Abstract. Introduction: Lipopolysaccharide (LPS) is a major cause of septic shock and death due to infection with Gram-negative bacteria. The purpose of this study was to quantify the effects of whole-body irradiation on lymphocyte populations during response to challenge with LPS. Materials and Methods: C57BL/6 mice (n=10/group) were irradiated whole-body with 3 gray (Gy) γ-rays in a single fraction at 0.8 Gy/min. LPS (E. coli serotype 0111:B4) at 1 mg/kg was injected intraperitoneally 10 days later and mice were euthanized at 60 min and days 1, 7, and 14 post-inoculation for analyses. Results: Significant interactions between radiation and LPS were noted in circulating and splenic lymphocyte subpopulations, including T-, B-, and NK-cells, particularly at the early time points. There were significant interactions on circulating, but not splenic, CD62L+ T-cell populations. However, there were no interactions on CD62L+ B-cells. Finally, there were significant interactions in both early and late blastogenic responses. Conclusion: The data support that response to infection with Gram-negative bacteria may be significantly compromised by exposure to ionizing radiation.

As medical advances and terrorist threats frequently make news headlines, exposure to radiation is quickly becoming an issue of public, private and government concern. Clearly, there are benefits to the use of radiation. Radiotherapy is a common treatment modality for cancer and other diseases. However, there are also equally clear hazards, such as the use of radioactive materials in acts of terrorism or war. As important as these concerns are, radiation exposure is not limited to terrestrial events. In a very real sense, understanding the effects of radiation on biological systems is crucial to the human race as a whole as we move beyond Earth’s protective atmosphere.

President George W. Bush unveiled his vision for the future of the United States space program in a speech given on January 14, 2004. Although the details of the overall proposal have since undergone significant changes, the President’s speech indicates that a manned mission to the Moon or Mars in the near future is a part of public policy. One of the many practical issues that need to be resolved before such a mission is possible includes developing methods to counter radiation effects (1, 2). This need was recently highlighted when, in the Fall of 2003, a series of Solar Particle Events (SPEs) reached Earth in fairly rapid succession. At least one of these radiation events appears to have been larger than any such recorded event to date in terms of energy released, fluence, and dose.

With current technology, exposure to radiation during long-term spaceflight missions is unavoidable. Eight to 12 months of a Mission-to-Mars will be in transit (1), during which astronauts will be susceptible to exposure via Galactic Cosmic Rays (GCRs) and SPEs (1, 3). According to the Reference Mars Mission, the estimated dose due to GCRs and quiescent solar activity is roughly 0.5-1 Sieverts (Sv) dose equivalent (1, 4). SPEs could add an additional ≥2 Sv to this dose, depending on shielding conditions (1, 5). Given lengthy mission durations of 2-3 years (1, 4, 6), during which access to health care facilities will be limited, elucidation of radiation effects, as well as the development of countermeasures, is critical to minimize the risk to astronauts.

Microbes that present the most serious threat of infection during long-term space missions include Gram-negative intestinal coliforms such as E. coli, Klebsiella, and Enterobacter, and ubiquitous bacteria in the environment such as Pseudomonas aeruginosa, as well as Gram-positive Enterococcus, Staphylococcus aureus and group A Streptococcus (7-9). These bacteria are very common in nosocomial infections that often progress to septicemia. The Centers for Disease Control and Prevention (CDC) places septicemia as the 10th leading cause of death in the United States with an estimated 34,000 deaths annually (10).
The data presented herein represent the first results from a series of studies from this laboratory exploring the effects of whole-body irradiation on the ability of the immune system to respond to an immune challenge. We utilized γ-ray radiation as a model for radiation exposure and then challenged the animals 10 days post-exposure (i.e., during the recovery phase of the hematopoietic-immune system). Our immune challenge was lipopolysaccharide (LPS), a surface molecule found on all Gram-negative bacteria and a major cause of septic shock. Mice were euthanized 60 min, 1 day, 7 days, and 14 days post-inoculation to capture various phases of the immune response. Leukocyte, erythrocyte, and thrombocyte data are presented in a separate paper (11).

