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# Analysis of Minocycline as a Radioprotectant

Shalini Mehrota  
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LOMA LINDA UNIVERSITY  
School of Medicine  
in conjunction with the  
Faculty of Graduate Studies

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Analysis of Minocycline as a Radioprotectant

by

Shalini Mehrotra

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A Dissertation submitted in partial satisfaction of  
the requirements for the degree of  
Doctor of Philosophy in Microbiology

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June 2012

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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## ABBREVIATIONS

|                   |                                    |
|-------------------|------------------------------------|
| ALS               | Amylotrophic Lateral Sclerosis     |
| ANOVA             | Analysis of Variance               |
| AP-1              | Activator Protein 1                |
| APC               | Allophycocyanin                    |
| ARS               | Acute Radiation Syndrome           |
| BBB               | Blood Brain Barrier                |
| Bik               | Bcl-2 Interacting Killer           |
| Bmp               | Bone Morphogenetic Protein (gene)  |
| CD                | Cluster of Differentiation         |
| CNS               | Central Nervous System             |
| COX-2             | Cyclooxygenase-2                   |
| CSF               | Cerebrospinal Fluid                |
| CXCL10            | C-X-C motif chemokine 10           |
| dH <sub>2</sub> O | Deionized Water                    |
| DMEM              | Dulbecco's Modified Eagle's Medium |
| DOD               | Department of Defense              |
| DOE               | Department of Energy               |
| EDTA              | Ethylenediaminetetraacetic Acid    |
| ELISA             | Enzyme-Linked Immunosorbent Assay  |
| FBS               | Fetal Bovine Serum                 |
| FDA               | Food and Drug Administration       |
| FITC              | Fluorescein Isothiocyanate         |

|                    |  |
|--------------------|--|
| Gdf                | Growth Differentiating Factor (gene)                     |
| G-CSF              | Granulocyte Colony Stimulating Factor                    |
| GM-CSF             | Granulocyte-Macrophage Colony Stimulating Factor         |
| Gy                 | Gray (1 joule of energy absorbed per kilogram of matter) |
| HCT                | Hematocrit   |
| HGB                | Hemoglobin   |
| HSCT               | Hematopoietic Stem Cell Transplantation                  |
| Hspa5              | Heat Shock 70 kDa Protein 5 (gene)                       |
| <sup>3</sup> H-TdR | Tritiated Thymidine                                      |
| ICAM-1             | Inter-cellular Adhesion Molecule 1                       |
| IFN- $\gamma$      | Interferon-gamma   |
| IL                 | Interleukin  |
| I.P.               | Intraperitoneal  |
| IR                 | Ionizing Radiation                                       |
| KC                 | Keratinocyte Chemoattractant                             |
| mAb                | Monoclonal Antibody                                      |
| MAPK               | Mitogen-activated Protein Kinase                         |
| MCH                | Mean Corpuscular Hemoglobin                              |
| MCHC               | Mean Corpuscular Hemoglobin Concentration                |
| MCP-1              | Monocyte Chemotactic Protein-1                           |
| MCV                | Mean Corpuscular Volume                                  |
| MHC II             | Major Histocompatibility Complex II                      |
| MIP-1 $\alpha$     | Macrophage Inflammatory Protein-1 alpha                  |

|         |   |
|---------|---|
| MMP     | Matrix Metalloproteinase  |
| MnSOD   | Manganese Superoxide Dismutase                                  |
| MPV     | Mean Platelet Volume  |
| NASA    | National Aeronautics and Space Administration                   |
| NF-kB   | Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells  |
| Nostrin | Nitric Oxide Synthase Trafficker                                |
| NK      | Natural Killer  |
| PBS     | Phosphate Buffered Saline                                       |
| PE      | R-phycoerythrin   |
| PerCP   | Peridinin Chlorophyll Protein                                   |
| PLT     | Platelet  |
| PTEN    | Phosphatase and Tensin Homolog                                  |
| qRT-PCR | Quantitative Real-time Polymerase Chain Reaction                |
| RANKL   | Receptor Activator of Nuclear Factor Kappa-B Ligand             |
| RANTES  | Regulated Upon Activation, Normal T-cell Expressed and Secreted |
| RBC     | Red Blood Cells   |
| RDW     | Red Blood Cell Distribution Width                               |
| ROS     | Reactive Oxygen Species   |
| RPE     | Retinal Pigment Epithelial Cells                                |
| RPMI    | Roswell Park Memorial Institute                                 |
| RSM     | Relative Spleen Mass  |
| SEM     | Standard Error of Mean  |



|               |                                    |
|---------------|------------------------------------|
| SGZ           | Subgranular Zone                   |
| SOD           | Superoxide Dismutase               |
| SPE           | Solar Particle Event               |
| SVZ           | Subventricular Zone                |
| TBI           | Traumatic Brain Injury             |
| Tc            | T Cytotoxic                        |
| TCR           | T Cell Receptor                    |
| Th            | T Helper                           |
| TNF- $\alpha$ | Tumor Necrosis Factor-alpha        |
| Treg          | T regulatory                       |
| VEGF          | Vascular Endothelial Growth Factor |
| WBC           | White Blood Cells                  |

## ABSTRACT OF THE DISSERTATION

Analysis of Minocycline as a Radioprotectant

by

Shalini Mehrotra

Doctor of Philosophy, Graduate Program in Biochemistry  
Loma Linda University, May 2012  
Dr. Daila S. Gridley, Chairperson

Exposure to radiation is increasing in a variety of settings including space exploration, diagnostic medical procedures and radiotherapy. Cells of the hematopoietic system, such as white blood cells (WBC), are especially sensitive to radiation and their decline can result in Acute Radiation Syndrome (ARS). Radiotherapy is often used for cancers of the central nervous system (CNS), but includes the risk for normal tissue damage, often leading to cognitive impairment. The literature suggests that *tetracyclines can be radioprotectors of the hematopoietic system with potential utility in radiation emergencies and anticancer radiotherapy*. Minocycline, a semisynthetic tetracycline derivative, has anti-inflammatory, free radical scavenging, anti-apoptotic and anti-angiogenic properties with exceptional penetration into the CNS. These qualities make *it a viable candidate for use in combination with radiotherapy for CNS tumors as a normal tissue radioprotectant and for hematopoietic recovery following whole-body irradiation*. This study was undertaken to determine the potential of minocycline as a radioprotective agent of the hematopoietic system and CNS in response to whole-body irradiation with 1, 2 and 3 Gy ( $\gamma$ -rays). C57BL/6 mice were injected with minocycline, 5 times beginning immediately before irradiation. Spleen, blood and brain were collected on days 4 and 32 post-irradiation. WBC and other cell populations were determined in

the blood and spleen while cytokines were quantified in CD3-activated splenocytes and homogenized brain supernatants. We also evaluated the impact of minocycline on DNA synthesis and viability of human glioblastoma cells versus astrocytes and microglia. Minocycline increased counts and percentages of splenic macrophages, granulocytes, natural killer (NK), T and CD8<sup>+</sup> T cells on day 4 and B cells on day 32. Minocycline up-regulated interleukin-1 $\alpha$  (IL-1 $\alpha$ ) which is radioprotective, as well as granulocyte-macrophage colony stimulating factor (GM-CSF) and G-CSF that accelerate neutrophil recovery at both time points post-exposure. Minocycline reversed the radiation-induced IL-10 decrease in the brain on day 4 while increasing vascular endothelial growth factor (VEGF), and lowering IL-1 $\beta$  on day 32. The drug did not protect glioblastoma cell lines from radiation but increased the viability of astrocytes at lower doses. These data support further testing of minocycline to counteract radiation insult to the hematopoietic system and CNS.

## CHAPTER ONE

### INTRODUCTION

#### **Radioprotectors**

Radiotherapy is commonly used to treat a wide range of cancer types. Treatment protocols aim to target cancer cells, while limiting damage to normal cells as much as possible. Radiation, however, can also cause damage to normal cells, resulting in adverse side effects depending on the sensitivity of the tissue and the dose of ionizing radiation (Hosseinimehr 2007). If the damage is sublethal, the cells may not function normally and could eventually progress to malignancy. Better tumor management using higher radiation doses can be accomplished if the surrounding normal tissues are protected against radiation insult. During space missions, normal cells and tissues of astronauts are also exposed to radiation above levels currently deemed as safe for the general population. A 2- to 3-year Mars mission could expose the astronauts to a total radiation dose up to 3.0 gray (Gy)(Simonsen, Cucinotta et al. 1993). As human space exploration proceeds and prevalence of radiation exposure due to the medical procedures (diagnostic and therapeutic) increases, there is an urgent need to identify safe and effective normal tissue radioprotectants.

