Low Dose, Low Dose Rate Photon Radiation Modifies Leukocyte Distribution and Gene Expression in CD4+ T Cells

Daila S. GRIDLEY1,2*, Asma RIZVI2, Xian LUO-Owen1, Adeola Y. MAKINDE2 and Michael J. PECAUT1,2

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A better understanding of low dose radiation effects is needed to accurately estimate health risks. In this study, C57BL/6 mice were γ-irradiated to total doses of 0, 0.01, 0.05, and 0.1 Gy (57Co; ~0.02 cGy/h). Subsets per group were euthanized at the end of irradiation (day 0) and on days 4 and 21 thereafter. Relative spleen mass and splenic white blood cell (WBC) counts, major leukocyte populations, and spontaneous DNA synthesis were consistently higher in the irradiated groups on day 0 compared to 0 Gy controls, although significance was not always obtained. In the spleen, all three major leukocyte types were significantly elevated on day 0 (P < 0.05). By day 21 post-irradiation the T, B, and natural killer (NK) cell counts, as well as CD4+ T cells and CD4:CD8 T cell ratio, were low especially in the 0.01 Gy group. Although blood analyses showed no significant differences in leukocyte counts or red blood cell and platelet characteristics, the total T cells, CD4+ T cells, and NK cells were increased by day 21 after 0.01 Gy (P < 0.05). Gene analysis of CD4+ T cells negatively isolated from spleens on day 0 after 0.1 Gy showed significantly enhanced expression of Il27 and Tcfcp2, whereas Inha and Socs5 were down-regulated by 0.01 Gy and 0.1 Gy, respectively (P < 0.05). A trend for enhancement was noted in two additional genes (Il1r1 and Tbx21) in the 0.1 Gy group (P < 0.1). The data show that protracted low dose photons had dose- and time-dependent effects on CD4+ T cells after whole-body exposure.

INTRODUCTION

Accidents such as those at Three Mile Island and Chernobyl, as well as threats of “dirty bomb” detonation by terrorists, have led to increased public awareness of the potential dangers of radiation.1) Radiation exposure above internationally accepted background levels also occurs in certain occupations, e.g., space flight, airline industry, nuclear power plants, and at nuclear medicine facilities. Although less recognized by the general public, normal tissues in cancer patients receiving radiotherapy are invariably irradiated to some degree. According to estimates of the U.S. Environmental Protection Agency, the background dose per person averages only about 0.36 cSv per year,2) and radiation workers generally receive less than 5 cSv/year.3) For crewmembers on the International Space Station, Cucinotta and colleagues have recently estimated an average effective dose of approximately 72 mSv.4) It is well established that exposure to sufficiently high doses of radiation can lead to necrotic or apoptotic cell death. Indeed, numerous studies over the last several decades have demonstrated that radiation causes chromosomal and DNA aberrations that can be lethal. However, the effects of prolonged exposure to low-dose/low-dose-rate radiation have been more difficult to detect and are still not yet clearly understood.

Cells of the immune system are among the most highly radiosensitive cells in the body and many studies have demonstrated that CD4+ T helper (Th) lymphocytes are especially vulnerable. Our previous results with rodent models exposed to various forms of radiation delivered to total doses of 2–3 Gy have demonstrated a dramatic reduction in T cells.5,11 Some of these studies have included 2 Gy simulated solar particle event (SPE) protons delivered over a 36-h period of time.12 There are several reports that some radiation-induced changes in T cells persist chronically. For example, investigations of atomic bomb survivors have revealed significant decreases in Th cells, but not T cytotoxic (Tc), B, or natural killer (NK) cells.13 Functionally, there were decreases in the frequency of T cells capable of secreting type I cytokines,14 T cell reactivity to mitogens15 and T
cell response to allogeneic cells. Signs of T cell impairment, including increased reactivation of the potentially oncogenic Epstein-Barr virus (EBV) and increased susceptibility to primary viral infections, have been reported in these individuals. Additional support for defective T cell function comes from studies of persons exposed to fallout from the nuclear power plant accident at Chernobyl. Some of these studies have revealed dose-dependent differences in gene expression patterns, with the up-regulated genes being primarily those associated with proliferation and apoptotic death in T cells and monocytes.