Materials and Methods

Animals. Female mice (C57BL/6J, 8-9 weeks old) were purchased from Charles River Breeding Laboratories (Wilmington, MA, USA) and shipped directly to the Loma Linda University (LLU) Animal Care Facilities (n=160 mice at 10/group/time point). Animals were acclimatized for 1-2 weeks before irradiation and maintained in a room controlled for temperature, humidity, with a 12:12 h light:dark cycle. Food and water were provided ad libitum. Female mice were used to minimize aggressive behavior and to compare with immune studies conducted previously in our laboratories. The animals were rapidly and humanely euthanized with 100% CO2 (12) at the appropriate time points. This study was approved by the Institutional Animal Care and Use Committee.

Whole-body γ-irradiation. Immediately prior to irradiation, the animals were placed individually into rectangular plastic aerated boxes (30 mm x 30 mm x 60 mm). Up to six mice were irradiated simultaneously to a total dose of 3 gray (Gy), delivered in a single fraction at a dose rate of 80 cGy/min, using a vertical beam from an AECL (Atomic Energy of Canada, Ltd., Commercial Products Division, Ottawa, Canada) Eldorado therapy unit containing a 60Co source. A plastic plate was placed immediately upstream of the boxes so that the front surface of each box was at the depth of maximum dose. Dose calibration was performed using a Capintec Model PRO6-G cylindrical thimble ionization chamber (Capintec, Inc., Ramsey, NJ, USA), in accordance with recommendations by the National Institute of Standards and Technology (NIST). Control animals were treated similarly under sham-irradiation conditions.

LPS injection. Ten days after irradiation, the mice were injected i.p. with E. coli LPS (O111:B4; Sigma Chemical Co., St. Louis, MO, USA) at 1 mg/kg in a volume of 0.1 ml 0.9% phosphate-buffered saline (PBS). Subsets of animals from each group were euthanized 60 min and 1, 7, and 14 days post-inoculation. The LPS dose selected is similar to those reported to induce significant immune modulation (13). The LPS dose was at 1 mg/kg in 0.1 ml 0.9% phosphate-buffered saline (PBS). Subsets of animals from each group were euthanized 60 min, 1 day, 7 days, and 14 days post-inoculation.

Flow cytometry analysis of lymphocyte populations. Samples of whole blood and spleen were evaluated using mixtures of fluorescence-labeled monoclonal antibodies (mAb; Pharmingen, San Diego, CA, USA), a direct-staining procedure, and a FACSCalibur flow cytometer (Becton Dickinson, Inc., San Jose, CA, USA). The mAb were conjugated to fluorescent isothiocyanate (FITC), R-phycocerythrin (PE), allophycocyanin (APC), or peridinin chlorophyll protein (PerCP). Antibody against the CD45 (30-F11) marker was used to identify leukocytes; lymphocyte gating was based on CD45 expression and side scatter. Specific lymphocyte populations and T-cell subsets were identified using mAb against the following molecules: CD3 (145-2C11, T-cells), CD4 (RM4-5, T helper or Th-cells), CD8 (53-6.7, T cytotoxic or Tc-cells), CD8 (53-6.7, T cytotoxic or Tc-cells), B220 (RA3-6B2, B-cells), and NK1.1 (PK136, natural killer or NK-cells). T- and B-cells were quantified separately using CD62L APC (mel-14) in concert with either CD3 or B220 markers. After accrual of at least 5,000 events per tube, analysis was performed using CellQuest software version 3.1 (Becton Dickinson). The number of each lymphocyte type was calculated: no. of cells in population/ml = no. of lymphocytes/ml x percentage of population.

Statistical analysis. Two-way analysis of variance (ANOVA) and Tukey’s pairwise multiple comparison test were used to determine significance at each time point (Systat software, version 10; Systat Software, Inc., Point Richmond, CA, USA). A p-value of <0.05 indicated significance. Outliers as defined by Systat, were excluded from the analysis.

Results

Circulating lymphocyte counts. (i) T-cells: There were main effects of radiation on T, Th, and Tc counts at all time points (p<0.005, Figure 1). Post hoc Tukeys indicate radiation-induced decreases were found in T and Th counts, regardless of LPS treatment, through day 7 (p<0.005). This decrease was only found in saline treated mice on day 14 (p<0.005). Similarly, radiation decreased Tc counts through day 14, independent of LPS exposure (p<0.005).

In contrast, LPS influenced T and Th-cell counts only through day 1 (p<0.005). Post hoc Tukeys indicate that LPS induced decreased in both 0 and 3 Gy groups (p<0.005). While the main effect of LPS on Tc counts continued through day 14 (p<0.05), post hocs indicate that the response of this population was somewhat complicated.
Like the other T-cell populations, there were LPS-induced decreases regardless of radiation through 60 min ($p<0.05$). However, LPS decreased Tc counts on day 1, and increased counts only in the unirradiated animals on day 7 ($p<0.05$). By day 14, there were no significant LPS-induced differences in individual radiation groups.