A radioprotectant is a compound that is administered before exposure to ionizing radiation to minimize radiation-induced toxicity to normal tissues. Thus, a radioprotective compound should protect cells from ionizing radiation if present before or during irradiation (Manori, Kushilevsky et al. 1986). Radioprotectants can serve as

useful adjuncts during radiotherapy if they can enhance the tolerance of normal tissues and not affect the radiosensitivity of tumors. Agents with these properties would facilitate the use of higher radiation doses to improve treatment outcome. This could be achieved if the radioprotector was well taken up by normal cells compared to tumor cells or protect the oxygenated tissue more than the hypoxic tumor mass (Maisin 1998). The biological use of many of the compounds currently under investigation is, however, limited due to high cytotoxicity (Ritter 1981). An ideal radioprotector should protect normal tissues without compromising the cytotoxic effect of radiation on tumor. In essence the radioprotector should not interfere with the damaging effects of ionizing radiation on tumors. This approach would increase therapeutic benefits of radiation treatment.

Numerous approaches to radioprotection have been tried, including use of compounds that act as free radical scavengers, facilitate DNA repair and induce production of growth factors and cytokines (Maisin 1998). However, currently, the only Food and Drug Administration (FDA)-approved radioprotectant for use during treatment of solid tumors is amifostine (formerly WR-2721); but this compound is well known to have side effects such as nausea, vomiting, and severe asthenia (Schuchter, Hensley et al. 2002). Clearly there is a need to develop new radioprotectors and mitigators for normal tissue.

### **Acute Radiation Syndrome (ARS)**

Exposure to ionizing radiation can cause a variety of physiological changes known as ARS. ARS occurs following significant whole-body or partial-body radiation exposure to doses >1 Gy administered at relatively high-dose rates (Waselenko,

MacVittie et al. 2004). Different ranges of total-body irradiation manifest as different forms of injury. The rapidly proliferating cells like the spermatocytes, hematopoietic cells and cells of the gastrointestinal tract, are the most susceptible to radiation injury (Waselenko, MacVittie et al. 2004). The main clinical types of ARS can be divided into hematopoietic, gastrointestinal and cerebrovascular syndromes. As a rule, these syndromes are manifested only when the entire body, or a substantial portion of it, is exposed to photons, e.g., X-rays and  $\gamma$ -rays, or a combination of photons and particle forms of radiation such as neutrons (Heslet, Bay et al. 2012). Each syndrome consists of a prodromal phase, latent phase, manifest illness and finally recovery or death. The prodromal phase generally lasts during the first 48 hours and can extend up to 6 days post-exposure. This early phase can be characterized by anorexia, nausea, vomiting, diarrhea, mild fever, conjunctivitis and skin erythema. During the latent phase, it appears as though the person has recovered. However, this is transient and can last from several days to a month. Manifest illness is comprised of severe depression of the immune system and can last for several weeks. Recovery is generally a possibility if the person survives this phase. Clinical management of ARS depends on the absorbed radiation dose and its distribution into the body. It often includes administration of broad-spectrum antibiotics (Kim, Pollard et al. 2009), as well as blood products and colony-stimulating factors to regenerate granulocytes.

### **Hematopoietic Syndrome**

One of the major factors in mortality following radiation is damage to the hematopoietic system. The hematopoietic syndrome can occur even at lower doses

compared to other syndromes because of the high radiosensitivity of the hematopoietic cells (Dorr and Meineke 2011) which reside primarily in the bone marrow. After total-body irradiation, injury to the hematopoietic system is clearly manifested in the form of depletion in circulating blood cell populations (Samarth, Goyal et al. 2004).

Hematopoietic syndrome can occur after total- or partial-body exposure to radiation doses above 1 Gy (Waselenko, MacVittie et al. 2004). It is associated with a dramatic decrease in peripheral WBC, platelet count, red blood cell (RBC) count and, if accompanied with the lack of prompt medical intervention that includes bone marrow transplantation, can lead to death (Stone, Moulder et al. 2004).

A reduction in the populations of circulating blood cells can lead to septicemia, hemorrhage, anemia, and death. Some novel radioprotective agents can stimulate, maintain and/or induce proliferation of progenitor cells from bone marrow. Among them are cytokines that can stimulate hematopoietic stem cells (Neta and Oppenheim 1988; Moreb, Zucali et al. 1989; Neta, Oppenheim et al. 1991; Neta 1997). Figure 1.1 shows the predicted time course of changes in various cell populations in response to 3 Gy radiation, as depicted by the International Atomic Energy Agency (Vienna, Austria).

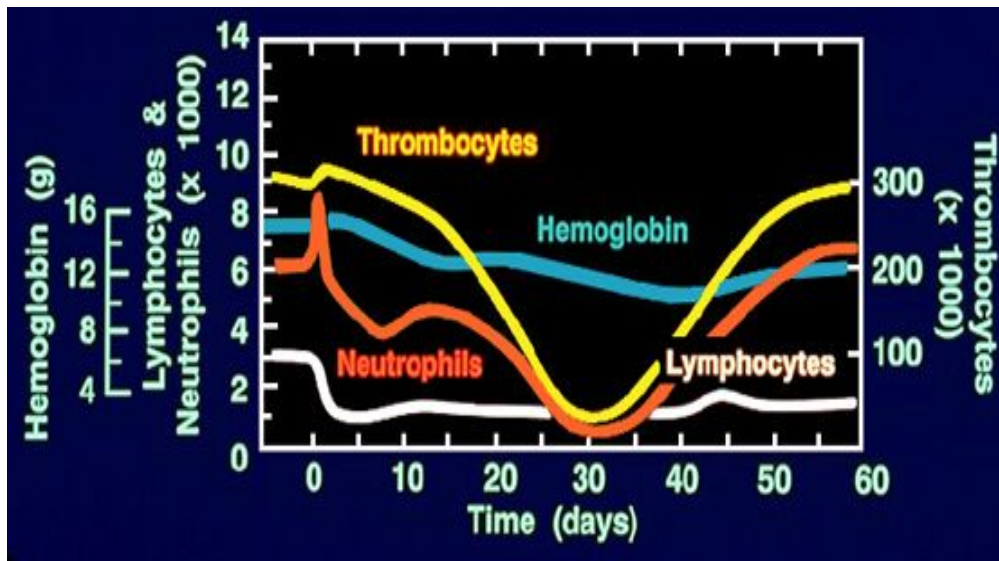


Figure 1.1. Predicted time course of changes in various cell populations in response to 3 Gy radiation. International Atomic Energy Agency (<http://www.iaea.org>).



## **Current Treatments for Radiation Injury to the Hematopoietic System**

A quantitative relationship exists between the severity of infections and the degree of neutropenia (Heslet, Bay et al. 2012). Management depends on the severity of hematopoietic syndrome and involves administration of erythrocyte concentrate, blood products and cytokines like G-CSF and GM-CSF. In more severe cases, bone marrow transplant is a treatment option (Dainiak 2010). However, transplantation is not feasible in emergencies involving a large number of people. Other radiation countermeasures are required due to this reason. Currently, there are no FDA-approved countermeasures for the management of ARS (Moroni, Coolbaugh et al. 2011).

## **Radiation and CNS Inflammation**

Radiation is a major treatment option for tumors located close to or within the CNS. Although the CNS is relatively radioresistant, recent studies have shown that the neural precursor stem cells are particularly sensitive to radiation and even relatively low doses of radiation can lead to impairment of adult postnatal neurogenesis. Neural precursor stem cells undergo apoptosis at clinically relevant doses that do not otherwise result in any overt tissue injury (Fike, Rola et al. 2007). Exposure of rats to high energy iron particles resulted in an alteration in behavior even after exposure to doses as low as 0.1 Gy (Rabin, Joseph et al. 2000). Eradication of the cancer, however is accompanied by damage to normal brain tissue and may impact cognition (Raber, Rola et al. 2004; Butler, Rapp et al. 2006; Meyers and Brown 2006). Neurocognitive deficits are an especially harmful side effect for children who undergo radiotherapy as a life-saving option. Irradiation of brain tissue often results in inflammation (Moore, Olschowka et al. 2004;

Moravan, Olschowka et al. 2011), i.e., migration of leukocytes such as neutrophils to the site of damage. It is also believed that radiation induces a neuroinflammatory milieu of pro-inflammatory cytokines and other factors, resulting in oxidative stress. This, in turn, is responsible for the pathogenesis of radiation-induced brain injury (Rola, Fishman et al. 2008; Ramanan, Zhao et al. 2010). An acute response to radiation in the brain involves an increase in inflammatory cytokines and mediators such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$ , inter-cellular adhesion molecule 1 (ICAM-1) and cyclooxygenase-2 (COX-2), as well as activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and activator protein 1 (AP-1) (Hong, Chiang et al. 1995; Raju, Gumin et al. 1999; Raju, Gumin et al. 2000). A chronic inflammatory response in the brain following radiation injury can lead to inhibition of neurogenesis (Monje, Mizumatsu et al. 2002). It has been estimated that 50% of adult and 100% of pediatric cancer survivors who receive whole-brain irradiation for metastatic disease will develop cognitive dysfunction (Ramanan, Zhao et al. 2010). This cognitive dysfunction is associated with decline of postnatal hippocampal neurogenesis (Monje, Mizumatsu et al. 2002). Patients who are subjected to cranial radiotherapy for management of primary and metastatic tumors form the most severe cases (~200,000/yr) (Abayomi 1996; Ramanan, Zhao et al. 2010).

