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Low-dose Gamma-rays and Simulated Solar Particle Event Protons Modify Splenocyte Gene and Cytokine Expression Patterns

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Ionizing radiation/Space flight/Lymphocytes/Immune system.

The goal was to investigate the T helper (Th) response in splenocytes of mice exposed to low-dose/low-dose-rate (LDR) γ-rays, simulated solar particle event protons (sSPE), or combination of both. C57BL/6 mice were exposed to LDR γ-radiation (^{57}Co) to a total dose of 0.05 Gray (Gy) at 0.024 cGy/h, either with or without subsequent exposure to 2 Gy sSPE protons. Expression of genes related to Th cells was evaluated immediately after exposure (day 0). On day 21, intra- and extracellular cytokine production was assessed after activation with anti-CD3 monoclonal antibodies (mAb) or phorbol 12-myristate 13-acetate/ionophore (PMA/I). Five genes were significantly modulated on day 0 in one or more of the irradiated groups compared to controls ($p < 0.05$): *Ccl11*, *Ccr5*, *Cd80*, *Inha*, and *Il9*. On day 21, numbers of cells positive for interferon-γ were high in the LDR + sSPE group versus 0 Gy and LDR γ-rays ($p < 0.05$), but there was no difference in IL-2 and TNF-α. Levels of secreted cytokines after anti-CD3 mAb activation were high for 5 (MIP-1α, GM-CSF, IFN-γ, TNF-α, IL-13) and low for 2 (IL-7, IL-9) in all irradiated groups. Priming with LDR photons had a significant effect on IFN-γ and IL-17 compared to sSPE protons alone; IL-2 was low only in the LDR + sSPE group. The cytokine patterns after anti-PMA/I activation were different compared to anti-CD3 mAb and with fewer differences among groups. The data show that total-body exposure to space-relevant radiation has profound effects on Th cell status and that priming with LDR γ-rays can in some cases modulate the response to sSPE.

INTRODUCTION

The impact of all aspects of the space flight environment on astronaut health must be considered in risk assessment models. Space travel, in general, has been shown to be immunosuppressive using a variety of endpoints including: reactivation of previous infections with Herpes simplex and Epstein-Barr viruses,[1,2] inhibition of mitogen-induced blastogenesis in leukocytes,[3–5] and decreases in the ability of leukocytes to produce interferon (IFN)-α/β.[6] Numerous additional immune abnormalities have been reported in leukocyte populations from humans after space flight, including changes in cytokine production and natural killer (NK) cell activity.[7–9] Delayed hypersensitivity skin test response, an immunological phenomenon under the control of T cells, can apparently also be inhibited by both short-term and long-term space flights.[10,11]

The mechanisms responsible for these changes are still undergoing considerable debate. However, exposure to radiation likely plays a significant role. Astronauts are exposed to increased levels of low-dose/low-dose-rate (LDR) radiation on virtually all missions, including residence on board the International Space Station (ISS). For mission profiles which extend beyond Low Earth Orbit (LEO), astronauts are exposed not only to the LDR radiation associated with galactic cosmic rays (GCR), but could also be exposed to relatively high doses of radiation during solar particle events (SPE).

Not surprisingly, recent studies using simulated SPE (sSPE) alone, or in combination with protracted low-dose exposure, have shown a variety of effects on immune and other body systems.[12–16] Furthermore, these studies and others suggest that T cells are among the cells that are at greatest risk. As these cells are vital for execution and regulation of both cell-mediated and innate immune processes, a clear understanding of the impact of radiation on this population is critical for predicting and successfully managing risk.

Although fortuitous from the research point of view, there are regrettably numerous terrestrial correlates for space radiation studies. T cell aberrations appear to be especially com-

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mon. For example, studies of atomic bomb survivors have shown significant decreases in mature T lymphocytes, T helper (Th) cells (although not T cytotoxic, B or NK cells),[17] frequency of T cells secreting type 1 cytokines,[18] T cell reactivity to mitogens,[19] and T cell response to allogeneic cells.[20] Signs of T cell impairment, including increased reactivation of the potentially oncogenic Epstein-Barr virus, have been reported in these individuals.[21–23] Additional data in support of defective T cell activities come from studies of people exposed to fallout from the nuclear power plant accident at Chernobyl.[24,25] However, an increasing number of reports indicate that exposure to LDR radiation may activate protective mechanisms against a subsequent high-dose radiation event. This process, also known as radioadaptation or hormesis, has been recently reviewed by Vaiserman.[26] In some studies, the protective mechanisms have included enhancement of lymphocyte functions.[27]

Although very important, most ground-based studies that include LDR radiation have focused on DNA damage/repair or gene expression alone.[28–32] It is the proteins, however, that are responsible for the biological effects; this is particularly true for cytokines. Collectively, cytokines are involved not only in immune responses, but also in hematopoiesis, wound healing, angiogenesis, and many other biological processes. Radiation/oxidative stress can alter the overall pattern of secreted cytokines, which are an integral part of host defense mechanisms and maintenance of homeostasis. Cytokines can activate (or in some cases inhibit) most immune populations, including T cells, B cells, and macrophages and influence target cell mobility, differentiation, and proliferation. For instance, $CD4^+$ (cluster of differentiation 4^+) T cells can be further differentiated into Th1, Th2, Th17 or T regulatory (Treg) cell lineages, depending on the local cytokine environment.[33] In turn, cytokines secreted by the Th subsets can activate or inhibit other surrounding immunocytes.[34] resulting in a complex, feedback-driven immunocyte-cytokine network. Studying a broad range of cytokines gives more comprehensive information than previously available.

