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EFFECT OF SUBLETHAL AND NON-LETHAL DOSES OF IONIZING RADIATION IN IFN-GAMMA KNOCKOUT MICE

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ABSTRACT

Immune interferon (interferon-γ, IFN-γ) is an important pro-inflammatory cytokine, wich presents augmented expression post-irradiation events due to tissue destruction and disseminated necrosis, observed mainly at hematopoietic sites. Blockade of IFN-γ would act as an accessory treatment of accidentally irradiated patients. IFN-γ knockout (GKO) mice were irradiated at non-lethal (4Gy) and sublethal (8Gy) doses and sacrificed by respiratory anaesthesia (CO_2) on days 2nd, 4th and 8th after irradiation events. Two microliters of tail blood were used to perform manual counts of circulating erythrocytes and platelets. Spleen and bone marrow cell suspensions were submitted to flow cytometry (FC) analysis to determine frequency of hematopoietic progenitor (HPC) CD34$^+$ cells located at main hematopoietic tissues. Increased counts of HPC were observed on day 2th in bone marrow samples (~4-fold at 4Gy and ~3-fold at 8Gy) and spleen (~3-fold at 4Gy, ~1-fold at 8Gy). CD34$^+$ cells are still present in spleen on day 4th after irradiation. These data showed the importance of IFN-γ in acute irradiation syndrome and may indicated its inactivation as a promising coadjuvant in hematopoietic recuperation protocols.

1. INTRODUCTION

Treatment and diagnosis using ionizing radiation (IR) had been important and very useful practices, with increasing usage in medical centers. The increasing in its use is accompanied by increasing of operational risks, leading to new studies of its effects in humans and subsequent development of new types of treatment of both accidentally or therapeutically exposed people. One of the most important (and most acute) effects of exposure to IR is hematopoietic syndrome, leading to bone marrow depletion, caused by direct destruction of hematopoietic stem cells (HSC`s) (1) and by disseminated inflammation at hematopoietic sites, due to necrosis (2). Inflammation can be upregulated by an interferon-gamma (IFN-γ) cascade, with macrophage activation and NO release to extracellular environment (3). These events constitute a very aggressive panorama, what may lead HSC populations to death (4), preventing from hematopoietic recovery. Blockade of IFN-γ activities (or its synthesis) may be a promising treatment candidate for accidentally exposed people, ranging from patients to workers.

2. MATERIALS AND METHODS

2.1. Experimental groups and irradiation procedures

All procedures were performed according to principles of animal welfare elsewhere described (5). Groups of male six-week old C57Bl/6j mice obtained from our colony, (Centro de Bioterismo da Fac.Medicina da Universidade de São Paulo – USP) and maintained at our own facilities, were γ-irradiated in a ^{60}Co panoramic source (Yoshizawa Kiko Co.) at the Center of Radiation Technology (IPEN/CNEN – SP) at 0, 4 or 8Gy (rate: 11,24 kGy/h), at 70cm from source and behind a 70% attenuator. Animals were immobilized in PVC capsules (6cm length, 2cm diameter) during irradiation events to prevent from heterogeneous exposure due to movimentation of mice during the event. Groups of interferon-gamma knockout mice (C57Bl/6j IFN-γ $^{-/-}$, GKO $^{-/-}$), acquired from Isogenic Mice Breeding Facility of Institute of Biomedical Sciences of University of Sao Paulo (ICB-USP), and were irradiated using same procedures. On days 2nd, 4th and 8th of experiment, tail blood was collected from specimens to perform counts (hematocytometer chamber) of total number of red blood cells and platelets, and mice were sacrificed by CO_2 inhalation to provide bone marrow and spleen cell suspensions.