### **Current Treatments for Radiation Injury to CNS**

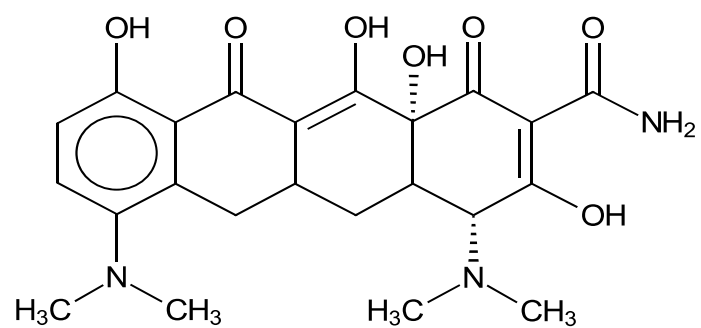
There are no preventative approaches or long-term therapies for radiation-induced cognitive impairment. Bevacizumab, a humanized monoclonal antibody against vascular

endothelial growth factor (VEGF), has very recently shown promise as a treatment option for radiation-induced necrosis in the brain (Matuschek, Bolke et al. 2011).

### **Minocycline and its Potential as a Radioprotectant**

Minocycline is a broad-spectrum semi-synthetic second generation tetracycline derivative. Tetracyclines are bacteriostatic compounds that exert their antimicrobial effect by inhibiting protein synthesis. Minocycline has a broad range of activity against both Gram-positive and Gram-negative bacteria. This drug is commonly used to treat infections due to *Neisseria meningitidis* and multi-drug resistant *Staphylococcus aureus*. It is readily absorbed in the gut when taken orally and patients have a low propensity for developing antibiotic resistance. Therefore, it is prescribed for long-term use in the management of acne and rosacea. On an average, a good safety record for long-term usage has been established for minocycline (Seukeran, Eady et al. 1997).

Figure 1.2 shows the chemical structure of minocycline.



**C<sub>23</sub>H<sub>27</sub>N<sub>3</sub>O<sub>7</sub>**

**M.W.=457.5**

Figure 1.2. Structure of Minocycline

### **Potential for Protective Effects of Minocycline in the Periphery**

Antibiotics have been employed as an important component in treating radiation injuries to minimize risk for systemic infections and facilitate recovery of tissues such as bone marrow and intestine (Thomas, Storb et al. 1976; Vriesendorp, Chu et al. 1994). Recent studies have demonstrated that minocycline possesses other potentially therapeutic effects that are distinct from its antimicrobial action. Of special importance is an *in vitro* study of 13 drugs by Epperly *et al.*; they showed that minocycline protected hematopoietic progenitor cells of the bone marrow from radiation damage (Epperly, Franicola et al. 2010).

*The above mentioned studies and other evidence support the testing of minocycline as a radioprotectant. The drug may maintain the integrity and accelerate the regeneration of hematopoietic progenitors in the bone marrow under conditions of oxidative stress.*

### **Potential for Protective Effects of Minocycline in the CNS**

Yrjanheikki et al. were the first to report that minocycline protected hippocampal neurons from death in a gerbil model of cerebral ischaemia (Yrjanheikki, Keinanen et al. 1998). Since then, minocycline has been reported to exert neuroprotective effects in animal models of traumatic brain injury, cerebral ischemia, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease and multiple sclerosis (Kim and Suh 2009; Guimaraes, Freire et al. 2010). It is small (495 kDa), well tolerated and highly lipophilic, properties which enable it to cross the blood-brain barrier (BBB) (Yong, Wells et al. 2004). It penetrates through the cerebrospinal fluid (CSF) more rapidly than

doxycycline and other tetracyclines (Macdonald, Kelly et al. 1973). Clinical trials with minocycline are underway for various neurodegenerative diseases. Importantly, some studies have demonstrated that minocycline protects retinal cells from light and oxidative stress-induced damage (Leung, Lindlief et al. 2007) and can also play a role in inhibiting angiogenesis (Maragoudakis, Peristeris et al. 1994). The retina of the eye is considered to be part of the CNS, since it forms as an outgrowth of brain tissue during embryonic development. Among the tetracycline derivatives, only minocycline possesses neuroprotective properties (Good and Hussey 2003). Krauss et al. have reported that minocycline, unlike tetracycline, possesses antioxidant and free radical scavenging properties similar to vitamin E (Kraus, Pasieczny et al. 2005). Neuroprotection by minocycline has also been attributed to its suppression of caspase activity (Stirling, Khodarahmi et al. 2004). However, the neuroprotective mechanisms of minocycline action have not yet been clearly defined. They can perhaps be attributed to the ability of minocycline to reduce microglial activation and proliferation and suppression of pro-inflammatory cytokines like TNF- $\alpha$  and interferon- $\gamma$  (IFN- $\gamma$ ). Microglial activation has been implicated in inflammatory processes that lead to neuronal cell death in neurodegenerative diseases (Dheen, Kaur et al. 2007). Indeed, microglial activation is the hallmark of brain pathology. Kim *et al.* reported that tetracyclines and fluoroquinolones can be robust protectors of the hematopoietic system and could be employed in scenarios of radiation injury (Kim, Pollard et al. 2009). Minocycline has been shown to exhibit neuroprotective actions by suppressing the activation and proliferation of microglial cells (Filipovic and Zecevic 2008). Use of a neuronal cell radioprotectant such as minocycline has potential to increase the possibility for cure without increasing risk for unwanted

complications. Blocking neuronal inflammation has been shown to restore neurogenesis (Monje, Toda et al. 2003). Owing to its anti-inflammatory and neuroprotective properties, in combination with the other mentioned non-antimicrobial properties, the testing of minocycline as a radioprotector/radiomitigator prompts further research.

***The above mentioned studies support the testing of minocycline as a potential drug for reducing radiation-induced CNS inflammation.***

### **Minocycline and Tumor Suppression**

It has been shown previously that tetracycline and its analogues can induce apoptosis in a number of cancer cell lines *in vitro* e.g., melanoma, osteosarcoma, breast, colorectal, and cervical carcinoma (Onoda, Ono et al. 2006). Sotomayor et al. demonstrated that minocycline enhanced tumor growth delay when administered along with therapies including melphalan, radiation, adriamycin and bleomycin (Sotomayor, Teicher et al. 1992). These investigators also reported that addition of minocycline to standard chemotherapy with cyclophosphamide actually resulted in delayed tumor growth (Sotomayor, Teicher et al. 1992). Teicher et al. found that minocycline has antitumor and cytotoxic properties which could be exploited in cancer treatment (Teicher, Dupuis et al. 1995). Zucker et al. reported that minocycline was capable of inhibiting melanoma cell activity *in vitro* (Zucker, Lysik et al. 1985). According to Du et al., minocycline inhibits the growth of tumor cells and attenuates ototoxicity when administered along with the chemotherapeutic drug cisplatin (Du, Zhang et al. 2011). Minocycline also exhibited antitumor effect on glioma cells and ovarian cancer cell lines (Markovic et al. 2011, Pourgholami et al. 2012).

*The above mentioned studies and evidence support the testing of minocycline along with radiation therapy as a combined treatment option for enhancing the cytotoxic effect of radiation on tumors.*

### **Purpose of this Study**

The increasing role of radiation therapy in cancer management along with the threat of nuclear or radiological terrorism urges the need for discovering agents for prophylaxis, mitigation, and treatment of radiation injury. The sensitivity of the hematopoietic system to ionizing radiation and the lack of agents to prevent or mitigate the damage gives utmost significance to our study. The purpose of the proposed study is to characterize a novel radioprotector with special emphasis on its impact on the hematopoietic system and CNS. The objectives are to characterize and identify minocycline-induced immunological effects in a mammalian model irradiated with space- and clinically-relevant doses and to determine if the drug affects glioblastoma and non-tumor cell response to radiation when exposed in culture. The conditions are unique and will provide new information relevant to a broad range of clinical applications.

In order to test the potential of minocycline in radioprotection, the following hypotheses were proposed: **1) Minocycline will protect the hematopoietic system and CNS against the damaging effects of whole-body irradiation; 2) The radioprotective effect of minocycline will be associated with increased capacity to produce anti-inflammatory factors; and 3) Minocycline will radioprotect normal CNS cells, but not glioblastoma cells, and may even exhibit tumoricidal properties.** These hypotheses were addressed in the following specific aims.



**Specific Aim 1: Determine if minocycline protects immune system cells against radiation in a mouse model.**

- A. Evaluate survival and distribution of leukocyte populations/subpopulations in blood and spleen.
- B. Characterize the cytokine profile of splenocytes to determine the capacity of minocycline to facilitate hematopoiesis post-irradiation.

**Specific Aim 2: Determine if minocycline protects the brain in an irradiated mouse model by reducing inflammation.**

- A. Perform analysis of cytokines/chemokines in the brain to determine if minocycline has potential to reduce inflammation.
- B. Assess gene expression by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), characterizing genes related to common cytokines and neurotoxicity.

