Abstract. Background/Aim: We previously found that minocycline enhanced the levels of several leukocyte populations and had the capacity to induce secretion of certain cytokines early after irradiation. In the current study we further determined the drug’s effect on hematopoietic recovery. Materials and Methods: Minocycline was injected intraperitoneally into C57BL/6 mice for 5 days, beginning immediately before exposure to 60Co γ-rays (1, 2, 3 Gy). Thirty-two days post-irradiation, spleen and blood were collected to quantify cell populations, cytokines in splenic T-cell supernatants after anti-CD3 activation, and chromosomal status based on spectral karyotyping. Results: While radiation resulted in significantly lower B-cell counts at 3 Gy in both blood and spleen, minocycline treatment increased the counts and/or percentages of splenic B-cells at 2 Gy and 3 Gy. In spleen supernatants, the drug-alone increased the levels of cytokines, including interleukin-1α (IL-1α) and IL-6 that are radioprotective, as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF that accelerate neutrophil recovery. In addition, minocycline suppressed the production of interferon-γ that can prevent hematopoiesis. Dose-dependent radiation-induced chromosomal abnormalities were present in splenic leukocytes. Conclusion: The data indicate that minocycline exerts a relatively long-term effect on parameters that influence hematopoietic recovery. Further testing of this drug as a countermeasure for acute radiation syndrome, is necessary to determine its full potential.

Exposure to ionizing radiation is increasingly common in a variety of settings, including space exploration, diagnostic medical procedures and radiotherapy. Unfortunately, the risks for exposure during military combat or acts of terrorism are also on the rise. Indeed, the September 11, 2001 attack on the United States, as well as the 2011 disaster at the Fukushima-Daiichi power plant in Japan, have certainly increased worldwide concern regarding radiation effects on human health. In addition, astronauts on space missions can become exposed to radiation doses within the range of 1-3 Gray (Gy) during a solar particle event (SPE); solar activity is expected to, once again, reach a maximum in 2013. Given the health risks associated with exposure in a variety of settings, there is an urgent need for discovery of safe and effective normal tissue radioprotectants and radiomitigators.

Radiation can cause pathologies collectively, referred to as acute radiation syndrome (ARS). The hematopoietic system is most sensitive to radiation (1) and one of the early manifestations of ARS is the hematopoietic syndrome. Indeed, declines in circulating white blood cell (WBC), lymphocyte, platelet (PLT) and red blood cell (RBC) counts are indicative of hematopoietic syndrome which can occur upon exposure to total-body radiation doses as low as 1-2 Gy. Management of hematopoietic syndrome includes administration of colony-stimulating factors that promote granulocyte and macrophage regeneration (G-CSF, GM-CSF), of blood products such as erythrocyte concentrate, and hematopoietic stem cell transplantation (HSCT), depending upon the severity of the case (2). Clinical management of radiation injury also includes administration of antibiotics, e.g. fluoroquinolones with broad-spectrum activity, to minimize the risk of systemic infection and facilitate recovery of tissues such as the bone marrow and gastrointestinal tract (3, 4). However, administration of antimicrobials, as well as antiemetics, antidiarrheal agents and analgesics, is primarily considered as supportive care.

Evidence in the literature suggests that tetracyclines can be robust radioprotectors of hematopoietic tissues, with potential utility in radiation emergencies and anticancer radiotherapy. Minocycine is a widely used semisynthetic, second-generation tetracycline derivative with broad-spectrum activity and long half-life after administration. Studies indicate that minocycline has properties that are completely distinct from its
antimicrobial action. It has anti-inflammatory, antiapoptotic, neuroprotective (5), and free-radical scavenging effects (6), and also possesses anti-tumorigenic potential (7). Given the properties of minocycline, we hypothesized that it would facilitate regeneration of immune cell populations following whole-body irradiation and thus have the potential to minimize serious complications associated with ARS. Our previous results obtained at day 4 post-irradiation were very promising, e.g. addition of minocycline treatment enhanced the counts and/or percentages of several leukocyte populations and increased the capacity to secrete cytokines with hematopoietic properties compared to radiation-alone (8). In the present study, we chose day 32 post-irradiation as our time point because recovery of immune cell parameters is close to normal at this time following the radiation doses used in our study. The goal was to determine if minocycline had any long-term effects when used alone or in combination with radiation, as opposed to a transient/short-lived effect on the assessed parameters.