Depression or dysfunction of the highly radiosensitive cellular components of the immune system, such as the CD4+ T cells, can lead to serious consequences, including increased risk for cancer. However, there are numerous reports that exposure to low dose radiation can result in radio-adaptation that can be beneficial in that it renders cells more resistant to a subsequent acute radiation event, as well as more resistant to cancer and certain other pathologies. In an interesting recent study, Jiang et al. found that low-dose X-radiation induced an adaptive response in normal cells, but not in tumor cells. Although proposed mechanisms include more efficient DNA repair and stimulated immunity, the underlying mechanisms remain unclear. A better understanding of the biological effects of protracted low dose radiation is needed in order to more accurately predict health risks and perhaps even employ radio-adaptation in certain clinical settings.

The major focus of the present study was to quantify the effects of low dose, low dose rate photons on CD4+ T cells in blood and spleen after total-body exposure in a mouse model. Our working hypothesis was that significant modulation would be induced in distribution of these cells and expression of genes associated with their functional status. We also proposed that at least some of the findings would be consistent with enhancement of the cell-mediated branch of the immune system in which the CD4+ T cells are of paramount importance.

MATERIALS AND METHODS

Animals
A total of 207 C57BL/6 female mice, 8–9 weeks of age, were purchased from Charles River Breeding Laboratories Inc. (Hollister, CA, USA). The animals were housed in large plastic cages (n = 10 maximum/cage) in a self-contained BioZone VentiRack (BioZone, Inc., Fort Mill, SC, USA) under standard vivarium conditions throughout the study. Cage dimensions were 46.5 cm, 15.5 cm, and 26.0 cm for length, height and width, respectively. CO2 euthanasia was performed according to the recommendations of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The Loma Linda University (LLU) Animal Care Facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and adheres to The Animal Welfare Act and Regulations implemented by the United States Department of Agriculture (USDA). The mice were kept under specific pathogen-free (SPF) conditions. This study was approved by the LLU Institutional Animal Care and Use Committee (IACUC) prior to initiation.

Experimental design
The mice were tested individually in two experimental runs. For the great majority of assays, there were 4 groups of mice: a) 0 Gy, b) 0.01 Gy, c) 0.05 Gy, and d) 0.10 Gy. The initiation of radiation exposure was timed so that all irradiated groups reached the total prescribed dose simultaneously. Subsets per group were euthanized on day 0 (within 1–2 h post-irradiation) and on days 4 and 21 thereafter; there were 13–22 mice/group/time point (n = 192 total). Gene expression analysis was done on day 0 and included 15 additional mice (n = 5/group) irradiated with 0 Gy, 0.01 Gy, and 0.1 Gy. Cell culture and processing were done under clean conventional conditions and included use of a class II laminar flow hood. All values obtained were used individually in the statistical analyses described below.

Low dose, low dose rate photon radiation
Irradiation was performed in the LLU Animal Care Facility in a room specifically designed for radiation

Fig. 1. White blood cell counts in spleen and blood. The data were obtained using an automated hematology analyzer. Each bar represents the mean ± SEM (n = 13–22 mice/group/time point). *One-way ANOVA at time of analysis, P < 0.05 for main effect of group.
Fig. 2. Major leukocyte populations in spleen and blood. The data were obtained using an automated hematology analyzer. Each bar represents the mean ± SEM (n = 13–22 mice/group/time point). *One-way ANOVA, P < 0.05 for main effect of group.

Fig. 3. Lymphocyte populations in spleen. The data were obtained using fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean ± SEM (n = 13–22 mice/group/time point). *One-way ANOVA, P < 0.05 for main effect of group.
research and animal housing. Total doses ranging from 0.01 to 0.1 Gy were delivered using $^{57}$Co plates (185 MBq activity; AEA Technology, Burlington, MA, USA) placed immediately beneath the cages (1 plate/2 cages) in a BioZone rack; dose rate was ~0.02 cGy/h. These flood sources are designed for quality control of gamma imaging cameras and provide high spatial uniformity. Dose calibration was performed using several thermoluminescent dosimeters (TLD) per cage; uniformity of dose was ±5%. Mice receiving 0 Gy were handled similarly, but were housed in a different room.

**Body and relative spleen masses**

At the times of euthanasia, mice were weighed and spleens were excised and weighed. Spleen mass relative to body mass (RSM) was calculated as follows: RSM = organ mass (mg)/body mass (g).