**Specific Aim 3: Determine if minocycline has a differential radioprotective effect on survival (DNA synthesis and viability) of glioblastoma versus non-malignant cells.**

- A. Determine if minocycline protects brain tumor cell lines (human U87MG, T98G, A172) from cytotoxic effects of ionizing radiation.
- B. Determine if minocycline protects non-tumorigenic cell lines present in the CNS (human astrocytes and microglia) from the cytotoxic effects of ionizing radiation.

CHAPTER TWO  
ANALYSIS OF MINOCYCLINE AS A COUNTERMEASURE  
AGAINST ACUTE RADIATION SYNDROME

(Accepted for publication in *In Vivo*)

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*Key Words:* Ionizing radiation, cytokines, lymphocytes, hematopoiesis.

*Running title:* Minocycline, radioprotection and Immune System.

## Abstract

Background/Aim: To evaluate the impact of an antibiotic, minocycline on several immune parameters in response to radiation in a mouse model. Materials and Methods: C57BL/6 mice were treated with minocycline (i.p.) for 5 days beginning immediately before radiation with 1- 3 Gy  $^{60}\text{Co}$   $\gamma$ -rays. Spleen and blood were collected on day 4 post-irradiation. Cell populations were determined in the blood and spleen. Splenocytes were activated with anti-CD3 antibody for 48 h and cytokines were quantified. Results: Minocycline increased the counts and/or percentages of splenic macrophages, granulocytes, NK, T and CD8<sup>+</sup> T cells (P<0.05 vs. radiation alone). Minocycline significantly increased IL-1 $\alpha$  and  $\beta$  which are radioprotective, as well as GM-CSF and G-CSF that accelerate neutrophil recovery (P<0.05 vs. radiation alone), while suppressing cytokines that could prevent hematopoiesis, e.g. MIP-1 $\alpha$ , TNF- $\alpha$  and IFN- $\gamma$ . Conclusion: These data indicate that minocycline should be further tested for restoration of the hematopoietic system after radiation exposure.

## Introduction

Radiation exposure is a highly significant concern in events of nuclear disasters. Although cytokines and growth factors have been extensively researched as potential radioprotectors and radiomitigators (Dainiak, Waselenko et al. 2003) very few effective countermeasures are available. Consequently, protection against radiation damage to normal tissues is very important to the Departments of Defense (DOD), Energy (DOE) and Homeland Security, as well as the National Aeronautics and Space Administration (NASA). Radiation in the space environment is above levels deemed to be safe on Earth and crew members could be exposed to doses as high as 1-3 Gray (Gy) during solar particle events (SPE) (Simonsen, Cucinotta et al. 1993). In addition, radiation therapy is widely employed as a treatment option for a wide range of malignancies. Bone marrow transplant recipients also receive doses of radiation that can result in hematopoietic syndrome. Despite advancements in treatment strategies, toxicity to normal tissue still remains a cause for concern.

Exposure to ionizing radiation can cause a variety of physiological changes collectively known as Acute Radiation Syndrome (ARS). One of the major factors in mortality in patients with ARS is damage to the hematopoietic system. Exposure to doses ~0.5-1 Gray (Gy) or above (gamma or X-rays) can compromise the hematopoietic system to some extent (Singh, Singh et al. 2011). Whole-body irradiation between doses ranging from approximately 1 to 8 Gy can lead to “hematopoietic syndrome” characterized by dramatic decrease in peripheral white blood cell (WBC), platelet and, red blood cell (RBC) counts and, if accompanied with the lack of prompt medical intervention that includes bone marrow transplantation, can rapidly lead to death (Singh, Singh et al.

2011). Hematopoietic progenitor stem cells have a restricted capacity of cell division after total-body irradiation above 2 to 3 Gy (Waselenko, MacVittie et al. 2004). Numerous complications are possible, including infections and internal hemorrhage. Medical management of ARS often includes administration of broad-spectrum antibiotics (Kim, Pollard et al. 2009), as well as blood products and colony-stimulating factors to regenerate granulocytes. Although there are currently no drugs approved by the Food and Drug Administration (FDA) for treatment of ARS, the use of growth factors like granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) is included as part of the standard therapy for ARS in the United States, resulting in improved function and numbers of macrophages and granulocytes (Heslet, Bay et al. 2012). Daily injections may be needed and side effects can occur. In more severe cases, bone marrow transplant is a treatment option. However, since this is not feasible in emergencies involving a large number of people, other radiation countermeasures are required.

Antibiotics have been employed as an important component in treating radiation injuries to minimize risk for systemic infections and facilitate recovery of tissues such as bone marrow and gastrointestinal tract (Vriesendorp, Chu et al. 1994). Minocycline, a semisynthetic, second generation tetracycline derivative, is often used to treat infections due to *Neisseria meningitidis* and multi-drug resistant *Staphylococcus aureus*. Recent studies have demonstrated that minocycline possesses other potentially therapeutic effects that are distinct from its antimicrobial action. It is reported to be neuroprotective and antiapoptotic (Stirling, Koochesfahani et al. 2005). Tikka *et al.* demonstrated radioprotection of neuronal cells through minocycline by the reduction in levels of lactate

dehydrogenase, DNA fragmentation and microglial cell numbers (Tikka, Usenius et al. 2001). Krauss *et al.* found that minocycline, unlike tetracycline, possesses antioxidant and free radical scavenging properties similar to vitamin E (Kraus, Pasieczny et al. 2005). Tetracyclines have been reported to have radioprotective properties for hematopoietic cells by Kim *et al.* (Kim, Pollard et al. 2009). In this context, we hypothesized that minocycline has potential to facilitate regeneration of immune cell populations following whole-body irradiation. We further hypothesized that minocycline would enhance capacity to secrete cytokines that facilitate hematopoiesis, thus minimizing potential for serious complications associated with ARS. Day 4 was selected for the analyses because ARS can result in death within a few days. Thus, an agent that protects close to the time of irradiation and/or rapidly enhances hematopoiesis would be of considerable value. In addition, immune depression at the day 4 time point is close to the nadir after 1-3 Gy whole-body irradiation in the C57BL/6 mouse model used in the present study.

## **Materials and Methods**

### **Animals and Experimental Design**

Female C57BL/6 mice (n = 80; 8-9 weeks of age; Charles River Breeding Laboratories, Inc. Hollister, CA, USA) were housed in large plastic cages (n=10/cage) and acclimatized for 5-7 days under standard vivarium conditions. Animals were divided into 8 groups (10 mice/group): (a) deionized water (dH<sub>2</sub>O) + 0 Gy (b) dH<sub>2</sub>O + 1Gy; (c) dH<sub>2</sub>O + 2 Gy; (d) dH<sub>2</sub>O + 3 Gy; (e) Minocycline + 0 Gy; (f) Minocycline + 1 Gy; (g) Minocycline + 2 Gy; and (h) Minocycline + 3 Gy. To characterize the early effects of the drug, animals were rapidly euthanized on day 4 post-irradiation using 100% CO<sub>2</sub> in

compliance with the recommendations of the National Institutes of Health and the Panel of Euthanasia of the American Veterinary Medical Association. The protocol used was approved by the Loma Linda University Institutional Animal Care and Use Committee.

### Drug Treatment and Irradiation

Minocycline was purchased from Triax Pharmaceuticals, LLC, Cranford, NJ, USA. Animals in the respective groups were given an intraperitoneal (I.P) injection of minocycline hydrochloride (45mg/kg in a volume of 0.1 ml) immediately before irradiation or I.P. injections of dH<sub>2</sub>O. The dose of drug and timing of the injections was based on previous reports (Yrjanheikki, Keinanen et al. 1998; Yrjanheikki, Tikka et al. 1999; Zhang, Lei et al. 2004). An Eldorado unit containing a Co-60 source was used to administer whole-body radiation at a dose rate of 1.58 Gy/min for a total dose of 1, 2 or 3 Gy to the mice in the respective groups. Non-anesthetized mice were placed individually into rectangular plastic aerated boxes (30 x 30 x 85 mm<sup>3</sup>). A second injection of minocycline (45mg/kg) or dH<sub>2</sub>O was administered to the appropriate groups immediately after irradiation. This was followed by 3 consecutive injections of minocycline (22.5 mg/kg) or dH<sub>2</sub>O on the following three days post-irradiation. Similar treatment was given to sham-irradiated groups, but without the radiation.

### Blood and Spleen Collection

Blood was obtained by cardiac puncture immediately after euthanasia in 1 ml tuberculin syringes containing [K<sub>2</sub>]EDTA. Spleens were harvested and single-celled suspensions were prepared by processing the spleens in complete RPMI 1640 medium

(Irvine Scientific, Santa Ana, CA, USA) using sterile applicator sticks. Spleens were washed and centrifuged to remove debris followed by lysis of RBC using 2 ml lysis buffer for 4 min at 4°C. Splenic leukocytes were suspended in 2 ml of RPMI 1640 medium for further analysis.