We recently evaluated $CD4^+$ T cells negatively isolated from spleens of mice exposed to LDR γ-rays, either with or without sSPE protons.[35] The LDR and sSPE doses were 0.01 Gy and 1.7 Gy, respectively. After $CD4^+$ T cell activation with anti-CD3 antibody, three cytokines in supernatants and five survival/signaling proteins (total and active forms) in cell lysates were evaluated. The most striking radiation effects were noted on day 21 post-irradiation and included: LDR γ-ray induced "normalization" of the high sSPE proton effect on lymphocyte specific protein kinase (Lck; total & active), increased production of interleukin-2 (IL-2) and IL-4 and decreased level of transforming growth factor-β1 (TGF-β1) in the LDR + sSPE group compared to sSPE alone.[35] In the present study, using higher LDR and sSPE doses (0.05 Gy γ-rays and 2 Gy sSPE protons, respectively), the impact of space-relevant radiation on the expression of 84 genes and 22 cytokines directly involved in immune responses of the Th1, Th2, and Th3 subsets were analyzed. Cytokine production by splenocytes was evaluated using two different cell activation techniques. The overall hypothesis was that protracted exposure to LDR γ-rays would modify the effect of sSPE protons on the pattern of at least some cytokines secreted by cells residing in the spleen, a major source of leukocytes that function in both humoral and cell-mediated immunity.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (n = 64), purchased from Charles River Laboratories (Hollister, CA) at 8–9 weeks of age, were housed in standard size, plastic cages (BioZone VentiRack™ BioZone, Inc., Fort Mill, SC) at a maximum of 8/cage. Standard vivarium conditions were maintained and there was free access to food and water. After 1–2 weeks of acclimatization, the mice were assigned to four groups: a) 0 Gy control, b) LDR, c) sSPE, and d) LDR + sSPE. Euthanasia was performed with 100% CO_2 according to recommendations of the National Institute of Health and the panel on Euthanasia of the American Veterinary Medical Association. The Loma Linda University (LLU) Institutional Animal Care and Use Committee approved this study.

Low-dose/low-dose-rate (LDR) photon irradiation

For LDR γ-irradiation, ^{57}Co plates (185 MBq activity; AEA Technology, Burlington, MA) were placed underneath the mouse cages (1 plate/2 cages in BioZone rack) and a total dose of 0.05 Gy was delivered at a rate of 0.024 cGy/hr over a period of 8–9 days. Cylindrical thimble ionization chambers were used for dose calibration, as recommended by the National Institute of Standards and Technology.

Simulated solar particle event (sSPE) proton irradiation

Mice were transported to the proton research room at LLU Medical Center and placed singly into plastic cubicles within cages of the BioZone VentiRack™. The aerated cubicles met the minimum size requirement for housing mice longer than 24 h and allowed easy movement. The mice were housed singly so that they would not shield each other during irradiations (e.g., during social activities such as sleeping), thus ensuring a consistent dose among individual mice. A total dose of 2 Gy protons was delivered over 36 h in 10 MeV energy increments (25 to 215 MeV). Dosimetry and details of the beam delivery system have been previously published.[36]

Gene expression analysis

On day 0 (1–2 h after radiation), spleens of non-irradiated

and irradiated mice (n = 3-4 mice/group) were harvested and quick-frozen in liquid nitrogen. RNA extraction and further analyses were performed at the SABiosciences service core in Frederick, MD, USA. Gene expression was evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) using the Mouse Th1-Th2-Th3 RT^2 Profiler^{TM} kit (SuperArray Biosciences, Fredrick, MD, USA). This array profiles the expression of 84 genes related to the three major subsets of CD4$^+$ helper T cells. The details of the method have been described previously.[37,38]

Body mass, relative spleen mass, and spleen processing for cytokines

Mice and spleens were weighed on day 21 post-irradiation. Spleen mass relative to body mass (RSM) was calculated: RSM = spleen mass (mg)/body mass (g). For intracellular and secreted cytokines, spleens were homogenized into single-celled suspensions with sterile applicator sticks and filtered to remove debris. Leukocyte counts were obtained using an automated analyzer (ABC Vet Hematology Analyzer; HESKA, Waukesha, WI, USA).

Intracellular cytokine staining

A BD intracellular cytokine kit (BD Biosciences, Bedford, MA, USA) was used for intracellular labeling of IFN-γ, IL-2, and tumor necrosis factor-α (TNF-α) according to the manufacturer's protocol. Briefly, splenocytes at 1-2 × 10^6/ml were plated into 6-well plates with each well containing a total volume of 6 ml. Cells were incubated for 4 h with leukocyte activation cocktail, harvested, and washed. After suspension in stain buffer at the concentration of 2 × 10^7/ml, the activated cells were dispensed into 96-well plates (50 µl/well), fixed, and permeabilized for subsequent intracellular staining. Purified blocking antibody cocktail, phycoerythrin (PE)-conjugated isotype control antibody cocktail and PE-conjugated antibodies against each of the three cytokines were added to their respective wells. Contents were then transferred to a correspondingly numbered tube using 200 µl of BD Pharmingen stain buffer and the final volume was brought to 400 µl using the same stain buffer. Data were then acquired and analyzed using flow cytometry (FACSCalibur^{TM}; Becton Dickinson, Inc.).