2.2. Hematopoietic cell suspensions used to Flow Cytometry Analysis

Spleens were asseptically removed and dissociated by mechanical disruption and sucessive, but gentle pippeting in PBS + 10% Fetal Bovine Serum (FBS)+ 5mM EDTA (pH 7,4). Cell suspension were passed through a Ficoll® cushion during centrifugation step (700 x g, 30min, RT) to collect polimorfonuclear fractions and mantained in same saline solution placed in ice until processing. Femurs were removed and internally flushed with PBS+FBS 10%+EDTA 5mM to dettach stem cells from bone cavitities. Suspensions were centrifuged as described. Cells from all samples (spleen and bone marrow) were counted and adjusted to 4x10^5 cells/mL in a total volume of 200µL, and reacted against monoclonal rat anti-mouse CD34 – phycoerythrin (MEC 14.7, Santa Cruz Biotechnologies) for 30 minutes on ice in dark. After this step, cells were suspended with cold EtOH 70% (800µL) and mantained at -20ºC until flow cytometry experiments. Acquisition of 50000 events was performed using FACSCalibur (BD Biosciences) and analysis using Summit Software (DAKO Cytomation). CD34$^+$ counts were acquired using gates shown in Fig.1. The positive capture gate was delineated using side scatter and log of fluorescence using a modified version of the Milan-Mulhouse protocol elsewhere described (6) and briefly detailed in Fig.1.

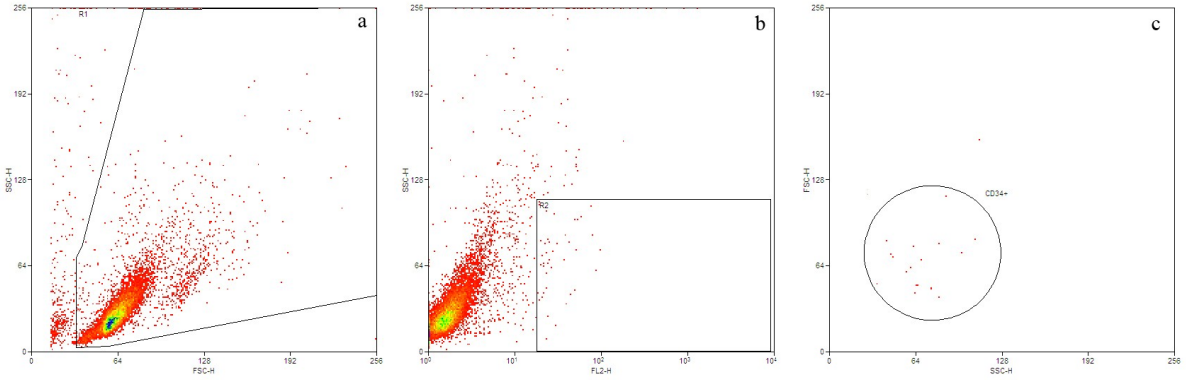

Figure 1: Example of used gates. (a) R1 containing integer cells, excluding debris. (b) R2 containing cells with lower side-scater values and higher fluorescence log values. (c) Final result, indicating positive cell population. The figure shows sample from a non-irradiated control.

3. RESULTS

Blood cell counts (red cells and platelets) of normal and knockout mice were distributed by dose and shown in Fig. 2 (4Gy) and 3 (8Gy).

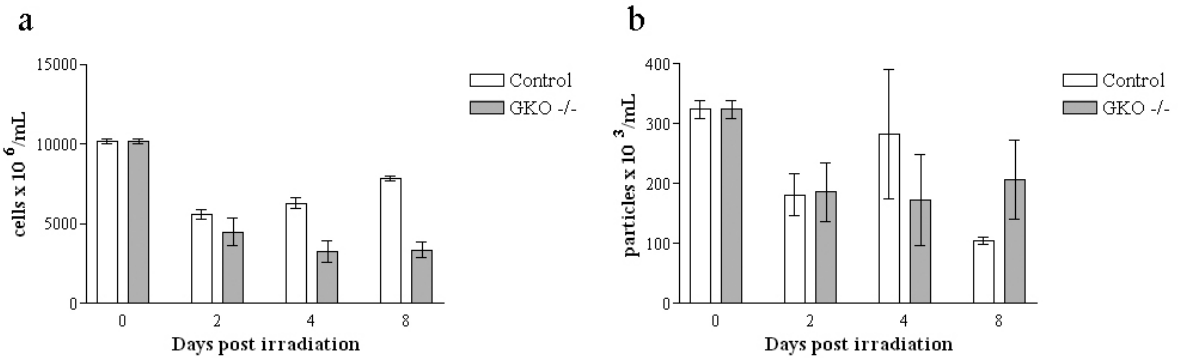

Figure 2: Variation of number of erythrocytes (a) and platelets (b) of peripheral blood in control wild-type and knockout mice irradiated at 4Gy.