#### Relative Spleen Mass (RSM)

Body weight was recorded for each mouse at the time of euthanasia and the spleens were weighed immediately after excision. Relative spleen mass was calculated as follows:  $RSM = \text{spleen mass (mg)}/\text{body mass (g)}$ .

#### Analysis of Cell Populations in Spleen and Blood

Spleen and blood cells were analyzed on an automated analyzer (HESKA<sup>TM</sup> Vet ABC- Diff Hematology Analyzer, HESKA Corp., Waukesha, WI, USA). WBC counts, as well as numbers and percentages of lymphocytes, granulocytes, and monocytes/macrophages were obtained. For the blood, additional values were obtained as follows: platelet (PLT) count, mean platelet volume (MPV), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular HGB concentration (MCHC), mean corpuscular volume (MCV), RBC distribution width (RDW), red blood cell (RBC) count and hemoglobin (HGB).

#### Flow Cytometry Analysis of Lymphocyte Populations in Spleen

The percentage of specific lymphocyte populations in the spleen was determined using monoclonal antibody (mAb) mixtures purchased from Pharmingen, San Diego, CA,



USA. A direct staining method was used to evaluate a minimum of 5,000 gated events for each sample with a FACSCalibur™ flow cytometer and CellQuest™ software version 3.1 (Becton Dickinson, Inc., San Jose, CA, USA). The mAb were conjugated with fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), allophycocyanin (APC), or peridinin chlorophyll protein (PerCP). First, the CD45 marker was used to identify the leukocytes; lymphocytes were then gated on CD45 and side scatter. mAb specific for CD3 (T cells), CD4 (T helper or Th subset), CD8 (T cytotoxic or Tc subset), CD19 (B cells), and NK1.1 (NK cells) were used for further analysis. T regulatory (Treg) cell analysis was carried out with a staining kit which included FJK-16s\*PE (anti-Foxp3), CD4\*FITC, and CD25\*APC (eBioscience, Inc., San Diego, CA, USA). The CD4<sup>+</sup>CD25<sup>-</sup> T cells, both with and without Foxp3, were quantified by gating on side scatter and the CD4<sup>+</sup> cells, followed by analysis of the CD25<sup>-</sup> versus FJK-16s<sup>-</sup> (Foxp3<sup>-</sup>) subset.

#### Activation of Splenocytes Using Anti-CD3 Coated Plates

The splenocytes were quantified after lysis of RBC using an automated hematology analyzer (HESKA Corp.). The concentration of splenocytes was adjusted to  $2 \times 10^6$  cells/ml with complete RPMI 1640 medium and 100  $\mu$ l/well was dispensed into 96-well plates coated with immobilized anti-CD3 antibody (anti-Mouse CD3 BioCoat™ T-Cell Activation, 96-well Assay Plates, BD Pharmingen, San Diego, CA, USA). One-hundred  $\mu$ l of RPMI 1640 was added to each well to make the total volume equal to 200  $\mu$ l/well. This procedure results in the activation of the T cell receptor (TCR)/CD3 signaling machinery. The plates were incubated at 37°C for 48 h and supernatants were harvested after 48 h and stored in -80°C until further analysis.

### Cytokine Analysis.

Spleen supernatants from -80°C were thawed and analyzed for 22 different cytokines and chemokines using the Mouse Cytokine/Chemokine Milliplex MAP Kit (Millipore, Billerica, MA, USA). The cytokines/chemokines evaluated were as follows: interleukin-1 $\alpha$  (IL-1 $\alpha$ ), IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, interferon- $\gamma$  (IFN- $\gamma$ ), IFN- $\gamma$ -induced protein 10 (IP-10), keratinocyte chemoattractant (KC, also known as CXCL1), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), regulated upon activation, normal T-cell expressed and secreted (RANTES) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Vascular endothelial growth factor (VEGF) was analyzed in the anti-CD3-activated supernatants using a VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

### Statistical Analysis

The data were analyzed using one-way or two-way analysis of variance (ANOVA). Tukey's test was performed for pair-wise multiple comparisons when indicated. Means and standard errors of means (SEM) are presented here; results from each mouse were included in the analyses. A *P* value of <0.05 indicated significance. SigmaStat<sup>TM</sup> software, version 2.03 (SPSS Inc., Chicago, IL, USA) was used to analyze the data.

## Results

### Relative Spleen Mass

As indicated in Figure 2.1, radiation generally decreased the spleen mass relative to body mass, i.e., the RSM ( $P < 0.05$ ). Although there was no minocycline x radiation interaction, treatment with the drug increased RSM regardless of radiation. In post-hoc analysis, minocycline significantly ( $P < 0.05$ ) increased RSM in all radiation groups except 3 Gy, where a trend for higher RSM was present ( $P < 0.1$ ).

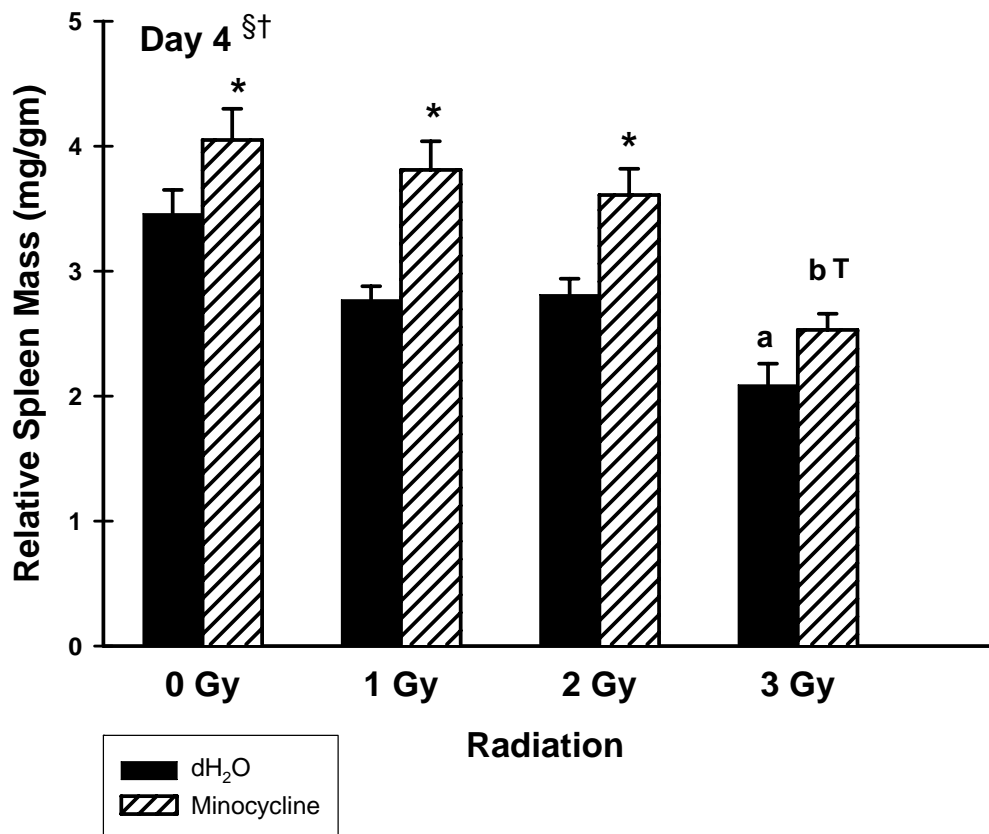


Figure 2.1 Relative Spleen Mass. Each bar represents the mean  $\pm$  SEM for n=10 mice/group. Two-way ANOVA: §, P<0.05 for main effect of radiation; †, P<0.05 for main effect of drug. Tukey test: \*, P<0.05 dH<sub>2</sub>O vs. Minocycline within each radiation dose; a, P<0.05 vs 0 Gy within dH<sub>2</sub>O groups; b, P<0.05 vs 0 Gy within Minocycline groups.

## WBC and Leukocyte Populations in Blood

Figure 2.2 shows the counts and percentages of lymphocytes, monocytes and granulocytes in the blood. Radiation generally reduced the counts of all three of these cell types ( $P < 0.05$ ), resulting in a significant radiation dose-dependent decrease in total WBC number. Addition of the drug did not significantly enhance any of the cell counts. The drug-induced decrease in lymphocytes was also apparent in the total WBC counts at 0 Gy ( $P < 0.05$  vs. dH<sub>2</sub>O) (Figure. 2). In terms of percentages, the granulocytes increased in the presence of the drug at all radiation doses while the lymphocyte percentages decreased ( $P < 0.05$ ). The changes in eosinophils counts and percentages were similar to the overall pattern of changes noted for granulocytes (Table 2.1).