**White blood cells (WBC) and major leukocyte types in spleen and blood**

These procedures have been previously described in detail. Briefly, spleens were gently processed into single-celled suspensions with sterile applicator sticks, washed, centrifuged to remove debris, and red blood cells (RBC) were lysed by incubation in 2 ml of lysing buffer for 4 min at 4°C. The spleen leukocytes were then suspended in 2 ml of complete RPMI 1640 medium (Irvine Scientific, Santa Ana, CA, USA) for further analyses. Whole blood was collected in [K$_2$]EDTA-containing syringes by cardiac puncture. An automated analyzer (HESKA™ Vet ABC-Diff Hematology Analyzer, Heska Corp., Waukesha, WI, USA) was used to quantify WBC, lymphocytes, granulocytes, and monocytes-macrophages. For blood, the analyzer also provided mean corpuscular volume (MCV, average RBC volume), mean corpuscular hemoglobin (MCH, average mass of HGB per RBC), mean corpuscular HGB concentration (MCHC, average HGB concentration per RBC), RBC distribution width (RDW, width of RBC based on cell number x cell size), platelet counts, and mean platelet volume (MPV).

**Spontaneous DNA synthesis**

This procedure is described in detail elsewhere. Aliquots of spleen leukocytes and blood were diluted with complete RPMI-1640 medium (Irvine Scientific) and dispensed into 96-well microtiter plates. $^{3}$H-thymidine ($^{3}$H-TdR; specific activity = $1.7 \times 10^{12}$ Bq/μmol; ICN Biochemicals, Costa Mesa, CA, USA) was added at $3.7 \times 10^{4}$ Bq/well and the plates were incubated for 3 h at 37°C in 5% CO$_2$. The counts per minute (cpm), volume tested, and number of WBC/ml were used to obtain cpm/10$^6$ cells.

![Fig. 4. Lymphocyte populations in blood. The data were obtained using fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean ± SEM (n = 13–22 mice/group/time point). *One-way ANOVA, $P < 0.05$ for main effect of group.](image_url)
Lymphocyte populations in spleen and blood

A 2-tube custom-made mixture (Pharmingen, San Diego, CA, USA) containing fluorescence-labeled monoclonal antibodies and a FACSCalibur™ flow cytometer (Becton Dickinson, Inc., San Jose, CA, USA) was used to identify CD3⁺ T, CD4⁺ Th, CD8⁺ Tc, CD19⁺ B, and NK1.1⁺ NK cells. Analysis of 5,000–10,000 events/tube was performed using CellQuest™ software (v3.1, Becton Dickinson). The percentages obtained were used together with cell numbers from the hematology analyzer to obtain numerical data for each lymphocyte population.

Negative isolation of CD4⁺ T cells

The BD™ IMag Mouse CD4 T Lymphocyte Enrichment Set – DM (Cat. No. 558131) and BD™ IMagnet (Cat. no. 552311) were used for negative selection of splenic CD4⁺ T lymphocytes according to the manufacturer’s protocol. This procedure uses monoclonal antibodies that bind to antigens on RBC and all leukocytes except CD8⁻/CD4⁺ T cells. The labeled cells are pulled toward the magnet (positive fraction), leaving the unlabeled cells in suspension so they can be drawn off and retained, thus resulting in a highly enriched suspension of CD4⁺ T cells. The samples were kept cold on wet ice during the entire procedure. CD4⁺ T cell samples were quick frozen at −70°C for gene expression analysis.

Gene expression in splenic CD4⁺ T cells

CD4⁺ T cells negatively isolated on day 0 from mice irradiated with 0 Gy, 0.01 Gy, and 0.1 Gy (n = 5/group) were evaluated for gene expression using RT² Profiler™ PCR Array Mouse Th1-Th2-Th3 (SuperArray Biosciences, Frederick, MD, USA). The details of this procedure have been previously reported. The 84 evaluated genes are associated with T cell-derived cytokines, CD4⁺ T cell markers, the T regulatory (Treg) cell network, T cell activation, and relevant transcription factors. Genes that differed significantly from 0 Gy controls, or for which a trend was observed, were noted.

Statistical analysis

Most of the data were analyzed at each time point using one-way analysis of variance (ANOVA), with the independent variable being radiation-exposure group; Tukey’s test was performed for pair-wise multiple comparisons. Gene expression data (fold-change compared to 0 Gy) were evaluated using Student’s t test. Means and standard errors of the mean (SEM) are presented; results from each mouse were included in the analyses. A P value of < 0.05 indicated significance, whereas P < 0.1 indicated a trend. SigmaStat™ software, version 2.03 (SPSS Inc., Chicago, IL, USA) was used for these analyses.

RESULTS

Body and relative spleen masses (RSM)

The only difference in body mass among groups was noted on day 0; the 0.1 Gy mice were heavier than the 0 Gy animals (21.4 ± 0.3 g vs. 20.1 ± 0.2 g, P < 0.05). Although all irradiated groups had higher RSM on day 0 than the non-irradiated controls (40.5 ± 2.0 to 41.7 ± 2.7 versus 38.9 ± 1.6) and lower RSM on day 4 (38.9 ± 1.3 to 39.4 ± 1.1 versus 40.0 ± 1.8), statistical significance was not obtained at any time point.