Given the fairly rapid LPS response, it is perhaps not surprising that there were significant radiation x LPS interactions on T, Th, and Tc counts only through day 1 ($p<0.005$). These were likely due to the limited response to LPS seen in irradiated mice.

(ii) B-cells: Radiation influenced B-cell counts at all time points ($p<0.005$, Figure 2), decreasing counts regardless of LPS at 60 min, only in the saline-treated mice on day 1, regardless of LPS on day 7, and only in the LPS-treated mice on day 14 ($p<0.05$).

There were main effects of LPS on B-cell counts at all time points except day 7 ($p<0.05$). However, post hocs indicate that LPS significantly changed counts only in the 0 Gy mice, where there was a decrease on day 1 ($p<0.005$), and an increase on day 14 ($p<0.005$).

There was a significant radiation x LPS interaction on NK-cell counts only on day 1 ($p<0.005$), due to a large LPS-induced increase in counts that was absent in the irradiated groups.
Splenic lymphocyte counts.

(i) T-cells: Radiation influenced T, Th, and Tc-cell counts at all time points ($p<0.05$, Figure 3). Post hoc Tukeys showed that there were significant radiation-induced decreases in all T-cell counts, regardless of LPS, through day 7 ($p<0.005$). However, at day 14, radiation-induced decreases were not significant in individual LPS groups in total T-cells, and significantly only in LPS-treated mice for Th and Tc counts ($p<0.05$). In contrast, there were no main effects of LPS on T, Th, and Tc-cell counts.

There were significant radiation x LPS interactions on all T-cell counts at 60 min for total T-cell counts ($p<0.05$). This was most likely due to the radiation-induced decreases in counts that diminished the slight LPS-induced increase noted in unirradiated mice.

(ii) B-cells: Radiation influenced B-cell counts at all time points ($p<0.005$, Figure 4). While there were significant radiation-induced decreases, regardless of LPS, through day 1 ($p<0.005$), radiation decreased counts only in the LPS-treated mice at the later time points ($p<0.05$).

In contrast, LPS influenced B-cell counts only at 60 min ($p<0.05$), and post hoc Tukeys revealed no LPS-induced changes in B-cell counts in either radiation group. Although there was no significant main effect at day 14, LPS increased counts in 0 Gy animals ($p<0.05$) and decreased counts in 3 Gy animals ($p<0.05$).

There were radiation x LPS interactions on days 1 ($p<0.05$) and 14 ($p<0.005$). In both cases, the LPS-induced increase in counts appears to have been reversed after radiation.

(iii) NK-cells: There were no main effects of either LPS or radiation on NK-cell counts at any time point (Figure 4), nor were there any significant interactions.

Circulating T and B memory cells. Because memory cells are typically developed after an immune challenge is cleared, we only assessed these populations 60 min after initiation of the challenge and fourteen days later.

(i) T-cells: There were main effects of radiation on CD3+/CD62L+ counts at both time points ($p<0.005$, Figure 5). However, post hoc Tukeys indicate radiation...
decreased counts regardless of LPS treatment only at 60 min \((p<0.005)\). Similarly, there were main effects of mitogen at only 60 minutes \((p<0.005)\), when post hoc tests indicate LPS decreased counts only in the 0 Gy mice \((p<0.005)\). The significant radiation x LPS interaction found at 60 min \((p<0.005)\) was likely due to the large radiation-induced decrease in this population that eliminated any further LPS response.

(ii) B-cells: Radiation influenced counts at both time points \((p<0.005, \text{Figure 5})\). Post hoc tests indicate radiation decreased counts, regardless of mitogen exposure at 60 min \((p<0.005)\), and in just the LPS groups on day 14 \((p<0.05)\). LPS did not influence CD19+/CD62L+ counts at either time point and there were no significant interactions.

Splenic T and B memory cells.

(i) T-cells: There were radiation effects on counts at both time points \((p<0.05, \text{Figure 6})\). In post hoc tests, radiation decreased counts, regardless of mitogen exposure at 60 min, and only in the LPS groups on day 14 \((p<0.05)\). There were no main effects of LPS, nor any significant interactions.