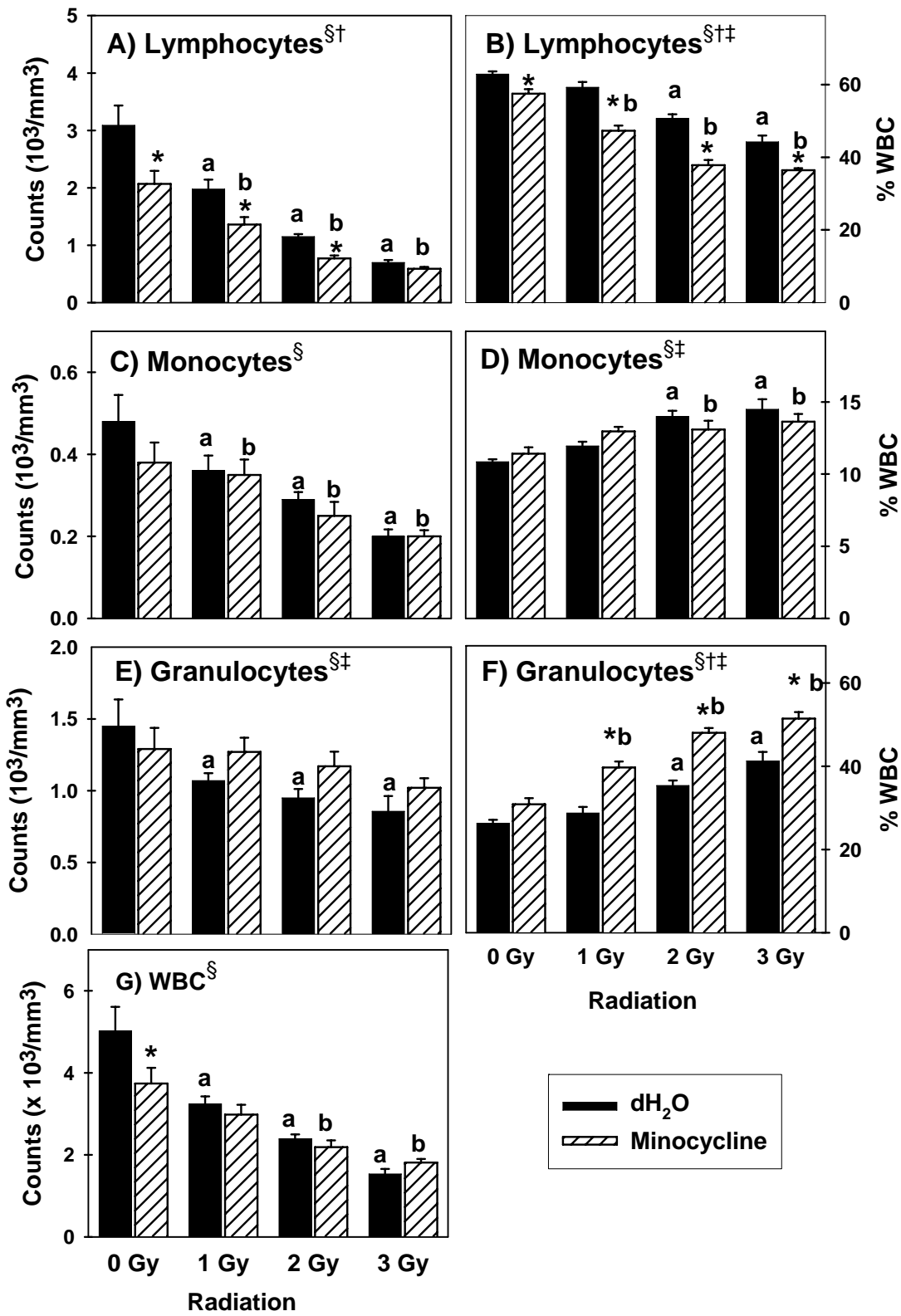


Figure 2.2 Leukocyte Populations in Blood. The data were obtained using an automated hematology analyzer. Each bar represents the mean  $\pm$  SEM for n=9-10 mice/group. Two-way ANOVA: §, P<0.05 for main effect of radiation; †, P<0.05 for main effect of drug; ‡, P<0.05 for interaction between radiation and minocycline, Tukey test: \*, P<0.05 dH<sub>2</sub>O vs Minocycline within each radiation dose; a, P<0.05 vs 0 Gy within dH<sub>2</sub>O groups; b, P<0.05 vs 0 Gy within Minocycline groups.

Table 2.1. Counts and Percentages of Eosinophils in Blood and Spleen. The data were obtained using an automated hematology analyzer (n=8-10 mice/group). Tukey test (within dH<sub>2</sub>O or Minocycline): \*,  $P < 0.05$  dH<sub>2</sub>O vs. Minocycline within each radiation dose; a,  $P < 0.05$  vs 0 Gy within dH<sub>2</sub>O groups; b,  $P < 0.05$  vs 0 Gy within Minocycline groups.

### Blood

| dH <sub>2</sub> O                                 |               |                |                           |                           | Minocycline     |                              |                             |                              |
|---|---------------|----------------|---------------------------|---------------------------|-----------------|------------------------------|-----------------------------|------------------------------|
| Test  | 0 Gy          | 1Gy            | 2 Gy                      | 3 Gy                      | 0 Gy            | 1 Gy                         | 2 Gy                        | 3 Gy                         |
| <b>EOS</b><br>(10 <sup>6</sup> /mm <sup>3</sup> ) | 0.1<br>± 0.01 | 0.08<br>± 0.01 | 0.1<br>± 0.01             | 0.07<br>± 0.01            | 0.07*<br>± 0.02 | 0.12* <sup>b</sup><br>± 0.01 | 0.12 <sup>b</sup><br>± 0.01 | 0.13* <sup>b</sup><br>± 0.01 |
| <b>EOS</b><br>(%)                                 | 2.3<br>± 0.2  | 2.4<br>± 0.2   | 4.2 <sup>a</sup><br>± 0.4 | 5.3 <sup>a</sup><br>± 0.3 | 2.8<br>± 0.1    | 4.2*<br>± 0.4                | 6.0* <sup>b</sup><br>± 0.8  | 7.5* <sup>b</sup><br>± 0.2   |

### Spleen

| dH <sub>2</sub> O                                 |                |                |                           |                             | Minocycline     |                           |                            |                            |
|---|----------------|----------------|---------------------------|-----------------------------|-----------------|---------------------------|----------------------------|----------------------------|
| Test  | 0 Gy           | 1Gy            | 2 Gy                      | 3 Gy                        | 0 Gy            | 1 Gy                      | 2 Gy                       | 3 Gy                       |
| <b>EOS</b><br>(10 <sup>6</sup> /mm <sup>3</sup> ) | 0.76<br>± 0.08 | 0.53<br>± 0.06 | 0.7<br>± 0.07             | 0.38 <sup>a</sup><br>± 0.07 | 1.02*<br>± 0.08 | 0.77*<br>± 0.1            | 1.02*<br>± 0.1             | 0.6 <sup>b</sup><br>± 0.04 |
| <b>EOS</b><br>(%)                                 | 2.2<br>± 0.1   | 2.2<br>± 0.1   | 3.2 <sup>a</sup><br>± 0.1 | 4.7 <sup>a</sup><br>± 0.1   | 2.2<br>± 0.1    | 3.2 <sup>b</sup><br>± 0.2 | 3.8* <sup>b</sup><br>± 0.2 | 4.6* <sup>b</sup><br>± 0.1 |



### WBC and Leukocyte Populations in Spleen

The distribution of lymphocytes, macrophages and granulocytes in the spleen is shown in Figure 2.3. Radiation generally caused a decline in the counts of these three leukocyte types ( $P<0.05$ ). Treatment with minocycline caused a significant increase in granulocyte and macrophage counts both in the 0 Gy and 2 Gy irradiated groups ( $P<0.05$ ), resulting in a significant main effect of drug. The pattern of changes in these leukocyte types was reflected in the total WBC counts (Figure. 2.3). A significant increase in granulocyte percentages was observed in the presence of minocycline in the 0 Gy, regardless of radiation ( $P<0.05$ ). The lymphocyte percentages decreased significantly in all groups ( $P<0.05$ ). Splenic eosinophil counts and percentages showed similar patterns as the granulocytes (Table 2.1).