Materials and Methods

Animals and experimental design. C57BL/6 female mice (n=80; 8-9 weeks old) were purchased from Charles River Breeding Laboratories, Inc., Hollister, CA, USA and acclimated for 5-7 days in large plastic cages (n=10/cage) under standard vivarium conditions. Animals were assigned to 8 groups, each consisting of 10 mice: a) deionized water (dH2O) + 0 Gy; b) dH2O + 1 Gy; c) dH2O + 2 Gy; d) dH2O + 3 Gy; e) minocycline + 0 Gy; f) minocycline + 1 Gy; g) minocycline + 2 Gy; h) minocycline + 3 Gy. Animals were rapidly sacrificed on day 32 after excision. The following formula was used to calculate spleen mass: RSM = spleen mass (mg)/body mass (g).

Blood and spleen collection. Cardiac puncture was performed immediately after euthanasia to collect blood in 1 ml tuberculin syringes containing K2-ethylenediaminetetraacetic acid (EDTA). Spleens were excised and processed into single-celled suspensions in complete RPMI-1640 medium (Irvine Scientific, Santa Ana, CA, USA) using sterile applicator sticks. After RBC lysis using 2 ml lysis buffer for 4 min at 4°C, the remaining splenic leukocytes were washed, centrifuged and suspended in 2 ml of RPMI-1640 medium. Cell populations collected from both of these body compartments were then analyzed as described below.

Analysis of cell populations in blood and spleen. An automated analyzer (HESKA™ Vet ABC- Diff Hematology Analyzer; HESKA Corp., Waunakee, WI, USA) was used to quantify total WBC and major leukocyte populations in blood and spleen, i.e. numbers and percentages of lymphocytes, granulocytes, and monocytes/macrophages. Although the spleen contains a reservoir of monocytes, in addition to macrophages (9), cells of this lineage within this body compartment are collectively referred to as macrophages hereafter for the sake of simplicity. Additional values were obtained for blood as follows: RBC count, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular HGB concentration (MCHC), RBC distribution width (RDW), PLT count, and mean platelet volume (MPV).

Flow cytometric analysis of lymphocyte populations in blood and spleen. The percentages of specific lymphocyte populations in the blood and spleen were determined using 2-tube monoclonal antibody (mAb) mixtures (Pharmingen, San Diego, CA, USA). T-Regulatory (Treg) cell analysis was carried out with a staining kit that included fluorescent mAb, specific for FJK-16s [anti-forkhead box P3 (Foxp3)], cluster of differentiation 4 (CD4), and CD25 (eBioscience, Inc., San Diego, CA, USA). The CD4+CD25+ T-cells, both with and without Foxp3, were quantified by gating on side scatter and the CD4+ cells, followed by analysis of the CD25+ versus FJK-16s (Foxp3+) subset. Additional details of these procedures have been previously described (8, 10).

Splenocyte activation using anti-CD3 mAb. The WBC were quantified in spleens after lysis of RBC, as described above, using a hematology analyzer (HESKA Corp.). After the cell density was adjusted to 2x10⁸ cells/ml with complete RPMI-1640 medium, the cells were dispensed into 96-well plates coated with immobilized anti-CD3 mAb (Mouse Anti-CD3 T-Cell Activation Plates; BD Pharmingen, San Diego, CA, USA). Each well contained 4x10⁵ cells in 0.2 ml of medium. After 48 h of incubation at 37°C in a humidified chamber, the supernatants were harvested and stored at –80°C until analysis for cytokines.