Activation of splenocytes with anti-CD3 mAb

Spleen cells were diluted to 2 × 10^6/ml using complete Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich Co., St. Louis, MO, USA). Aliquots of 100 µl were then dispensed into 96-well plates coated with immobilized anti-CD3 monoclonal antibody (mAb) for T cell activation (BD Biosciences). Total volume was 200 µl/well after adding another aliquot of 100 µl complete RPMI medium. Supernatant was harvested after 48 h of incubation at 37°C and frozen for later analysis.

Activation of splenocytes with PMA/I

Single-celled suspensions of spleen leukocytes were diluted in the complete RPMI 1640 medium to 2 × 10^6/ml. Aliquots of 100 µl were then plated in 96-well plates. Phorbol 12-myristate 13-acetate (PMA) at the concentration of 50 ng/ml and ionomycin (I) at 500 ng/ml were added (both from Sigma-Aldrich). The total volume of 200 µl/well was again achieved by adding additional 100 µl of the complete RPMI 1640 medium. Supernatant was harvested after 48 h of incubation at 37°C and frozen until analysis.

Quantification of secreted cytokines by multiplex

Cytokines were quantified in the supernatants harvested from splenocytes activated with either anti-CD3 mAb or PMA/I by using a 22-plex mouse cytokine/chemokine kit from Millipore (Billerica, MA, USA) according to the manufacturer's protocol. Data were acquired using the Luminex 100 system and analyzed with Masterplex QT® software (Linco Research, Inc., St. Charles, MO, USA).

Statistical analysis

Gene expression data (fold-change compared to 0 Gy) were evaluated using Student's *t* test. For the other data, one-way analysis of variance (ANOVA) was used and, when indicated, post-hoc Tukey's test was utilized for pair-wise multiple comparisons to identify significant differences ($P < 0.05$) between individual groups (SigmaStat^{TM} software, version 2.03, SPSS Inc., Chicago, IL, USA). Data are presented as mean and standard error of mean (SEM).

RESULTS

Gene expression

Table 1 presents the significantly up-/down-regulated genes at the end of irradiation on day 0 compared to the 0 Gy control group ($p < 0.05$). Of the 84 genes evaluated, enhanced expression was evident in *Ccl11* (sSPE group), *Cd80* (LDR and LDR + sSPE groups), and *Inha* (LDR

Table 1. Significantly up-down-regulated genes at end of irradiation (day 0).

Gene	LDR	sSPE	LDR + sSPE	Official name
Ccl11	–2.00	1.66	--	Chemokine (C-C) motif ligand 11
Ccr5	1.74	--	--	Chemokine (C-C) motif receptor 5
Cd80	2.24	--	2.67	CD antigen 80
Il9	--	--	–4.76	Interleukin 9
Inha	3.10	--	--	Inhibin alpha

Numbers indicate fold-change compared to 0 Gy control group ($p < 0.05$). LDR: low-dose/low-dose rate γ-rays; sSPE: simulated solar particle event protons. The "--" indicates no statistically significant difference from 0 Gy.

Table 2. Body and relative spleen mass on day 21 post-irradiation.

Groups	Body mass (g)	Relative spleen mass
Control	22.2 ± 0.4	39.7 ± 1.4
LDR	22.7 ± 0.5	41.7 ± 1.1[a]
sSPE	22.9 ± 0.4	36.7 ± 1.2
LDR + sSPE	21.7 ± 0.3	38.0 ± 1.2

LDR: low-dose/low-dose-rate γ-rays; sSPE: simulated solar particle event protons; Relative spleen mass (RSM) = spleen mass (mg)/body mass (g). [a]$P < 0.05$ versus sSPE.

group); reduced expression was noted in *Ccl11* (LDR group) and *Il9* (LDR + sSPE group).

Body mass and relative spleen mass

These data are shown in Table 2. On day 21, there were no significant differences in body mass among groups. The RSM, however, was significantly increased in the LDR γ-irradiated, but only when compared the sSPE protons alone ($p < 0.05$).

Intracellular cytokine staining

Splenocytes from mice exposed to radiation were activated by PMA/I and then treated with brefelidin A so the cytokines were trapped in the Golgi apparatus. Cells were then incubated with phycoerythrin (PE)-conjugated antibodies against IFN-γ, IL-2 and TNF-α and analyzed using flow cytometry. Figure 1 shows the number of cells staining intracellularly for each of the three cytokines. There was a significant increase in cells positive for IFN-γ in the LDR + sSPE group compared to the 0 Gy and LDR groups ($p < 0.05$). Number of cells stained for IL-2 and TNF-α showed no significant differences among groups.

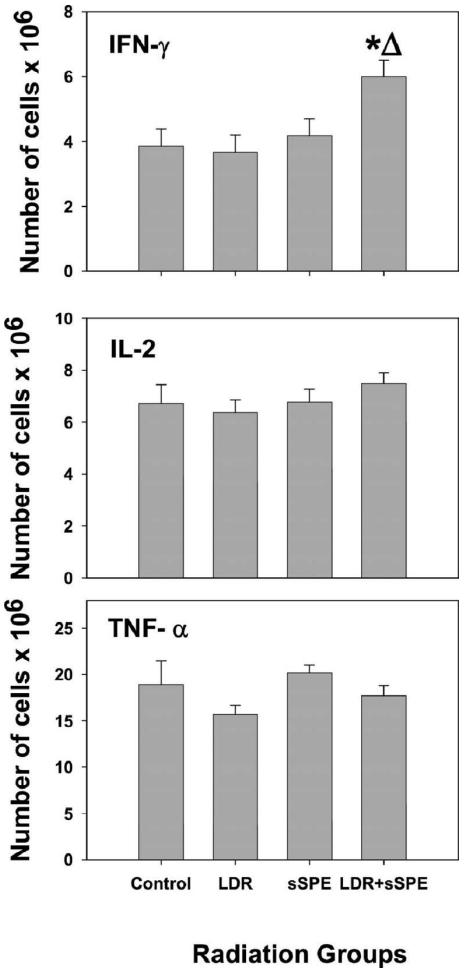

Fig. 1. Number of splenocytes expressing intracellular cytokines on day 21 post-irradiation. Activation cocktail that included PMA/I and a golgi apparatus blocker were used and the cells were quantified using flow cytometry. Values represent mean ± SEM for n = 12–13 mice/group. *$p < 0.05$ vs. 0 Gy Control; Δ$p < 0.05$ vs. LDR. IFN: interferon; IL: interleukin; TNF: tumor necrosis factor.