(ii) B-cells: There were radiation effects on counts at only 60 min \((p<0.005, \text{Figure 6})\), when radiation decreased counts regardless of mitogen exposure \((p<0.005)\). There were no main effects of LPS, nor were there any significant interactions.

Spontaneous blastogenesis.

(i) Blood: There were significant main effects of radiation on at all time points \((p<0.05, \text{Figure 7})\). Radiation increased blastogenesis regardless of mitogen exposure at 60 min \((p<0.05)\), but there were no significant radiation effects on individual LPS groups on either day 1 or 14. There were main effects of in vivo LPS exposure on in vitro spontaneous blastogenesis in the blood at all time points \((p<0.05)\). Post hoc Tukeys show LPS increased blastogenesis only in the 3 Gy treated animals at 60 min \((p<0.05)\), regardless of radiation exposure on day 1 \((p<0.005)\), and in neither radiation group on Day 14. There were no significant interactions at any time point.

(ii) Spleen: There were significant main effects of radiation at all time points \((p<0.05, \text{Figure 7})\). Radiation increased blastogenesis at 60 min and day 1, regardless of mitogen exposure \((p<0.005)\). By day 14, the influence of radiation was minimal. There were main effects of mitogen on day 1 \((p<0.005)\), when LPS-induced increases occurred regardless of radiation exposure \((p<0.05)\). There were significant radiation x LPS interactions on day 1 \((p<0.005)\) due to a radiation-induced enhancement of the LPS response.
Discussion

There were no surprising main effects of either treatment alone on any of the parameters characterized. To summarize, *in vivo* treatment with LPS generally decreased circulating T- and B-cell counts early on, and increased counts at later time points due to clonal expansion. Circulating NK counts remained elevated after LPS treatment throughout the experiment. In contrast, the main effects of LPS on splenic lymphocyte counts were minimal. Immediately after exposure to LPS, the number of circulating memory cells decreased, followed by a slight increase in the spleen at later time points. LPS generally increased spontaneous blastogenesis at the early time points, returning to control levels by day 14. Radiation generally decreased all lymphocyte counts, except for NK-cells, in both compartments at all time points, approaching control levels by day 14. Radiation also increased *in vitro* spontaneous blastogenesis at the early time points.

Given that both mitogen exposure and radiation can influence immune function, interactions between these two factors on many of our measured endpoints are to be expected. Because we were primarily interested on the influence of radiation on the inflammatory response to an immune challenge, we will focus the rest of the discussion on statistical interactions.

Bacterial infections are typically resolved within 1-2 hours through innate immune mechanisms. However, chronic infections will necessarily involve lymphocyte subpopulations (17-19). Sepsis is already known to lead to an increase in lymphocyte apoptosis (20, 21), and subsequently most sepsis patients are lymphopenic (22). Further dysregulation of these populations by radiation could potentially limit the ability to respond to chronic bacterial infections.

There were radiation x LPS interactions in many of the circulating lymphocyte populations, particularly at the early time points. Clearly, T- and B-cell counts were still very low ten days after exposure to γ-rays, when the mice received the LPS injections (*i.p.*). However, 60 minutes after the injections, all T-cell counts further decreased in the blood. This was presumably due to trafficking into the peritoneal cavity, in response to chemotactic signals released by LPS-activated macrophages, and into lymphoid organs for antigen presentation. T-cell counts remained low, and B-cell counts were similarly reduced, even 24 hours after the injections. In all cases, the drastic reductions in cell counts after radiation severely limited the number of cells that could traffic out of the blood, resulting in reliable statistical interactions. There were similar radiation x LPS interactions found in the spleen.

Almost immediately after exposure to LPS, there were significant radiation x LPS interactions on circulating, but not splenic, memory T-cell counts. The *i.p.* injection of LPS generally decreased this population, presumably due to trafficking into the peritoneal cavity as part of the general inflammatory response. However, as with overall T-cells, radiation decreased the entire memory T-cell population to the point that the subsequent challenge with LPS had little additional effect. This is somewhat surprising because the literature suggests memory T-cells are radioresistant compared to their naïve counterparts (23-25). Additionally, memory cells appear to be enriched in the CD8+ T-cell pool 14 days after exposure to radiation (25). One possible explanation for this may be that the memory T-cells that survived the initial radiation exposure were recruited in the recovery process, either as part of clonal expansion or damage surveillance, thus depleting the population available to respond to the immune challenge 10 days later. Another explanation may be a short-term delay in the ability of irradiated memory T-cells to proliferate in response to an immune challenge. Grayson *et al.* found that memory T-cells were unable to proliferate in response to a viral challenge 18 h after exposure to radiation, but proliferative capacity returned as overall recovery from radiation progressed (25).