Cytokine analysis. Immediately after thawing, splenic supernatants were analyzed using the Mouse Cytokine/Chemokine Milliplex MAP Kit purchased from Millipore, Billerica, MA, USA. The 22 cytokines/chemokines were: interlukin-1α (IL-1α), IL-1β, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, G-CSF, GM-CSF, interferon-γ (IFN-γ), IFN-γ-induced protein-10 (IP-10), keratinocyte chemoattractant (KC), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), regulated upon activation, normal T-cell expressed and secreted (RANTES) and tumor necrosis factor-α (TNF-α). In order to quantify vascular endothelial growth factor (VEGF), an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) was used.
Spectral karyotyping for chromosome abnormalities. A portion of the spleen from five mice per group was used to generate metaphase spreads (after RBC lysis) according to standard procedures that included activation with phytohemagglutinin (PHA), a mitogen that induces T-cell proliferation. Chromosomes in 10 metaphases per sample were evaluated using SKY Spectral Laboratory Reagents and Concentrated Antibody Detection kits (Applied Spectral Imaging, Inc., Carlsbad, CA, USA). All chromosomes were visualized and analyzed using a HiSKY Complete Cytogenetic Imaging System (Applied Spectral Imaging, Inc.), interfaced with a Case Data Manager Version 6.0.0.3, an Olympus B-50 fluorescence microscope, and a CCD 130QDS camera; the software, microscope, and camera were obtained from the Genomic Centre for Cancer Research and Diagnosis, Winnipeg, MB, Canada. The procedures and analyses were carried out by a board-certified cytogeneticist.

Statistical analysis. The data were analyzed using SigmaStat™ software, version 2.03 (SPSS Inc., Chicago, IL, USA). Two-way analysis of variance (ANOVA) and, when indicated, Tukey’s test, were carried out to obtain pair-wise multiple comparisons. The data are presented as means and standard errors of means (SEM). p-Values less than 0.05 were selected to indicate significance.

Results

Body and RSM. Overall, the values for body mass ranged from 21.2±0.2 g (dH2O + 3 Gy) to 22.3±0.3 g (Minocycline + 0 Gy). RSM ranged from 3.6±0.2 (dH2O + 3 Gy) to 4.2±0.2 (Minocycline + 0 Gy). No statistical difference in these two parameters was observed, regardless of minocycline treatment or irradiation.

WBC and leukocyte populations in blood. As shown in Figure 1, the WBC count ranged from 3.3×10^3/mm^3 to 5.7×10^3/mm^3. Radiation had a main effect on the WBC counts due to decreasing counts with increasing dose. In post-hoc analysis, significantly lower WBC counts were found at 3 Gy in both drug-treated and untreated groups (p<0.05). Numbers for lymphocytes, monocytes, and granulocytes were generally reduced (p<0.05) after exposure to radiation and drug addition did not have a significant impact (Figure 1).

Although radiation had a main effect on both monocyte and granulocyte percentages (p<0.05), post-hoc analysis indicated this was due only to increases noted at the higher doses. These proportional changes were reversed for lymphocytes. Addition of minocycline brought the lymphocyte percentages close to normal in the 3 Gy-treated group, i.e., minimizing the decrease that was caused by radiation-alone. Similarly, the drug brought the monocyte percentage close to normal at 3 Gy, preventing the radiation-induced increase. The drug increased the granulocyte percentage only at 2 Gy, while radiation increased the granulocyte percentage only at 3 Gy (p<0.05). This resulted in a drug × radiation interaction for granulocytes (p<0.05).

RBC and PLT characteristics in blood. The results of erythrocyte and thrombocyte characteristics are listed in Table I. Radiation had main effects on RBC counts and HCT, generally reducing both parameters in irradiated groups (p<0.05). There was no effect of radiation on HGB and no drug effect or interactions for RBC, HCT, and HGB. Although radiation very slightly increased MCV (p<0.05), post-hoc analysis showed no differences between groups. The lower MCV value in the minocycline plus 3 Gy-treated group compared to the group that received only 3 Gy (p<0.05) was likely due to the very small SEM obtained with the drug (the hematology analyzer gives out only whole integers). Although there was no radiation or drug effect on MCH, a drug × radiation interaction existed. This interaction was likely due to increase of MCH at 3 Gy in the non-drug treated group and a subsequent decrease in its 3-Gy drug-treated counterpart. Neither drug nor radiation affected the MCHC. Radiation generally increased RDW (p<0.05); post-hoc analysis showed that the 1 Gy- and 3 Gy-treated groups (without drug) had higher RDW values compared to those treated with 0 Gy (p<0.05). The drug had a main effect on RDW (p<0.05); post-hoc testing showed that the 3-Gy group treated with minocycline, had higher RDW compared to the 0-Gy group treated with the drug.