Secreted cytokine concentrations after splenocyte activation

Figure 2 shows that concentrations of MIP-1α, GM-CSF, IFN-γ, TNF-α, and IL-13 activated by anti-CD3 mAb were significantly increased in all irradiated groups compared to 0 Gy control ($p < 0.001$). However, after PMA/I activation, different patterns were observed. MIP-1α was decreased in the LDR and LDR + sSPE groups and IFN-γ and TNF-α were decreased in the LDR group ($p < 0.005$ vs. 0 Gy); GM-CSF and IL-13 were unaffected

Figure 3 shows that radiation exposure, regardless of regimen, resulted in significantly decreased production of IL-7 and IL-9 after anti-CD3 mAb activation ($p < 0.005$). Low levels of IL-9 also occurred in all three irradiated groups after PMA/I activation, whereas the concentration of IL-7 was below detectable levels in all groups.

Cytokines that generally showed high dependence on radiation regimen are presented in Fig. 4. After anti-CD3 mAb activation, RANTES was increased in sSPE and LDR + sSPE groups ($p < 0.05$ and $p < 0.005$, respectively), MCP-1 was low in the LDR group ($p < 0.05$ vs. sSPE), IL-2 was low compared to 0 Gy and sSPE ($p < 0.005$ for both), and IL-17 was high in the sSPE group ($p < 0.005$ vs 0 Gy and LDR + sSPE). After PMA/I activation, RANTES was low in all irradiated groups ($p < 0.005$) and MCP-1 was increased in the LDR + sSPE group ($p < 0.05$), but there was no radiation effect on IL-2 and IL-17 concentrations.

None of the radiation regimes had any effect on the concentrations of IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-15, G-CSF, IP-10 (IFN-gamma-inducible protein 10), and KC (keratinocyte chemoattractant) (data not shown).