WBC and major leukocyte populations in spleen and blood

Figure 1 shows that the WBC counts were higher in the...
spleen on day 0 in all irradiated groups compared to the non-irradiated controls (one-way ANOVA, $P < 0.05$ for main effect of group). Tukey’s test revealed that the 0.1 Gy group had increased numbers ($P < 0.05$), whereas the 0.01 Gy group had a trend ($P < 0.1$). There were no differences among groups in WBC counts in the blood (Fig. 1).

Splenic lymphocytes and monocytes/macrophages were elevated at this early time point, with the 0.01 Gy group having higher numbers than the 0 Gy controls ($P < 0.05$); a trend for increased granulocytes was also noted ($P < 0.1$) (Fig. 2). However, the circulating granulocyte count on day 0 was low in the 0.01 Gy group ($P < 0.05$ vs. 0 Gy) and there was a trend for low granulocytes in the 0.05 Gy group ($P < 0.1$ vs. 0 Gy) (Fig. 2). On days 4 and 21 numbers of all three leukocyte types were similar to the controls in both blood and spleen.

There were no significant differences in the percentages of each major leukocyte population in either body compartment at any of the time points and RBC and platelet characteristics in blood were similar among groups (data not shown).

Table 2. Genes not affected by radiation in CD4+ T cells on day 0

| Gene   | Name and brief description |
|--------|---------------------------|
| Bcl6   | B-cell leukemia/lymphoma 6; negative transcription factor |
| Ccl5   | Chemokine (C-C motif) ligand 5; recruits T cells, eosinophils, basophils |
| Ccl7   | Chemokine (C-C motif) ligand 7; recruits monocytes, macrophages, eosinophils |
| Ccl11  | Small chemokine (C-C motif) ligand 11; recruits eosinophils |
| Ccr2   | Chemokine (C-C motif) receptor 2; receptor for monocyte chemoattractant protein-1 |
| Ccr3   | Chemokine (C-C motif) receptor 3; present on activated memory T cells |
| Ccr4   | Chemokine (C-C motif) receptor 4; present on Th1 & Th2 cells |
| Ccr5   | Chemokine (C-C motif) receptor 5; present on memory/activated Th1 cells |
| Ccr10  | Chemokine (C-C motif) receptor 10; recruits Treg cells to mucosal layers |
| Cda4   | CD4 antigen; co-receptor for Th cell activation during antigen presentation |
| Cda7   | CD27 antigen; member of TNF receptor superfamily; helps regulate B-cell activation |
| Cda8   | CD28 antigen; co-receptor for T cell activation |
| Cda9   | CD40 antigen; member of TNF receptor superfamily; co-stimulating protein |
| Cda10  | CD80 antigen; provides co-stimulatory signal for T cell activation and survival |
| Cda16  | CD86 antigen; provides co-stimulatory signal for T cell activation and survival |
| Cebpb  | CCAAT/enhancer binding protein (C/EBP), beta; transcription factor |
| Crebbp | CREB binding protein; enhances CREB transcriptional activity |
| Csg2   | Colony stimulating factor 2 (granulocyte-macrophage); growth factor |
| Cta4   | Cytotoxic T-lymphocyte-associated protein 4; receptor on Th cells, prevents function |
| Cxcr3  | Chemokine (C-X-C motif) receptor 3; involved in Th1 cell migration |
| Gata3  | GATA binding protein 3; transcription factor; induces Th1, suppresses Th2 pathway |
| Gfi1   | Growth factor independent 1; transcriptional repressor and splicing control factor |
| Icos   | Inducible T-cell co-stimulator; expressed especially on Th2 cells |
| Ifng   | Interferon gamma; Th1-derived multifunctional cytokine; suppresses Th2 activity |
| Igf9f6 | Immunoglobulin superfamily, member 6; Th1-related; down-regulated by activation |
| Il2    | Interleukin 2; stimulates growth, differentiation & survival of Tc cells; needed for development of T cell memory and maturation of Treg cells |
| Il2ra  | Interleukin 2 receptor, alpha chain; part of high-affinity IL2 receptor |
| Il4    | Interleukin 4; Th2 cytokine that promotes development of Th0 cells to Th2 |
| Il4ra  | Interleukin 4 receptor, alpha; binds to IL4 & IL13; regulates IgE production |
| Il5    | Interleukin 5; Th2 cytokine; stimulates antibody production |
| Il6    | Interleukin 6; T cell cytokine with pro- & anti-inflammatory effects |
| Il7    | Interleukin 7; T cell cytokine that acts as hematopoietic growth factor |
| Il9    | Interleukin 9; T cell cytokine that stimulates proliferation and prevents apoptosis |
| Il10   | Interleukin 10; T cell cytokine that down-regulates expression of Th1 cytokines |
| Il12b  | Interleukin 12B; cytokine that functions in Th1 cell development; acts on NK cells |
| Il12rb2| Interleukin 12 receptor, beta 2; helps form high affinity IL12 binding sites |
### Table 2. (Continued.)