Radiation generally increased the PLT count and MPV (p<0.05). Although the changes primarily occurred in the 2-Gy- and 3-Gy-treated groups, there were no significant radiation-dependent differences in post-hoc comparisons between individual groups. Similarly, although the drug also had a significant impact on the PLT count (p<0.05), this effect reached significance only in the 3-Gy-treated groups (p<0.05) and there were no significant drug×radiation interactions.

WBC and leukocyte populations in spleen. Figure 2 shows that the WBC counts in the spleen ranged from 38.3×10^3/mm^3 to 49.9×10^3/mm^3. Radiation had a main effect on the counts (p<0.05), causing a gradual decline with increasing dose, but significantly lower WBC counts were present only in the dH2O plus 3-Gy-treated group compared to the dH2O plus 1-Gy-treated group (p<0.05) in post-hoc analysis. The distribution of lymphocytes, macrophages and granulocytes is also shown in Figure 2. Radiation generally caused a decline in lymphocyte counts, resulting in a significant main effect (p<0.05), although this did not reach significance in post-hoc analysis. There were no significant drug effects or interactions on the number of lymphocytes. Although lymphocyte percentages were significantly low at 0 Gy in the presence of minocycline (p<0.05), this effect did not result in a significant main effect or interaction. The macrophage and granulocyte counts were not significantly altered by radiation or drug. In contrast to lymphocytes and granulocytes, radiation at 3 Gy did increase macrophage percentages, resulting in a significant main effect (p<0.05). Granulocyte percentages were unaffected by either radiation or drug.
Figure 1. Major leukocyte populations in blood. An automated hematology analyzer was used to obtain data for n=9-10 mice/group. Each bar represents the mean±SEM (standard error of mean). Two-way analysis of variance: §p<0.05 for main effect of radiation; ‡p<0.05 for drug × radiation interaction. Tukey test: *p<0.05 for deionized water (dH2O) versus minocycline within each radiation dose; †p<0.05 versus 0 Gy within dH2O-treated groups; ‡p<0.05 versus 0 Gy within minocycline-treated groups.
Flow cytometric analysis of lymphocyte populations in blood. Radiation generally caused a decline in the circulating T-, B- and CD8+ T-cytotoxic (Tc) cell counts (Figure 3), resulting in a significant main impact of radiation \((p<0.05)\). Addition of the drug had no effect based on two-way ANOVA. However, post-hoc analysis showed that T- and B-cell counts were no longer depressed in the 3-Gy-treated group that had received minocycline. Although radiation generally reduced the CD4+ T-helper (Th) cell counts (trend, \(p<0.1\)), no significance was obtained in post-hoc analysis. The CD4:CD8 ratio generally increased with increasing radiation dose \((p<0.05)\). The natural killer (NK) cell counts were not significantly affected by either drug or radiation. In terms of percentages (Figure 4), radiation alone reduced the levels of B- and Tc cells \((p<0.05)\). Post-hoc analysis, however, revealed that the percentage of B-cells in the 3-Gy-treated group was closer to normal when minocycline treatment was added. T- and NK cell percentages were not significantly altered by radiation. Percentages of Th cells generally increased with increasing radiation dose, but there was no significant impact of the drug.

Flow cytometric analysis of lymphocyte populations in spleen. Radiation had no significant impact on T-, B- or NK cell counts (Figure 5). However, the number of T-cells was significantly reduced in the 3-Gy-treated group that had received minocycline. Although radiation generally reduced the CD4+ T-helper (Th) cell counts and CD8+ Tc cell counts (Figure 3), resulting in a significant main effect of radiation \((p<0.05)\). Minocycline reversed the radiation effect at 2 and 3 Gy,\(^*\) resulting in a main effect of drug and a drug × radiation interaction \((p<0.05)\). The data were obtained using an automated hematology analyzer \((n=8-10\) mice/group). Mino: Minocycline. Two-way analysis of variance: \(^1p<0.05\) for main effect of radiation; \(^2p<0.05\) for main effect of drug; \(^3p<0.05\) for drug X radiation interaction. Tukey test within deionized water \((dH_2O)\) or minocycline treatment: \(^*p<0.05\) for \(dH_2O\) versus minocycline within each radiation dose; \(^*p<0.05\) versus 0 Gy within \(dH_2O\)-treated groups.