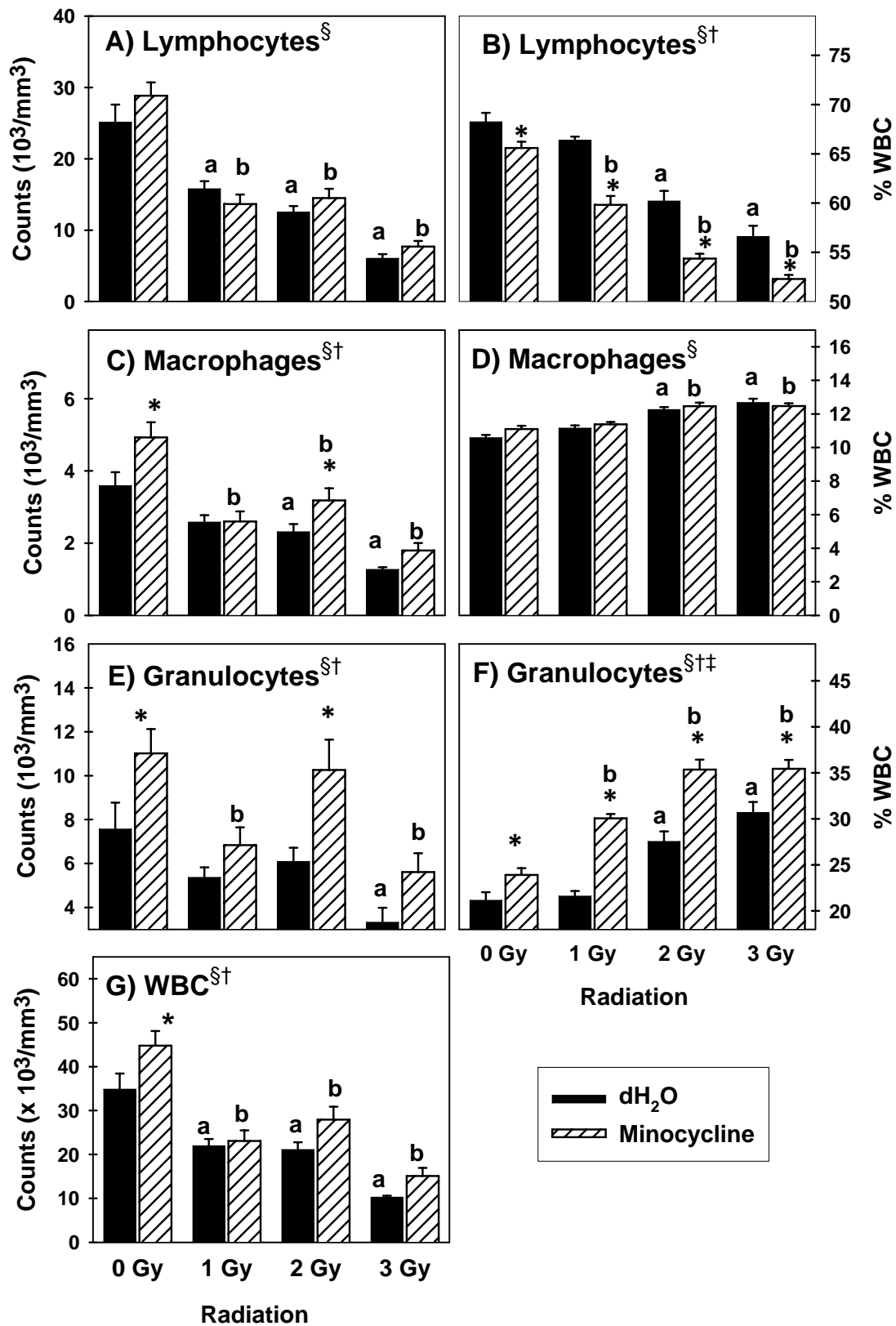


Figure 2.3. Leukocyte Populations in Spleen. The data were obtained using an automated hematology analyzer. Each bar represents the mean  $\pm$  SEM for n=9-10 mice/group. Two-way ANOVA: §, P<0.05 for main effect of radiation; †, P<0.05 for main effect of drug, Tukey test: \*, P<0.05 dH<sub>2</sub>O vs Minocycline within each radiation dose.

## Flow Cytometric Analysis of Lymphocyte Populations in Spleen

Radiation caused a decrease in the T, B and NK cell counts ( $P < 0.05$ , Figure. 2.4). There were main effects of minocycline on the T cell and NK cell populations ( $P < 0.05$ ). In the post-hoc analysis, addition of the drug significantly increased the T cell counts at 0 Gy, 2 Gy and 3 Gy ( $P < 0.05$  vs. the counterpart groups without drug). Minocycline alone increased the B cell counts in the 0 Gy group ( $P < 0.05$ ). Although drug-treated groups showed consistently higher NK cell counts, significance was found only at 2 Gy ( $P < 0.05$ ).

Consistent with the total T cell count, radiation caused a decline in the CD4<sup>+</sup> Th and CD8<sup>+</sup> Tc cells ( $P < 0.05$ , Figure. 2.4). While there was no significant minocycline impact on these two subsets, the high counts in the 0 Gy and 2 Gy groups ( $P < 0.05$ ) resulted in a main effect of the drug ( $P < 0.05$ ). Both radiation and minocycline generally increased CD4:CD8 ratios ( $P < 0.05$ ). However, for radiation this was primarily due to changes in the 2 and 3 Gy groups. Treatment with the drug generally mitigated or abolished the radiation-induced increases at the higher doses ( $P < 0.05$ ), resulting in a significant drug x radiation interaction ( $P < 0.05$ ).

When looking at the percentages (Figure. 2.5), radiation caused a decrease in B cells with corresponding increases in the T and NK cell populations ( $P < 0.05$ ). Since minocycline caused a significant proportional increase in B cells at 0 Gy ( $P < 0.05$ ), there was a significant main effect of the drug ( $P < 0.05$ ) and a drug x radiation interaction ( $P < 0.05$ ). Radiation-induced increases in NK cell percentages were generally augmented when mice were treated with minocycline. However, in post-hoc analysis, the drug-

induced increase reached significance only in the 2 Gy group ( $P<0.05$ ), resulting in a drug x radiation interaction ( $P<0.05$ ).

Overall, radiation increased Th cell percentages while decreasing Tc cells ( $P<0.05$ , Figure. 2.5). The drug did not significantly impact the proportion of Th cells. Although, the drug caused an increase in Tc cell percentages ( $P<0.05$ ), significance was found only at 2 Gy in post-hoc analysis ( $P<0.05$ ); this led to a significant drug x radiation interaction ( $P<0.05$ ).

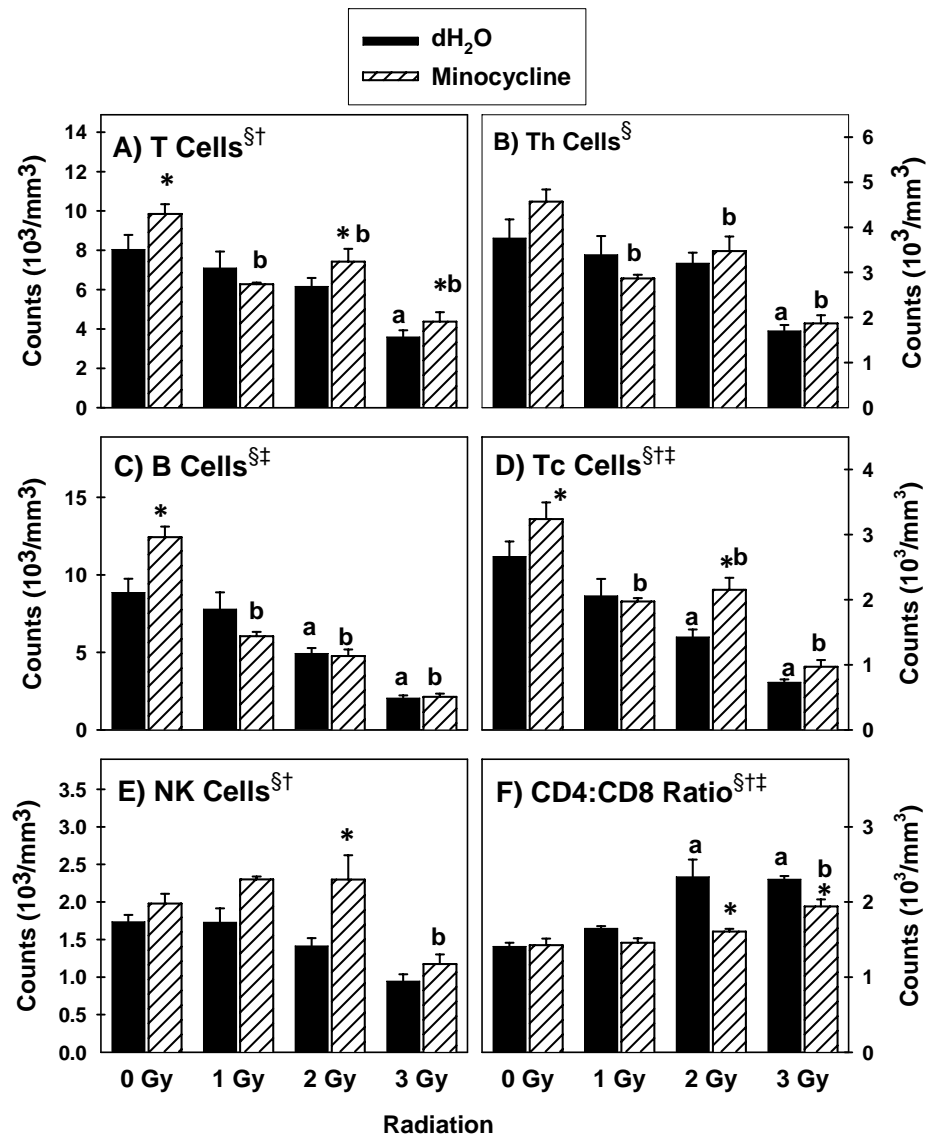


Figure 2.4. Lymphocyte Populations in Spleen. The data were obtained using fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean  $\pm$  SEM for  $n=5-10$  mice/group. Two-way ANOVA: §,  $P<0.05$  for main effect of radiation; †,  $P<0.05$  for main effect of drug. Tukey test: \*,  $P<0.05$  dH<sub>2</sub>O vs. Minocycline within radiation doses; ‡,  $P<0.05$  for interaction between radiation and minocycline. Tukey test: \*,  $P<0.05$  dH<sub>2</sub>O vs. Minocycline within each radiation dose; a,  $P<0.05$  vs 0 Gy within dH<sub>2</sub>O groups; b,  $P<0.05$  vs 0 Gy within Minocycline groups.