| Gene     | Name and brief description                                                                                                                                 |
|----------|----------------------------------------------------------------------------------------------------------------------------------------------------------|
| IL13     | Interleukin 13; Th2 cytokine involved in B cell maturation; up-regulates MHC class II                                                                       |
| IL13ra1  | Interleukin 13 receptor, alpha 1; primary IL13 binding subunit                                                                                               |
| IL15     | Interleukin 15; cytokine that activates T cells; similar to IL2                                                                                              |
| IL17a    | Interleukin 17A; T cell cytokine that regulates activities of NF-κB & MAP kinases                                                                             |
| IL18     | Interleukin 18; Th1 cell pro-inflammatory cytokine; induces IFN-γ production                                                                                |
| IL18bp   | Interleukin 18 binding protein; inhibitor of pro-inflammatory cytokine IL18                                                                               |
| IL18r1   | Interleukin 18 receptor 1; essential for IL18 signal transduction                                                                                           |
| IL23a    | Interleukin 23, alpha subunit p19; associates with IL12B to form IL23                                                                                       |
| IL27ra   | Interleukin 27 receptor, alpha; forms part of IL27 receptor                                                                                                 |
| Ifi1     | Interferon regulatory factor 1; activates IFN-α & -β transcription                                                                                           |
| Ifi4     | Interferon regulatory factor 4; regulates Th2 cytokine production                                                                                             |
| Jak1     | Janus kinase 1; functions in signal transduction pathways                                                                                                |
| Jak2     | Janus kinase 2; functions in signal transduction pathways                                                                                                |
| Jak3     | Janus kinase 3; functions in signal transduction pathways                                                                                                |
| Janb     |Jun-B oncogene; inhibits cell proliferation; induces senescence; is tumor suppressor                                                                     |
| Ma6      | Avian muscle-specific fibrosarcoma (v-maf) A542 oncogene homolog; positive transcription factor that interacts with c-Myb |
| Mapk8    | Mitogen-activated protein kinase 8; functions in immediate-early gene expression                                                                              |
| Mapk9    | Mitogen-activated protein kinase 9; plays key role in T cell differentiation                                                                                |
| Nfatc1   | Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1; transcription factor; member of nuclear factor of activated T cells (NFAT) family  |
| Nfatc2   | Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2; transcription factor; member of nuclear factor of activated T cells (NFAT) family  |
| Nfatc2ip | Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein; has role in inducible expression of T cell cytokine genes    |
| Nfatc3   | Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3; member of nuclear factors of activated T cell DNA-binding transcription complex |
| Nkb1     | Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105; encodes protein which undergoes co-translational processing; results in DNA binding subunit of NF-κB |
| Ptprc    | Protein tyrosine phosphatase, receptor type, C; signaling molecule that regulates many cell processes (growth, differentiation, mitotic cycle, oncogenic transformation) |
| Pcgf2    | Polycomb group ring finger 2; negative regulator of transcription; tumor suppressor                                                                           |
| Sftp4d   | Surfactant associated protein D; member of collectin family; has lectin-like activity                                                                      |
| Socs1    | Suppressor of cytokine signaling 1; negative regulator of cytokine signaling                                                                               |
| Socs3    | Suppressor of cytokine signaling 3; negative regulator of cytokine signaling                                                                               |
| Spp1     | Secreted phosphoprotein 1; enhances production of IFN-γ and IL12; decreases IL10                                                                          |
| Stat1    | Signal transducer and activator of transcription 1; up-regulates type I & II interferons                                                                  |
| Stat4    | Signal transducer and activator of transcription 4; essential for T cell response to IL12; regulates Th cell differentiation                                 |
| Tgfb3    | Transforming growth factor, beta 3; growth factor; key regulator of wound healing                                                                       |
| Tlr4     | Toll-like receptor 4; functions in innate & Th1 immunity                                                                                                  |
| Tlr6     | Toll-like receptor 6; functions in innate & Th1 immunity                                                                                                  |
| Tme1d    | Transmembrane emp24 domain containing 1; interacts with IL1 receptor-like 1                                                                               |
| Tnf      | Tumor necrosis factor; multi-functional cytokine; pro-inflammatory; induces apoptosis                                                                      |
| Tnfrsf4  | Tumor necrosis factor receptor superfamily, member 4; activates NF-κB; suppresses apoptosis                                                                |
| Tnfrsf8  | Tumor necrosis factor receptor superfamily, member 8; helps activate NF-κB                                                                                |
| Tnfrsf9  | Tumor necrosis factor (ligand) superfamily, member 4; provides co-stimulation to T cells                                                                   |
| Tyk2     | Tyrosine kinase 2; implicated in IFN-γ, IL6, IL10 & IL12 signaling                                                                                           |
| Yy1      | YY1 transcription factor; activates & suppresses many diverse promoters                                                                                     |