Flow cytometric analysis of CD4+CD25+Foxp3+ T-cells in spleen. There was no significant impact of drug or radiation on the counts of either CD4+CD25+ or CD4+CD25+Foxp3+ T-cells (Figure 7). In post-hoc analysis, addition of minocycline caused a significant increase in the percentage of CD4+CD25+ cells, but only in the 0-Gy-treated group that did not receive radiation \((p<0.05\) versus the 0 Gy group without drug). This resulted in a significant main effect of the drug and a drug × radiation interaction on CD4+CD25+ T-cell percentages \((p<0.05)\). There was also a significant drug-induced increase in the percentage of CD4+CD25+Foxp3+ cells in the 3-Gy-treated group compared to that treated with 3-Gy alone \((p<0.05)\). However, this was not enough to result in any main effects or interactions.

Cytokines in splenic supernatants. Concentrations of cytokines that were generally increased in the groups that received minocycline are presented in Figure 8. Minocycline had a main

| Table 1. Summary of erythrocyte and platelet characteristics in blood. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | 0               | 1               | 2               | 3               |
| RBC \((10^7/\mu l)\) | 9.0±0.1 \(dH_2O\) | 8.2±0.2 \(dH_2O\) | 8.5±0.2 | 8.3±0.1 \(dH_2O\) |
| HGB \((g/dl)\)     | Minocycline | 8.7±0.2 | 8.5±0.2 | 8.6±0.2 | 8.3±0.2 |
| MCHC \((\%\)      | 33.3±0.03 \(dH_2O\) | 33.3±0.01 33.3±0.02 33.3±0.01 |
| RDW \((\%)        | 41.1±0.3      | 37.2±0.5 33.8±0.7 30.8±1.3 |
| MCV \((\mu m3)\)   | 46.0±0.2      | 46.0±0.2 46.0±0.2 47.0±0.2 |
| MCH \((pg)\)       | 15.1±0.1      | 15.3±0.1 15.5±0.1 15.1±0.1 |
| MPV \((\mu m3)\)   | 5.2±0.05      | 5.1±0.02 5.2±0.03 5.4±0.10 |

\(^1p<0.05\) for main effect of radiation; \(^2p<0.05\) for main effect of drug; \(^3p<0.05\) for drug X radiation interaction. Tukey test within deionized water \((dH_2O)\) or minocycline treatment: \(^*p<0.05\) for \(dH_2O\) versus minocycline within each radiation dose; \(^*p<0.05\) versus 0 Gy within \(dH_2O\)-treated groups.

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Figure 2. Major leukocyte populations in spleen. An automated hematology analyzer was used to obtain data for n=9-10 mice/group. Each bar represents the mean±SEM (standard error of mean). Two-way analysis of variance: $p<0.05$ for main effect of radiation; Tukey test: $p<0.05$ for deionized water (dH$_2$O) versus minocycline within each radiation dose; $p<0.05$ versus 0 Gy within dH$_2$O-treated groups.
effect on IL-4, IL-10 and IL-13, whereas radiation had a main effect on IL-4, IL-10 and IP-10 ($p<0.05$). A drug × radiation interaction was noted for G-CSF, IL-1α, IL-4, IL-6 and IL-10. In post-hoc analysis, minocycline increased the levels of G-CSF, GM-CSF, IL-1α, IL-4, IL-6, RANTES and IL-13 only in the non-irradiated groups ($p<0.05$). The drug-induced enhancement in these seven cytokines was no longer present when the drug was combined with radiation, regardless of dose.
Among the drug-treated groups, IL-4 and IL-6 concentrations were significantly lower in all irradiated groups compared to the 0-Gy-treated group that received minocycline ($p < 0.05$). However, the drug significantly increased the level of IL-10 in both the 0-Gy- and 1-Gy-treated groups compared to their respective counterparts that did not receive drug ($p < 0.05$). A similar, although less pronounced, drug effect was noted for IL-17, i.e. a trend ($p < 0.1$) for an increase was present in the non-irradiated and 1-Gy-treated groups.