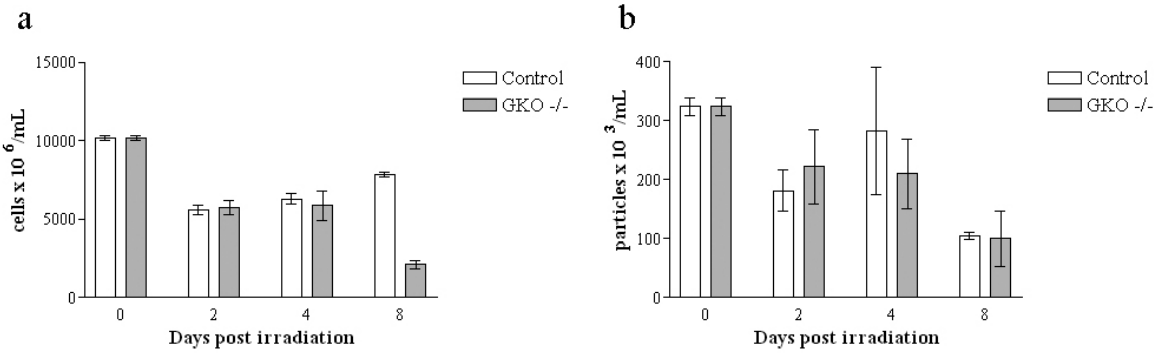

Figure 3: Variation of number of erythrocytes (a) and platelets (b) of peripheral blood in control wild-type and knockout mice irradiated at 8Gy.

Relative results of $CD34^{+}$ were given in percentage proportional to the absolute number of events acquired using controls. Fig. 4 shows results on spleen cells and Fig. 5 on bone marrow suspensions.

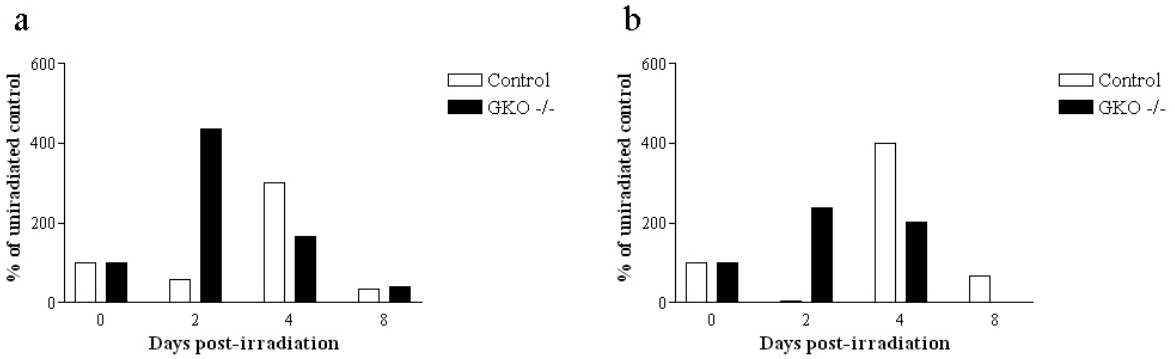

Figure 4: Variation of relative number of CD34$^+$ cells in spleen cells of control wild-type and knockout mice. (a) 4Gy. (b) 8Gy.

