysis, cytokine release assay, and antioxidant activity tests. In addition, CD28 and CD314 receptor expressions were also analyzed.

Here, we detected that 1, 3 and 5 Gy of IR mainly induces necrotic cell death in PBMCs 24 h after exposure. Previous studies have shown that IR exposure can induce both necrotic and apoptotic cell death in immune cells<sup>(18,19)</sup>. For example, Falcke *et al.* showed that a single dose of IR ( $\geq 2$  Gy) exposure induced necrotic cell death primarily in peripheral blood lymphocytes<sup>(17)</sup>. However, another study showed that low dose of radiation treatment induces a relative maximum of apoptosis in PBMC in the dose range between 0.3 Gy and 0.7 Gy<sup>(20)</sup>. Hence, in our study the main reason underlying the induction of necrotic cell death in PBMC might be the use of higher doses of IR than that of other studies (figure 1A-B).

Secretion of proinflammatory cytokines such as IL-2, IFN- $\gamma$  and TNF- $\alpha$ , and activation of Granzyme B are particularly important in defining T-cell and NK-cell responses<sup>(22)</sup>. Therefore, we examined whether the secretion of IL-2, IFN- $\gamma$ , and TNF- $\alpha$ , as well as Granzyme B activation is modulated by IR exposure, and IR exposure is associated with PBMC's secretion. Here we showed that IR exposure significantly increased the levels of TNF $\alpha$ , IL2 and INF $\gamma$  (figure 2A-D). In a previous study, low-dose (0.05 Gy) IR exposure was shown to increase IL-2, IFN- $\gamma$  and TNF- $\alpha$  levels in human PBMCs obtained from healthy donors<sup>(23)</sup>. Conversely, another study conducted with extremely high doses (5 to 150 Gy) of IR exposure showed that cytokine secretion of PBMCs are impaired by use of 50 Gy dose of IR or beyond<sup>(24)</sup>.

In our study, we also measured the endogenous antioxidant enzyme "catalase" activity. The enzyme activity increased with as low as 1 Gy of IR dose, after 24 h exposure to radiation in PBMCs, whereas decreased with 3 Gy or 5 Gy. However, in a previous study catalase activity was shown to increase with as low as 3 Gy of IR after 48-72 exposure to radiation<sup>(21)</sup> (figure 3). The difference between previous data and ours may be due to the use of different time intervals to measure catalase activity after cells are exposed to IR. In our study, catalase activity was measured 24 hours after exposure to IR, whereas in the previous study, the measurement was made 48-72 hours after exposure. Another possible explanation might be the impairment of the antioxidant response of red blood cells due to high-dose IR exposure which accordingly results in decreased catalase activity. Currently, our data are limited and more research is needed to further clarify the effects of IR exposure on antioxidant response.

CD28 is an important receptor involved in T cell activation by mediating stimulation of dendritic cells

and T cell interaction via co-stimulatory molecules such as CD80 and CD86<sup>(25)</sup>. We further validated our findings by showing increase of CD28 expression with 1 and 3 Gy doses of IR exposure which was further decreased with 5 Gy (figure 4A). In a previous study, Silveira *et al.* showed that 300 cGy (3 Gy) IR exposure increased CD28 expression in peripheral blood lymphocyte but decreased it by 600 cGy (6 Gy)<sup>(12)</sup>. Our data are in agreement with previous data confirming that 1-3 Gy IR exposure stimulates the activation of T cells at the molecular level. Following, we investigated the effect of IR on NKG2D receptor expression in order to shed light on immunomodulatory effects of IR exposure. Our data confirmed that 1-3 Gy of IR exposure increased NKG2D receptor expression in PBMCs (figure 4B). A previous study has also shown radiation therapy increases the expression of the NKG2D ligand in a dose-dependent manner<sup>(26)</sup>. Since NK cell activity is regulated by a complex balance between receptors on NK cells and their corresponding ligands on tumor cells it is particularly important to investigate on the receptor expression in response to IR treatment. Thus, our data provide important evidence by showing 3 Gy of IR exposure increases NKG2D expression in PBMCs. These data suggest that increasing doses of IR exposure may induce T and NK cell activated immune response through increased CD28, NKG2D expression and cytokine release.

## CONCLUSION

This study demonstrates that exposure to IR affects the expression levels of immune- receptors, the release of immunomodulatory cytokines, and the cytotoxic response in PMBCs in a dose dependent manner. Our results suggest that, even low doses of radiation exposure may modulate immune response via inducing T and NK cell activation.

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**Conflicts of interests:** The authors declare that they have no conflict of interest.

**Author contribution:** AK, NO, MKE and BD designed the study, performed statistical analysis and prepared the manuscript.

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