The cytokines that generally were low at concentrations in the groups treated with the drug or for which there was no effect of either drug or radiation, are shown in Figure 9. Minocycline-alone had a main effect on concentrations of IFN-γ, IL-7 and VEGF, whereas a main effect of radiation
was noted for IL-7, VEGF and MIP-1α \( (p<0.05) \). Post-hoc analysis showed that minocycline treatment resulted in low IFN-γ-only when compared to the non-treated 0-Gy group \( (p<0.05) \). IL-7, however, was low in all drug-treated groups, regardless of radiation dose \( (p<0.05 \text{ versus respective groups without drug}) \). Post-hoc analysis showed no significant differences among groups in the levels of MIP-1α, IL-2, IL-12(p70), KC and TNF-α.
Spectral karyotyping of leukocytes in spleen. Table II summarizes chromosomal characteristics for each group. Although there were 2-3 metaphase spreads with chromosomal aberrations in samples from non-irradiated mice, with and without minocycline, the karyotype was normal in the great majority (Figure 10). Although the minocycline-treated group had relatively few chromosome aberrations after 1-Gy exposure compared to the group that received 1 Gy-alone (8.2% versus 16.0%, respectively), the drug did not have much impact on the detrimental effect of radiation when it was delivered at the higher doses. At 2 Gy-alone, 12.2% of metaphases had aberrations, while 14.0% had...
aberrations in the minocycline plus 2 Gy-treated group; at the highest dose, the percentages were 30.6% (3 Gy) and 27.1% (minocycline plus 3 Gy). Abnormalities included whole chromosome additions/deletions and translocations that involved both short and long arms. Overall, the greatest effect of radiation was noted on chromosomes 3, 4, 9, 10, 16, 17 and 18, regardless of drug treatment. Examples of abnormal karyotypes are presented in Figure 11 (1 Gy, with and without drug) and Figure 12 (3 Gy, with and without drug).

Discussion

Leukocytes and lymphoid organs are well-known to be highly radiosensitive. Bone marrow failure, as a consequence of radiation exposure, can result in severe leukopenia which, in turn, can greatly influence morbidity and mortality (11). In our previous study, conducted on day 4 post-irradiation, major findings were that minocycline treatment significantly increased counts and/or percentages of splenic macrophages, granulocytes, total T-cells, CD8+ T-cells and NK cells, and modified splenocyte capacity to secrete numerous cytokines in irradiated mice (8). The present study focused on the relatively late effects of minocycline on hematopoietic and immune parameters in an irradiated mouse model, i.e. day 32 after exposure. Another goal was to determine if the drug caused any unexpected changes in cell profiles and cytokine production patterns. All aspects of study design in the previous and present studies were similar except for the time point for analyses and the addition of spectral karyotyping to determine whether minocycline had any effect on radiation-induced chromosomal aberrations.