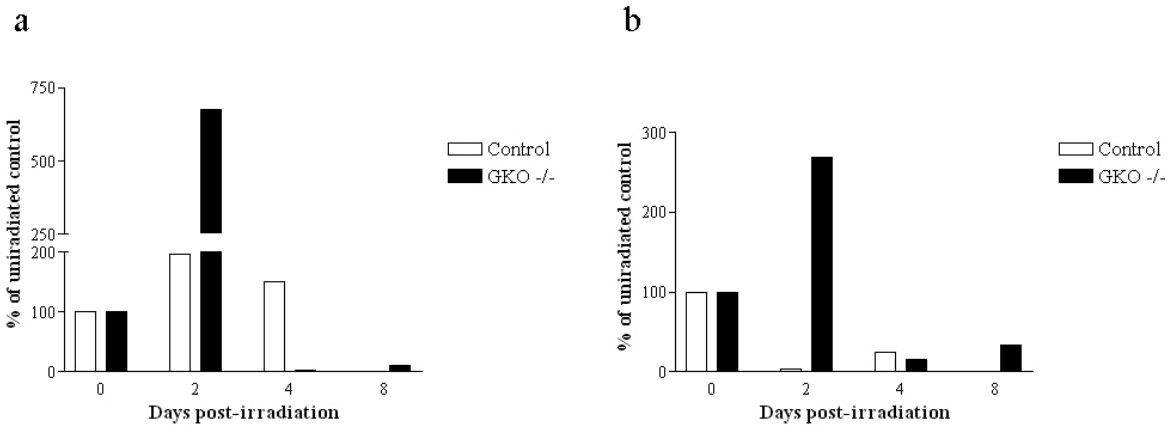

Figure 5: Variation of relative number of CD34$^+$ cells in bone marrow cells of control wild-type and knockout mice. (a) 4Gy. (b) 8Gy.

Control wild-type mice showed similar counts of red blood cells (RBC), at all tested doses comparing with knockout mice. Differences were observed on days 4th (4Gy) and 8th post-irradiation (8Gy), when w.t. (wild-type) controls showed higher counts (Fig 2a and Fig 3a) .Platelet counts were similar among tested groups, with remarkable difference on day 8th (4Gy), when k.o. mice showed higher counts (Fig. 2b and 3b). CD34$^+$ counts were higher on day 2nd (all doses) and lower on day 4th in k.o. mice. (Fig 4a and 4b). No cells were detected at day 8th, 8Gy in spleens of k.o. group. In bone marrow suspensions, was observed high increase at day 2nd p.i. , followed by decrease on day 4th(all doses). At day 8th, only samples from GKO$^{-/-}$ group showed CD34$^+$ cells in analysed samples.

4. CONCLUSIONS

Radiation-induced macrophage activity is probably the most important inflammatory event in accidentally or experimentally exposed, due to high radioresistance of macrophages (~100Gy) and activation of an auto-induced nitric oxide (NO) - IFN-γ cascade (7). Previous experiments showed that inhibition of NO production (data not shown) increases the prevalence of CD34$^+$ cells in hematopoietic organs. Experiments using GKO$^{-/-}$ mice were another form to determine whether these cascades are important to hematopoietic recovery from radiation exposure. Absence of statistical difference between blood counts among

control and test groups may suggest that, even improving the chemotaxis of HSC`s to hematopoietic organs after radiation exposure (8), the inhibition (or absence) of fully functional IFN-γ does not seem to have influence in classical hematologic parameters. This fact may suggest that the restablishment of normal levels of blood cellularity must involve another pathways comitted with cytokine and/or adhesion molecules expression (9, 10, 11, 12, 13) and/or with some specific HSC populations (14, 15, 16). Expected disseminated radiation damage in splenic cell populations (17) was not observed in test-group mice. Inversely, on day 2nd after radiation, samples from BM and spleen presented very high counts of $CD34^+$ comparing to controls in all tested doses, showing a possible improvement of compensation mechanisms after tissue destruction by IR (18). Surface expression of the sialomucin $CD34^+$ (19) is one of the most important and directly targeted parameter to evaluate homing of HSC`s to hematopoietic tissues, and inhibition of pro-inflammatory cytokines (20) may be a suitable method to change the traffic (21) of the HSC`s to hematopoietic sites. Results show that lack of IFN-γ increases the amount of homing of HSC`s at hematopoietic organs.

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