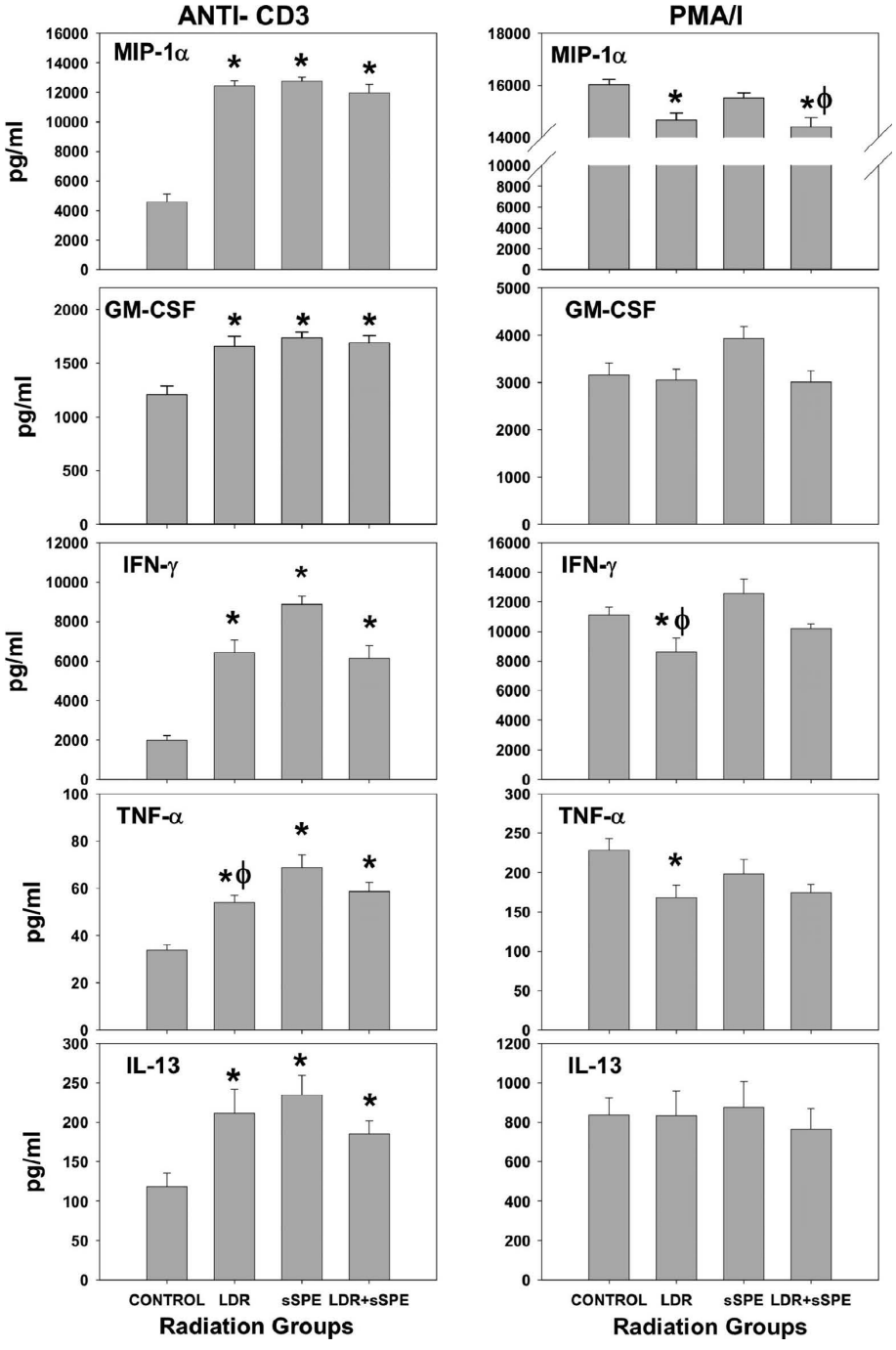

Fig. 2. Increased cytokines in splenocyte supernatants in all irradiated groups after anti-CD3 monoclonal antibody (anti-CD3 mAb) activation on day 21 post-exposure. Levels of the same cytokines obtained after phorbol 12-myristate 13-acetate/ionomycin (PMA/I) activation are shown in the right panels. Cytokines were quantified using the Luminex 100 system. Values represent mean ± SEM for 12–13 mice/group. $^{*}p < 0.005$ vs. 0 Gy Control, $\phi p < 0.005$ vs. sSPE. MIP: macrophage inflammatory protein; GM-CSF: granulocyte-macrophage colony stimulating factor; IFN: interferon; TNF: tumor necrosis factor; IL: interleukin.

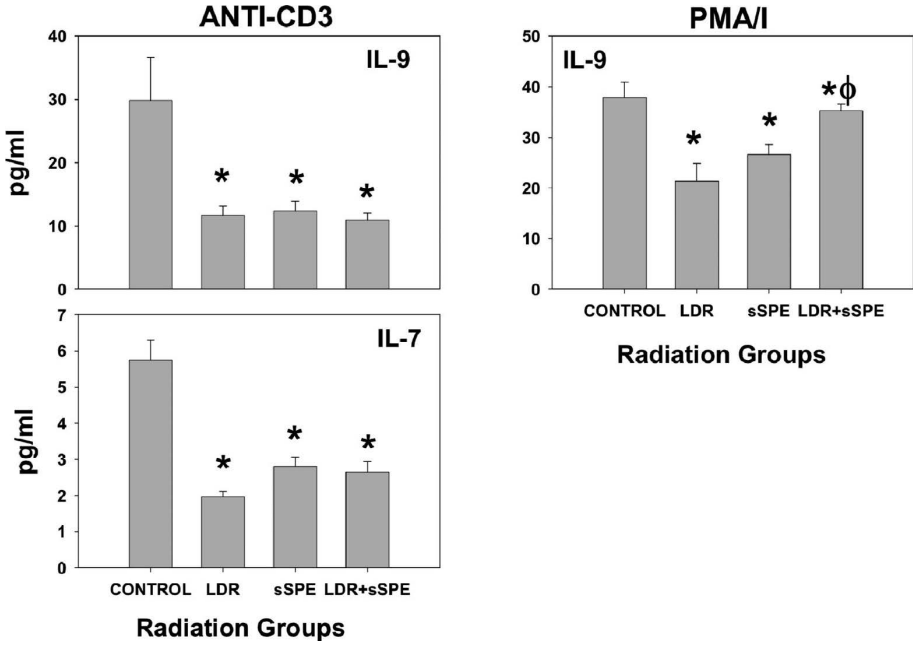

Fig. 3. Decreased cytokines in splenocyte supernatants in all irradiated groups after anti-CD3 monoclonal antibody (anti-CD3 mAb) activation on day 21 post-exposure. The level of the IL-9 obtained after phorbol 12-myristate 13-acetate/ionomycin (PMA/I) activation is shown in the right panels; IL-7 was below the level of detection in all groups after PMA/I. Cytokines were quantified using the Luminex 100 system. Values represent mean ± SEM for 12–13 mice/group. $^{\star}p < 0.005$ vs. Control, $\phi p < 0.005$ vs. sSPE. IL: interleukin.

DISCUSSION

Gene analysis was performed on spleens collected within 1–2 h after irradiation (day 0) to determine if there were immediate effects related directly to Th lymphocyte functions. Of the 84 genes analyzed, significant changes were noted in five, and the radiation regimen had a strong influence on whether an effect was induced. Two of these were chemokine genes, *Ccl11* and *Ccr5*, related to leukocyte trafficking. The expression of *Ccl11*, encoding a protein known as eotaxin, was decreased in spleens from the LDR γ-irradiated group, increased in the sSPE proton-irradiated group, and unaffected in the group receiving LDR + sSPE radiation. Eotaxin facilitates eosinophil migration and has been associated with allergies such as asthma[39] and inflammatory bowel disease.[40] The lack of enhancement of the gene encoding eotaxin in the LDR + sSPE group, when enhancement did occur after sSPE alone, suggests that low-dose priming may decrease risk for eosinophil-associated pathologies.

Expression of the other chemokine gene, *Ccr5*, was significantly enhanced only in LDR γ-irradiated mice. The *Ccr5* gene codes for CD195, a receptor that binds to several different ligands, thus facilitating migration of $CD34^+$ progenitors during hematopoiesis and directing antigen-presenting dendritic cells and memory Th1 cells to sites of tissue damage.[41,42] Interestingly, this gene is also involved in recruitment of $Foxp3^+$ Treg cells that prevent chronic and/or excessive inflammation.[43] These characteristics of *Ccr5* suggest that LDR exposure may enhance hematopoiesis and result in efficient repair of tissue damage with decreased risk for serious pro-inflammatory side effects.