Other early interactions occurred in circulating NK-cells. In unirradiated animals, there were sharp increases in circulating NK numbers one day after an LPS challenge. This early response is crucial to both innate and humoral immunity as NK-cells are the primary producers of an important inflammatory cytokine, interferon-γ (IFN-γ), after exposure...
to LPS or heat-inactivated *E. coli* (26). Furthermore, NK-cells, macrophages, and B-cells are all involved in a cytokine circuit that is activated during microbial infections (27). Although not characterized here, NK-cells have a direct impact on B-cell switching to IgG2a (27). The lack of early LPS-induced increases in circulating NK populations in irradiated animals, coupled with the large radiation-induced decrease in B-cell counts, could have both acute and long lasting dysregulation.

Indeed, the only significant long-term interaction on lymphocyte phenotype counts occurred in splenic B-cells on day 14. As with the T-cells, radiation caused an early decrease in overall B-cell counts. By day 7-14, B-cells appear to have recovered in mice that did not receive the LPS challenge. Because these mice were not treated with LPS, the recovering population likely included the full repertoire of B-cells. In contrast, splenic B-cell counts in irradiated mice that also received the LPS challenge were considerably lower than controls on day 14.

The relatively high dose of LPS, a prototypic thymus independent-1 (TI-1) antigen, suggests that this interaction was due to a combination of polyclonal expansion, recovery from radiation, and apoptosis. Generally, polyclonal expansion of B-cells peaks 7-10 days after exposure to an antigen and stimulation with LPS will lead to the preferential proliferation of B-cells that recognize LPS. We believe that the DNA repair mechanisms activated by radiation are switched off and/or superseded by the antigen response (e.g. upon stimulation with LPS, resources are reallocated away from DNA repair and dedicated to responding to the more immediate immune challenge). During periods of heightened proliferation, lymphocytes are particularly vulnerable to apoptotic death due to radiation-induced DNA damage and genomic instability, presumably through the activation of p53 (28, 29). An increase in vulnerability to apoptosis during proliferation has clearly been shown in T-cells (30-32) and a similar phenomenon may also occur in B-cells. We believe that the LPS-induced proliferation ultimately led in an increase in apoptosis, and therefore a decrease in counts. As antigen presentation and antibody production is initiated in the lymphoid organs, it should come as no surprise that these effects appeared primarily in the spleen.

Given the long-term interaction in B-cells, it might be surprising that there were no interactions in memory B-cell populations in either compartment at either time point. However, the lack of an interaction in this population at day 14 would also be consistent with the idea that the LPS-specific B-cells undergo radiation-induced apoptosis and are therefore unavailable for development into memory cells. This could have some interesting consequences for astronauts exposed to antigens requiring a B-cell response shortly after exposure to radiation. Radiation would increase apoptotic death in responding populations and therefore inhibit the development of immunological memory.

One day after LPS injection, radiation appears to synergistically augment spontaneous blastogenesis in the spleen, resulting in a response that is greater than a summed effect of the individual factors. We have already shown that exposure to radiation leads to an acute increase in spontaneous blastogenesis (14, 16, 33). This synergistic response would suggest that leukocytes were also primed for division by pre-exposure to radiation. The addition of LPS to the system initiated an exaggerated inflammatory response that took advantage of the primed leukocytes. The relatively short time course of the LPS response seen in the irradiated animals is consistent with the idea that feedback control mechanisms are still in place.

While it is likely that the proliferating populations are lymphocytes and not monocytes, because sepsis is generally due to an exaggerated inflammatory response to a bacterial infection (18, 21), an synergistic increase in blastogenesis could suggest that there is a lower bacterial load threshold for which septic shock might occur (34, 35).

**Conclusion**

Together, these data suggest that the ability to respond to a bacterial immune challenge is altered by radiation. Acutely, general and memory lymphocyte populations are not available for trafficking into the site of infection, or into the lymph nodes for antigen-presentation and activation. There may also be a disruption of the NK-macrophage-B-cell cytokine circuit necessary for coordination of the inflammatory response in chronic infections. This is complicated by reports that radiation may also activate latent bacteria (36) and viruses (37). Clearly, additional work is required to discern potential mechanisms and countermeasures for radiation-induced disruptions in the response to an immune challenge.

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