Information available at www.genecards.org and http://en.wikipedia.org
Lymphocyte populations in spleen and blood

As shown in Figure 3, radiation had a depressive effect on all three lymphocyte populations in the spleen at one or more times of assessment (one-way ANOVA \( P < 0.05 \)). T cell counts were decreased in the 0.05 Gy group on days 4 and 21, B cells were low in the 0.05 Gy group on day 4 and in the 0.01 Gy group on day 21, and NK cells were low in the 0.01 Gy group on day 21 (\( P < 0.05 \) vs. 0 Gy). Further analysis showed that the decrease in T cells was due to low numbers of CD4+ T cells (not CD8+) (Fig. 3). However, a normal CD4:CD8 ratio was generally maintained except on day 21 when the 0.1 Gy group had a higher ratio compared to both of the other irradiated groups (\( P < 0.05 \)) (Fig 3).

Figure 4 shows that T cell counts in blood from the 0.01 Gy group were high on day 21 (\( P < 0.05 \) vs. 0 Gy, \( P < 0.1 \) vs. 0.05 Gy and 0.1 Gy), but B cells were unaffected. NK cells were increased at this same early time point in the 0.01 Gy group (\( P < 0.05 \) vs. 0 Gy). In Figure 4, one can also see that the high T cell count in the 0.01 Gy group on day 21 was primarily due to increased numbers of CD4+ T cells (\( P < 0.05 \) vs. 0 Gy). The CD4:CD8 ratio was similar among groups, except for a trend for group effect on day 0 (one-way ANOVA \( P < 0.1 \)) when all irradiated groups had lower ratios than the 0 Gy controls (Fig. 4).

Spontaneous DNA synthesis in spleen and blood

Basal DNA synthesis in both spleen and blood are shown in Figure 5. There were no significant differences in this measurement in the spleen. However, a significant effect did occur on day 21 in the blood (one-way ANOVA \( P < 0.05 \)), most likely because the 0.01 Gy group had higher cpm values than the 0.05 Gy group.

Gene expression in splenic CD4+ T cells

Table 1 presents the genes that were affected in the CD4+ T cells isolated from the spleen, either significantly or for which a trend was observed. On day 0, there was a significant radiation effect on expression of 4 genes. Il27 and Tcfcp2 were up-regulated in the 0.1 Gy group (\( P < 0.05 \) vs. 0 Gy). A trend for enhanced expression was noted for Il1rl and Tbx2 after 0.1 Gy (\( P < 0.1 \) vs. 0 Gy). Inha was down-regulated in the 0.01 Gy group and Socs5 was down-regulated in the 0.1 Gy group (\( P < 0.05 \) vs. 0 Gy). Genes not affected by radiation are presented in Table 2.

**DISCUSSION**

The results show that differences in body mass among groups were minimal and transient. The slight, but statistically insignificant, changes in spleen mass relative to body mass (RSM) were consistent among all irradiated groups, i.e., high on day 0, low on day 4. These data, together with observation of normal grooming and other activities both during and after irradiation, suggest that overall health was maintained.