Cd80 gene expression was up-regulated in mice exposed to LDR photons, either with or without sSPE irradiation, but not sSPE protons alone. The gene codes for CD80 (also known as B7.1) that is present on dendritic cells and activated monocytes. CD80 provides an essential co-stimulatory signal for T cell activation during antigen presentation.[44] Thus, the findings suggest that low-dose background irradiation may result in more efficient presentation of antigens to T cells, perhaps resulting in some degree of enhanced protection against infectious agents when SPE-induced depression in white blood cell (WBC) counts occurs.

The *Inha* gene, encoding inhibin alpha (inhibin A), was up-regulated in the LDR group, but not in the sSPE and LDR + sSPE groups. Inhibins are members of the TGF-β (transforming growth factor-β) superfamily that have been studied for decades in the context of reproductive endocrinology. However, inhibin A is now known to affect differentiation, proliferation, and apoptosis in a variety of cell types.[45] Especially pertinent are reports that it enhances T cell maturation.[46] Hence, the increased expression of the *Inha* gene in the LDR group may be an early signal for increased pro-

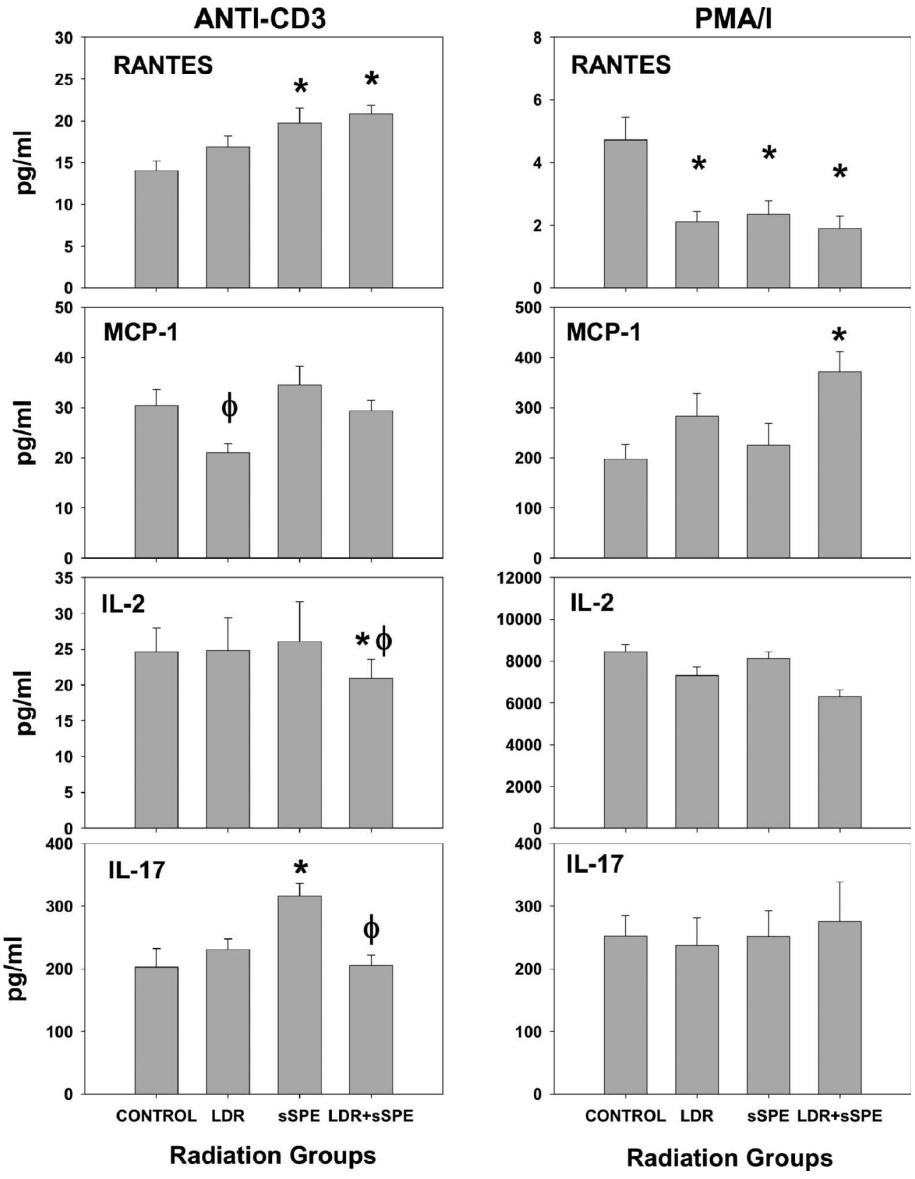

Fig. 4. Cytokines/Chemokines in splenocyte supernatants in all irradiated groups after activation with monoclonal anti-CD3 antibodies or Phorbol 12-myristate 13-acetate/ Ionomycin (PMA/I). Cytokine concentrations were quantified using the Luminex 100 system. Values represent mean ± SEM for 12–13 mice/group. Tukey: $^{*}p < 0.005$ vs. Control, $\phi p < 0.005$ vs. sSPE. RANTES: regulated upon activation, normal T-cell expressed and secreted; MCP: monocyte chemoattractant protein; IL: interleukin.