Figure 7. T-Regulatory cells in spleen. The data for 5-10 mice/group are based on fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean±SEM (standard error of mean). Two-way analysis of variance: ‡p<0.05 for drug×radiation interaction. Tukey test: *p<0.05 for deionized water (dH2O) versus minocycline within each radiation dose.
Figure 8. Quantitative analysis of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1α (IL-1α), IL-4, IL-6, IFN-γ-induced protein-10 (IP-10), regulated upon activation, normal T-cell expressed and secreted (RANTES), IL-13, IL-10, and IL-17. Data were obtained from supernatants after activation of splenocytes with anti-CD3 monoclonal antibody. The mean±SEM (standard error of mean) is presented for n=8-10 mice/group. Two-way analysis of variance: §p<0.05 for main effect of radiation; †p<0.05 for main effect of drug; ‡p<0.05 for drug×radiation interaction. Tukey test: *p<0.05 for deionized water (dH₂O versus minocycline within each radiation dose; bp<0.05 versus 0 Gy within minocycline-treated groups; Tp<0.1 for dH₂O versus minocycline within each radiation dose.
In the present study, the distribution of most of the major cell types was equivalent to normal. However, complete restoration of some subpopulations was still lacking. B-Cell counts were significantly reduced in both the blood and spleens from the 3-Gy-treated group (without drug) compared to the non-irradiated group. This contradicts the findings of Kajioka et al. that B-cell counts in blood after 3 Gy whole-body irradiation of the same strain of mice were no longer significantly depressed by day 29 after exposure (12). However, it should be noted that the mean value for B-cell counts was ~35% less than that of the non-irradiated controls and the mice were stimulated with sheep RBCs (a procedure that may have enhanced the recovery process in the study by Kajioka et al.). In the spleen, minocycline reversed/eliminated the 3-Gy radiation-induced B-cell decrease, thereby suggesting that the drug could be helpful in maintenance/recovery of this cell type.
Although splenic T-cell counts were equivalent to normal, circulating T-cell numbers were still low in the 3-Gy-treated groups that did not receive the drug. Similarly, although CD4+ Th cells recovered in both compartments (blood and spleen), the CD8+ Tc cell counts in the blood at 3 Gy were significantly lower compared to that of the dH2O plus 0-Gy group (<0.05). This is consistent with the literature where Tc cells are regarded as being more radiosensitive compared to the Th cell subset (12, 13). Interestingly, it has been proposed that the level of radiation-induced apoptosis in the CD4+ and CD8+ T-cell populations has the potential to identify patients who are hypersensitive to radiation, and thus may be used to help predict risk for development of late toxicities (14).

Our previous study in which assessments were conducted on day 4 post-irradiation (8) showed that treatment with minocycline increased the splenic production capacity for radioprotective cytokines and growth factors, but suppressed the production of cytokines that could limit hematopoiesis. The results of the present study show that the drug on its own significantly increased the potential to produce G-CSF, GM-CSF, IL-1α, IL-4, IL-6, IL-10, IL-13, and RANTES even at day 32 after irradiation. Although, IL-1α and IL-6 are radioprotective (15) and G-CSF and GM-CSF help in restoring granulocytes after neutropenia, all of the minocycline-induced increases in these cytokines were no longer evident in the irradiated groups that had been treated with the drug. From our previous observations at day 4 post-irradiation, minocycline significantly increased the production of these cytokines, even in the irradiated groups, suggesting that the impact of the drug on the cytokines was acute and transient with respect to radiation response. Health implications, if any, of the changes seen with drug-alone at day 32 remain to be determined.

As noted above, the drug increased the levels of IL-10, a Th2 cell-derived anti-inflammatory cytokine. There is also evidence that IL-10 can prevent the development of fibrosis (16), which is a late consequence of radiation exposure. Nelson et al. reported that IL-10 treatment resulted in reduction of inflammation and fibrosis scores in patients with hepatitis-related liver fibrosis (17). The increased levels of IL-10 observed even at day 32 post-irradiation indicate that the drug may have some benefit in this regard. IL-10 also stimulates humoral immunity (18) and inhibits the suppression of hematopoiesis induced by IFN and TNF-α (19). Other investigators have shown that minocycline increases the expression of IL-10 mRNA and reduces TNF-α mRNA (20).

There was a trend for drug-induced enhancement of IL-17 levels in splenic supernatants from the 0-Gy- and 1-Gy-treated groups. IL-17, a cytokine secreted by activated CD4+ and

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<th>Group</th>
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<th>Aberration</th>
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<td>dH2O+0 Gy</td>
<td>40,XX (47/50)</td>
<td>39,XX,−7 41,XX,+4 38,XX,−2,−17</td>
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<tr>
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<td>Mino+2 Gy</td>
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The data were obtained using spectral karyotyping. Analysis was performed on 48-50 metaphase spreads/group, i.e. 7-10 metaphases on each of 5 mice/group. Numbers in parentheses under ‘Normal’ indicate the number of metaphase spreads. Each aberration was noted in at least one metaphase/group. Nomenclature: add, additional material of unknown origin; del, deletion; der, derivative chromosome; p, short arm; q, long arm; t, reciprocal translocation; X, female chromosome; (), surround structurally altered chromosomes and breakpoints; +/-, numerical aberrations.
CD8+ T-cells, induces the production of hematopoietic cytokines (21, 22). It has been shown to stimulate fibroblast production of IL-6 and G-CSF, both of which assist in hematopoiesis (23). These findings related to IL-17 support the possibility that minocycline may be a useful enhancer of hematopoietic recovery.