Perhaps not surprisingly, results regarding leukocyte populations were not consistent in both body compartments. A striking example is the lack of radiation effect on WBC counts in peripheral blood on day 0, whereas low counts were found in the spleen. The differences may be related to regenerative capacity of the resident populations. Cells circulating in blood reflect the status of stem and progenitor cells in the bone marrow that are routinely involved in hematopoiesis, whereas the major functions of the spleen include removal of senescent red blood cells, as well as other unwanted materials, and assistance in immune defense against infectious agents. Changes in cell migration patterns in response to radiation-induced damage may be another contributing factor. The differences seen in splenic WBC counts (Figs. 1 and 2) in the 0 Gy control mice sacrificed at different time points are likely to reflect the high sensitivity of the immune system to numerous external/internal factors and emphasize the importance of processing control and test animals simultaneously under the same conditions. This difference may be due to day-to-day variations in our automated hematology analyzers. However, it is also possible that this is a stress effect. We attempted to keep the time of euthanization constant across all time points (starting at 0800). To do this on day 0, we had to remove the irradiation plates prior to transporting the animals from the animal care facility to our laboratory. This slight difference in handling may have contributed to the differences in counts. Indeed, inclusion of controls at each time of analysis is very important in all immunological studies to take into account day-to-day variability so that differences, if any, due to a test agent can be detected.

Analysis of lymphocyte populations showed that radiation had a depressive effect on splenic T, B, and NK cell counts at one or more time points of assessment. By day 21, the greatest effect on all three populations was seen in the 0.01 Gy group; the decrease in T cells was due to reduction in the CD4+ subset. The low CD4+ T cell number in the spleen may have triggered homeostatic mechanisms in order to regain a normal balance in the lymphocyte subsets. The elevated CD4+ T cell count in the blood at the same 21-day time point supports this possibility. Loss of lymphocytes, as well as other WBC, results in activation of hematopoietic mechanisms that stimulate self-renewal. The process includes cytokines such as stem cell factor, a protein essential for proliferation and differentiation of stem and progenitor populations. Interleukins, colony-stimulating factors, and other cytokines, of course, also participate. Although the underlying mechanisms remain to be elucidated, it further appears that exposure to 0.01 Gy may have arrested lymphocyte progression through the cell cycle more effectively than either the 0.05 Gy or 0.1 Gy doses. Radiation is well known to arrest cells in the G1/G2 phases so that DNA repair can take place. If successful repair occurs, the cell can proceed.
that long-lasting depletion in the Th1 subset exists concomitantly with excessive proliferation of the CD4+ Th2 subset and that antigenic stimuli may be necessary to reveal radiation-induced immune disturbances. Compared to the large fluctuations seen in lymphocyte populations after acute high dose radiation, the present data demonstrate that perturbations after protracted low dose whole-body irradiation are relatively modest when based on cell counts.

Analysis of gene expression was performed on CD4+ Th cells negatively isolated from spleens of mice on day 0 after irradiation with 0.01 and 0.10 Gy. The altered gene profiles were strikingly different in the two irradiated groups. After 0.01 Gy, there were no up-regulated genes and only Inha was significantly down-regulated. The Inha gene encodes the alpha subunit of inhibin, a member of the transforming growth factor beta (TGF-β) superfamily. Inhibin has been studied mostly in the context of pregnancy, especially as a predictor of successful conception. Its immunomodulatory effects include inhibition of interferon-γ (IFN-γ) and interleukin-12 (IL-12) production by Th1 and dendritic cells and up-regulation of IL-4 and IL-10 production by Th2 cells.

Thus, the down-regulation of Inha suggests that exposure to 0.01 Gy radiation may promote a shift toward the Th1 phenotype and thus also enhance cell-mediated immunity. In contrast, much higher radiation doses have generally been associated with a shift favoring the Th2 subset. Given the association of Inha with the TGF-β superfamily and IFN-γ, it is of interest to note that expression of the Tgfb3 and Ifng genes were not significantly affected in our analyses. In future studies, expression of genes for other members of the TGF-β superfamily such as TGF-β1 and TGF-β2, as well as genes for IFN-γ receptors should be assessed.

There were two significantly up-regulated genes in the group that received the higher dose of 0.10 Gy. The increased Il27 encodes IL-27, a cytokine in the IL-12 family. Although it was first described as a pro-inflammatory cytokine that helps initiate Th1 cell responses, more recent studies have shown that it suppresses IL-2 production and inhibits Th1, Th2, and Th17 cells while expanding the population of inducible regulatory T cells. IL-27 may also play a key role in controlling sepsis by directly suppressing granulocyte production of endotoxin-induced unstable oxygen radicals. Thus, the up-regulation of Il27 seen here may minimize the risk for septic shock, should infection with a Gram-negative bacteria occur. Tcfcp2 was also significantly up-regulated after 0.1 Gy. The CP2 protein derived from this gene is a factor in the nuclear transcription factor (NTF) family that participates in regulation of cell maturation. The factor is also known to be rapidly increased in response to antigenic and mitogenic stimuli and to be a critical trans-activator of IL-4 in Th2 cells.