duction of T cells. In our previous studies of LDR photon effects, exposure to low doses resulted in high WBC counts in the spleen.[37] This type of response could be related to inhibin A and interpreted as radioprotective. However, our previous study also showed that the *Inha* gene was down-regulated in $CD4^+$ T cells isolated from spleens,[37] indicating that the enhancement in *Inha* expression noted here was likely occurring in cells other than $CD4^+$ T lymphocytes. Modulatory effects of inhibin A on cytokines include inhibition of IFN-γ and IL-12 production by Th1 and dendritic cells and enhanced production of IL-4 and IL-10 by Th2 cells.[47,48]

The *Il9* gene for IL-9 was significantly down-regulated only in the LDR + sSPE group. Although IL-9 is important in mucosal immunity, it is also strongly linked to development of asthma, chronic allergic inflammation, and other similar diseases.[49–51] Over-expression of IL-9 has also been reported to facilitate cell transformation and tumorigenesis, e.g., thymic lymphoma in mice and Hodgkin's disease in humans.[52] Hence, its down-regulation in the combination group could reflect a protective response against pathologies

such as hypersensitivity reactions and/or cell transformation that may eventually lead to cancer.

In addition to determining immediate post-irradiation gene effects, another goal was to determine if early (day 0) changes correlated with intracellular and extracellular cytokine production that was quantified later on day 21 post-exposure. The above-described changes in gene expression generally did not predict cytokine production at the later time point. This was not entirely surprising, since our previous studies have shown that gene patterns are highly time-dependent after protracted exposure to 0.01 Gy and 0.1 Gy doses of γ-rays.[37,38)]

On day 21, intracellular expression of three cytokines associated with the Th1 (IFN-γ, IL-2) and Th2 (TNF-α) subsets was evaluated. The number of splenocytes positive for IFN-γ was increased in the combination group (LDR + sSPE) compared to 0 Gy and LDR photons, while numerical values for IL-2 and TNF-α positive cells were similar among all groups. IFN-γ has numerous immunoregulatory activities, including promotion of Th1 cell differentiation by up-regulating transcription factor T-bet;[53)] antiviral and antitumor properties are also prominent.[54)] It has been reported that peripheral blood mononuclear cells isolated from astronauts after space missions have a lower number of $CD4^+$ (but not $CD8^+$) T cells intracellularly stained for IFN-γ; decreases in $CD3^+$, $CD4^+$, and $CD8^+$ T cells stained for IL-2 were also noted.[55)] These results were obtained after mitogen-induced activation and the investigators state that their findings may not be due to factors during flight, but instead due to the stress of landing followed by efforts to regain homeostasis.

Two different methods were implemented to quantify splenocyte capacity to secrete cytokines. The purpose was to determine whether the radiation regimens differentially affected signaling pathways that lead to cytokine secretion. Anti-CD3 mAb was used to mimic activation that occurs during antigen presentation *in vivo* via engagement of the T cell receptor complex (TCR/CD3) and PMA/I, essentially a non-specific mitogen, was used to activate cells while bypassing TCR/CD3 engagement.

The concentration of IFN-γ was increased on day 21 in supernatants of anti-CD3 mAb-activated cells from all three irradiated groups. The enhancing effect was most pronounced with sSPE protons, resulting in a significantly higher level than in the LDR and LDR + sSPE groups. The pattern after PMA/I activation was strikingly different; supernatants from the LDR group had decreased IFN-γ and levels in the sSPE and LDR + sSPE groups were similar to 0 Gy. The pattern for level of secreted IFN-γ is also different compared to the number of cells positive for intracellular IFN-γ even though PMA/I was used for activation in both assessments (Figs. 1 and 2). This discrepancy suggests that the molecular mechanisms involved in synthesis versus secretion capacity for IFN-γ were differentially affected by radiation. In addition, although the number of cells staining positive for intracellular IFN-γ were significantly increased in the LDR + sSPE group, this did not translate into a high level of the cytokine in the supernatant. These findings are not entirely surprising, since radiation may have altered the function of components involved in cytokine synthesis and secretion. Previous findings suggest that protein tyrosine kinase activity and reactive oxygen species are needed for radiation-mediated intracellular signaling that leads to cytokine production.[56)] More recently, Anderson *et al*. in a study of inflammatory arthritis in mice, demonstrated that level of TNF-α secreted by macrophages in response to lipopolysaccharide activation was dependent on genes encoding tristetraprolin (TTP) and T cell intracellular antigen-1 (TIA-1) that decrease levels of inflammatory cytokines by promoting mRNA degradation and protein translation, respectively.[57)] Thus, radiation-induced changes in either TTP or TIA-1 status, as well as protein tyrosine kinase activity and oxygen radicals, could certainly influence levels of secreted cytokines. Overall, we found that IFN-γ levels after PMA/I activation were much higher than after anti-CD3 mAb, likely due to production of this cytokine by multiple cell types, e.g., Th1, T cytotoxic, and NK cells. IFN-γ, a major cytokine with roles in both innate and adaptive immunity, is especially important in defense against microbes, as well as tumor control.[58)] We have previously noted increased IFN-γ production with anti-CD3 mAb-activated splenocytes from mice shortly after return from a mission on the Space Shuttle Endeavour.[59)]

TNF-α levels in supernatants followed essentially the same pattern described above for IFN-γ, i.e., enhancement in all irradiated groups after anti-CD3 mAb activation and depression in the LDR irradiated group after PMA/I activation. Also similarly to IFN-γ, the patterns for secreted TNF-α and the number of cells positive for the cytokine intracellularly did not correlate well, again suggesting that the radiation regimens may have had a differential effect on production versus secretion capabilities. TNF-α is secreted by the Th1 and Th2 subsets, neutrophils, NK cells, endothelium, and mast cells. It induces antitumor activity, is cytotoxic for many types of transformed cells, but also participates in development of septic shock.[60)] Up-regulation of TNF-α after radiation exposure has been reported by other investigators as well.[61)]