While radiation increased the production of VEGF at 3 Gy (p<0.05 versus 0 Gy), minocycline suppressed its production at both 1 and 3 Gy. Since VEGF is an angiogenic cytokine which facilitates tumor growth and metastasis, treatment with the drug may limit these possibilities during radiotherapy, as well as in populations with not yet diagnosed cancer that are exposed to radiation. Recent reports do, indeed, indicate that minocycline has antitumor properties (24, 25). However, IL-7, which helps in the development of T- and B-lymphocytes that have potential to directly or indirectly attack tumor cells, was significantly down-regulated in all groups that received minocycline. A similar pattern was observed for this cytokine on day 4 post-irradiation in our previous study (8). IL-7 is also known to promote the secretion of Th1 cell-derived pro-inflammatory cytokines such as IFN-γ and TNF-α and thus perpetuate responses associated with this T-cell subset (26). Reduced levels of IFN-γ observed in the drug-treated group that received 0 Gy could be due to inadequate activation of Th1 cells. It has been reported that minocycline can suppress T-cell activation (27, 28).

Radiation is well-known to damage DNA which can result in pathologies that include immune dysregulation and cancer such as leukemia and lymphoma (29). Furthermore, chromosome aberrations are generally regarded as sensitive indicators of radiation exposure and it has been proposed that they may be useful as predictors of cancer risk (30). We, therefore, performed a pilot experiment on splenic WBC using spectral karyotyping. The changes seen in a few metaphase spreads from the non-irradiated groups (with and without drug) are not surprising for cells that are cultured in vitro. The presence of radiation-induced abnormalities, however, was very obvious, especially at 3 Gy. Overall, in the irradiated groups (without drug), analysis demonstrated that 10 chromosomes were deleted and four chromosomes were added to the genome in one or more of the metaphases and a variety of partial additions/deletions and translocations were noted. With the possible exception of the 1-Gy dose, minocycline did not provide any obvious radiation protection, thereby indicating that sub-lethally irradiated cells were transformed to some extent regardless of drug treatment. Immunological pathologies associated with some of the chromosomes most affected by radiation include acute T-cell lymphoblastic leukemia, B-cell leukemia/lymphoma, T-cell immunodeficiency, asthma and systemic lupus erythematos (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/books/NBK22266/).

**Conclusion**

Although minocycline increased the production of several anti-inflammatory, immunosuppressive cytokines, such as IL-4, IL-6 and IL-10 and reduced IFN-γ, it also up-regulated pro-inflammatory cytokines such as G-CSF, GM-CSF, IL-1α, IL-17 and RANTES. Whether it is beneficial that treatment with the drug increased production capacity in the spleen for immunosuppressive cytokines on its own even at day 32 post-
irradiation, remains to be determined. An immunosuppressive environment could lessen the ability to fight infections. Kielian et al. indicated that minocycline has immune modulatory properties that balance deleterious versus beneficial inflammation (31). However, the drug also counteracted radiation-induced declines in the B-cell population in the spleen, which would promote humoral immunity. The few differences among groups in RBC and platelet characteristics were very minimal, essentially indicating full recovery. Data from the spectral karyotyping suggested that the drug may minimize the risk for chromosomal aberrations when the radiation dose is 1 Gy or less. This possibility, however, would have to be confirmed. Overall, the study brings new knowledge on the properties of minocycline, a drug that has potential as a normal tissue radioprotectant. Further research should be carried out to determine its effectiveness in humans during cancer radiotherapy and for the management of ARS.
Figure 12. Examples of chromosomal aberrations in splenic leukocytes from 3-Gy-irradiated mice. The data were obtained using spectral karyotyping. Top panel: Deionized water (dH2O) plus 3 Gy, karyotype 39,XX,der(3)t(3-14),-9,-12,+19. Bottom panel: Minocycline plus 3 Gy, karyotype 38,XX,der(3)t(3_13),-13,-18. *Whole chromosomes deleted or added.

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