There were two additional genes that approached significance (P < 0.1) for up-regulation after exposure to 0.1 Gy. Il11r1 encodes the type I IL-1 receptor that is found on the surface of a variety of cell types including T cells. IL-1 is a macrophage-derived pro-inflammatory cytokine that assists in T cell activation. The receptor for IL-1 plays an important role in inflammation by activating nuclear factor-kappaB (NF-kB) and may also be involved in the development of autoinflammatory pathologies and sickness behavior via neuromodulation in the central nervous system. Thbx21 (T-box 21, also known as T-bet/Tbx21) was the other gene with a trend toward significant up-regulation after 0.1 Gy exposure. This gene encodes a transcription factor that activates IFN-γ production in the Th1 subset and promotes development of Th1 cells while inhibiting Th2 cells. Expression of the gene for IFN-γ, however, was not enhanced at the time of analysis in the present study.

Sox5, encoding suppressor of cytokine signaling 5, was the only down-regulated gene in CD4+ T cells from the 0.1 Gy group. The suppressor of cytokine signaling (SOCS) proteins are negative regulators of the JAK-STAT signal transduction pathway that is triggered by many cytokine-receptor interactions. These regulators are crucial in preventing excessive inflammation. The modestly decreased expression in Sox5 suggests that regulation of inflammatory processes may be somewhat compromised in this irradiated group. However, it must be noted that the expression of other genes encoding SOCS proteins (Sox1 and Sox3) was similar to the 0 Gy controls.

Although, to our knowledge, this is the first study of gene expression that has focused specifically on the CD4+ T cell subset after in vivo low dose γ-irradiation, a number of investigators have found changes in lymphoid cell gene expression patterns after exposure to low dose radiation. For example, whole-body irradiation of mice to total doses of 0.2 Gy and 2 Gy by Amundson and colleagues revealed dose-dependent differences in the gene profiles, as well as differences among cells residing in the spleen, thymus, and liver. Sakamoto-Hojo et al. found altered gene expression in human lymphocytes obtained from hospital personnel chronically exposed to low dose radiation. In a more recent study, Fachin and co-workers using cultured human lymphocytes exposed to γ-rays to total doses of 0.1, 0.25,
and 0.5 Gy found that the affected genes were involved in oxidative stress response/DNA repair, metabolism, cell growth/differentiation, and transcription regulation; radiation dose-dependent differences in the genetic profile were also noted.49) The results presented here reflect the biological impact of low-dose, ionizing radiation delivered from a $^{57}$Co source over relatively long periods of time. Co-57 consists of a mixture of gamma photons of various wavelengths and two discrete energies (122 keV and 136 keV) that are essentially equivalent to orthovoltage X-rays. In tissue, they will generate electrons with a broad spectrum of energies ranging from approximately 100 keV down to the ionization threshold (12 eV). Based on published Monte Carlo track structure studies, one can assume that the electron track-ends contribution of Co-57 γ-ray, Co-60 γ-ray, and 250 kV X-ray irradiations, are similar and the relative biological effectiveness of these radiation qualities relative to each other is about 1.0 +/- 10%.50)

Clearly, both the total dose and dose rate of radiation, as well as radiation quality, are important in the biological outcome. Radiation delivered rapidly can kill any cell, if the total dose is high enough. Predictions of health risks for low-dose, low-dose rate radiation have been based primarily on the linear-no-threshold model for decades. However, there are increasing reports that protracted low-dose radiation can result in radioresistance.23,24) In contrast, other studies have found that low-dose/low-dose-rate exposure can kill cells more effectively than high dose-rate irradiation, can sensitize certain cell types to a subsequent high-dose-rate exposure, or have no effect.51) Much research remains to be done to clarify the biological consequences and the underlying mechanisms responsible for the reported effects.

In summary, the data show significant perturbations in CD4+ Th lymphocytes after protracted low dose, whole-body exposure to photons. Radiation dose- and time-dependent changes were evident; overall, greater radiation effects were noted in the spleen than in the blood. Alterations in CD4+ T cell gene expression, although strikingly different in the 0.01 Gy and 0.10 Gy groups support a shift in the Th1/Th2 balance toward the Th1 phenotype, thus suggesting that cell-mediated responses against virus-infected and other aberrant cells may be enhanced. Our long-term goals include studies that facilitate a better understanding of the dynamics of low dose radiation-induced immune modulation and its relationship to the development of disease.

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