MIP-1α (macrophage inflammatory protein 1 alpha), one of the molecules to which CD195 (protein product of the *Ccr5* gene discussed above) binds, was increased in all irradiated groups when cells were activated by anti-CD3 mAb. However, the amount produced was less than with PMA/I. This may be at least partly explained by the fact that PMA/I can activate both T cells and macrophages while anti-CD3 mAb activates only T cells. Exposure to LDR photons, alone or in combination with sSPE, decreased the production of MIP-1α by PMA/I-activated cells, a pattern also noted for the *Ccr* gene. Since MIP-1α can inhibit hematopoietic stem cell proliferation, this could be advantageous, i.e., with

decreased MIP-1α production regeneration of WBC would be facilitated. Indeed, use of MIP-1α analogs in pre-clinical studies has resulted in enhanced leukocyte recruitment during tumor radiotherapy, as well as improved tumor control.[62]

RANTES (regulated upon activation, normal T-cell expressed and secreted), another target for the product of the *Ccr5* gene, plays an important role in T lymphocyte recruitment and inflammation. Similarly to MIP-1α, RANTES was increased in all irradiated groups when cells were activated by anti-CD3 mAb, but decreased after PMA/I. Cell culture studies by other investigators have demonstrated that exposure to 2 Gy X-rays up-regulates RANTES production.[63]

MCP-1 (monocyte chemoattractant protein-1) is a chemokine that recruits monocytes, T lymphocytes, eosinophils, and basophils to sites of tissue damage. Its expression has been shown to increase after radiation, resulting in inflammation.[43] Given that this chemokine is secreted by cells of the monocyte-macrophage lineage, and not by T cells, there was less of it produced when cells were activated by anti-CD3 mAb compared to PMA/I. After PMA/I activation, MCP-1 concentration was high in supernatants from the LDR + sSPE group (but not sSPE group), indicating that pre-exposure to LDR photons resulted in enhancement. This result is consistent with a shift towards the Th2 type of immune response, as it has been shown that MCP-1 drives T cell commitment towards the Th2 subset by directly enhancing IL-4 production.[64]

The level of GM-CSF in supernatants of anti-CD3 mAb-activated splenocytes were significantly increased in all irradiated groups, an effect not seen with PMA/I. Overall, the amount produced was greater with PMA/I than with anti-CD3 mAb. These data support the possibility that even the very low dose of 0.05 Gy increased the need to regenerate cells of the granulocytic and monocytic lineages. GM-CSF is frequently used following radiation accidents to facilitate recovery of hematopoietic function.[65]

The different radiation regimens had no effect on the level of IL-2 after anti-CD3 MAb activation. However, the cytokine was significantly decreased after PMA/I activation in the LDR + sSPE group. This suggests that the dose of 0.05 Gy was not radioprotective, but instead may be harmful if there is a subsequent SPE. However, it should be noted that the level of IL-2 produced after anti-CD3 mAb activation was 300-fold less than the amount produced when cells were activated with PMA/I. This could mean that a signal from CD3 alone was not enough to activate the cells to full potential, e.g., additional signals may be needed from surrounding macrophages activated by PMA/I. IL-2 and its receptor (IL-2R) are mainly produced by Th1 cells when they are stimulated by antigen. Binding of IL-2 with its receptor leads to clonal proliferation of T cells. NK cells, B cells, cytotoxic T cells and macrophages can be activated by IL-2, and IL-2 is also necessary for Treg cell development in the thymus. Radiation can compromise T cell-mediated immunity by severely decreasing production of IL-2.[61,66,67]

There was a significant increase in IL-13 in supernatants of splenocytes activated by anti-CD3 mAb in all three irradiated groups on day 21. However, after PMA/I activation, there were no significant differences. The increase in IL-13 concentration after anti-CD3 mAb could again be explained by the fact that this cytokine can be secreted by a variety of cell types. Hence, the data suggest that exposure of LDR, sSPE or LDR + sSPE could induce an allergic inflammatory response mediated by T cells, especially the Th2 subset,[68] but the effect cancels out with the involvement of other cells.

IL-9 was significantly decreased in all irradiated groups, regardless of anti-CD3 mAb or PMI/I activation. These data indicate that the mechanisms leading to IL-9 synthesis and/or secretion are highly radiosensitive and that pre-exposure to low-dose γ-rays to the total dose of 0.05 Gy did not have any radioprotective effect. These findings were somewhat surprising, since IL-9 is a Th2 cell-derived cytokine and space studies have shown a shift towards a Th2 type immune response.[69]

In conclusion, the data show that total-body exposure to space-relevant radiation induced numerous changes in cytokine production capacity, as well as early gene expression. Overall, radiation had a greater effect on cytokine levels when cells were activated with anti-CD3 mAb compared to PMA/I, suggesting greater radiosensitivity of the CD3/TCR signaling pathway. In some cases, priming with LDR γ-rays modified the effect of sSPE protons alone. Body mass, a sensitive indicator of overall health, was similar among all groups. Finally, it must be stressed that although many radiation-induced changes were noted that certainly could have meaningful immunological/biological effects, it is not possible at this time to make definitive conclusions. Further studies are obviously needed to determine if the observed changes have a significant impact on disease risk.

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