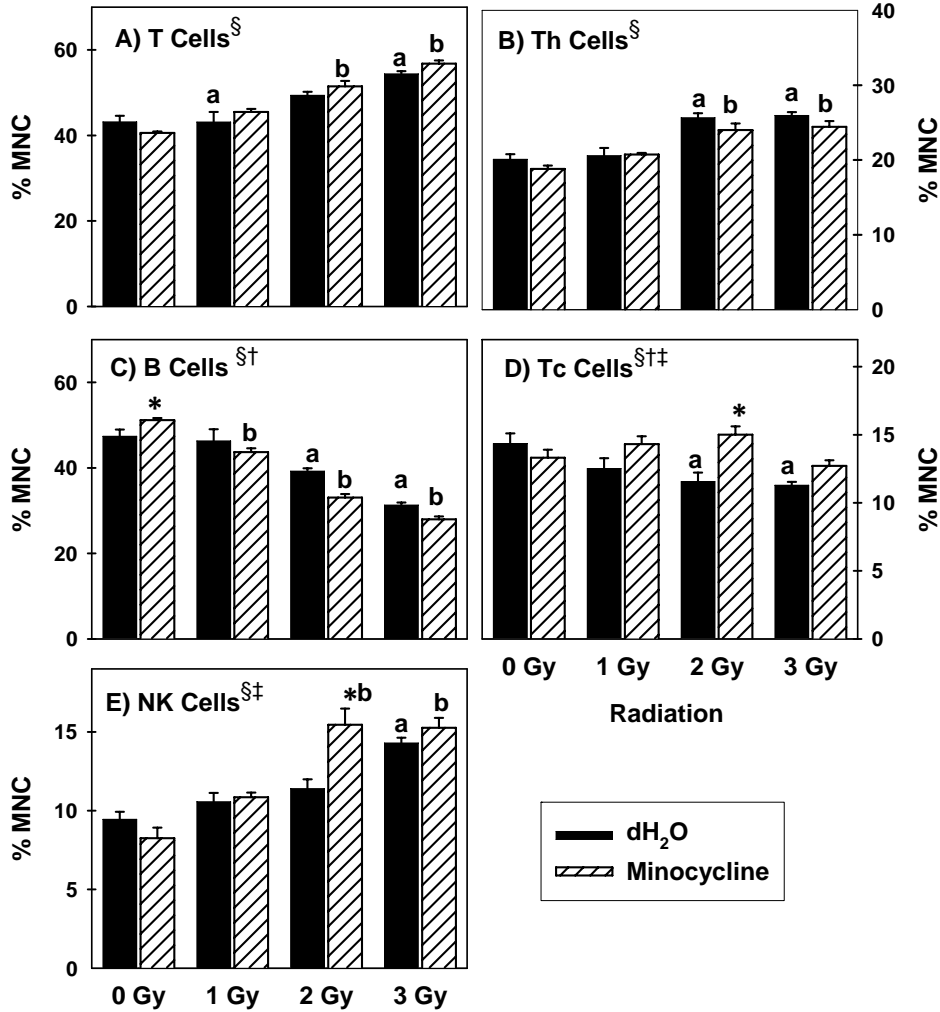


Figure 2.5. Lymphocyte Populations in Spleen. The data were obtained using fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean  $\pm$  SEM for n=5-10 mice/group. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; †,  $P < 0.05$  for main effect of drug; ‡,  $P < 0.05$  for interaction between radiation and minocycline. Tukey test: \*,  $P < 0.05$  dH<sub>2</sub>O vs Minocycline within radiation doses; †,  $P < 0.05$  for interaction between radiation and minocycline. Tukey test: \*,  $P < 0.05$  dH<sub>2</sub>O vs Minocycline within each radiation dose; a,  $P < 0.05$  vs 0 Gy within dH<sub>2</sub>O groups; b,  $P < 0.05$  vs 0 Gy within Minocycline groups.

### Flow Cytometric Analysis of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T Cells in Spleen

Radiation generally increased the CD4<sup>+</sup>CD25<sup>+</sup> T cell counts (Figure. 2.6). Addition of minocycline caused a significant increase at 0 Gy and 2 Gy ( $P<0.05$ ). This resulted in significant main effects of radiation and drug on these cells ( $P<0.05$ ). Radiation also caused a significant decrease in the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cell counts ( $P<0.05$ ). Although minocycline-treated groups had consistently higher counts, significance was found only at 0 Gy ( $P<0.05$ ). This resulted in significant main effects of radiation and drug on the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Treg population ( $P<0.05$ ).

While radiation generally increased the percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells with increasing dose, addition of minocycline significantly enhanced the effect of radiation at 1 Gy and 2 Gy ( $P<0.05$ ; Figure. 2.6). This resulted in main effects of radiation and drug on CD4<sup>+</sup>CD25<sup>+</sup> T cell percentages ( $P<0.05$ ). Radiation at 2 Gy and 3 Gy increased the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Treg cell percentages significantly ( $P<0.05$ ). At 1 Gy, the percentage of these cells was higher compared to the group with no drug ( $P<0.05$ ).



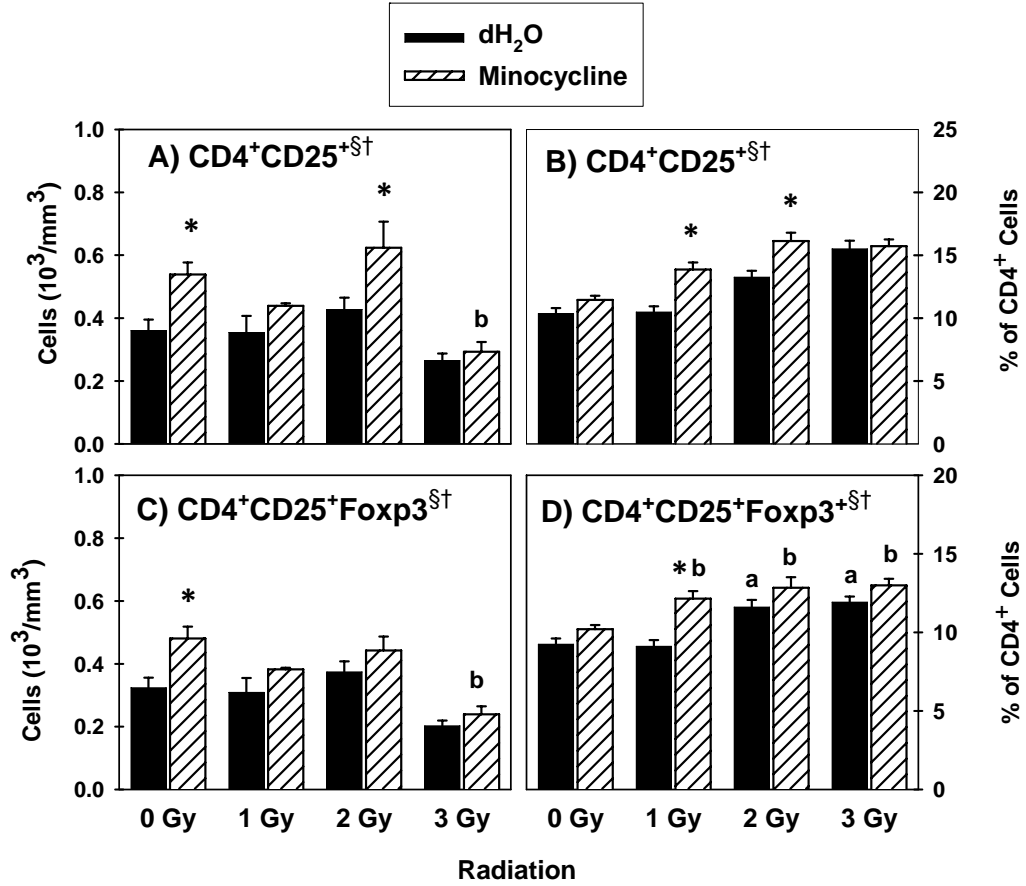


Figure 2.6. T Regulatory Cells in Spleen. The data were obtained using fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean  $\pm$  SEM for  $n=5-10$  mice/group. Two-way ANOVA: §,  $P<0.05$  for main effect of radiation; †,  $P<0.05$  for main effect of drug; ‡,  $P<0.05$  for interaction between radiation and minocycline. Tukey test: \*,  $P<0.05$  dH<sub>2</sub>O vs Minocycline within radiation doses; †,  $P<0.05$  for interaction between radiation and minocycline. Tukey test: \*,  $P<0.05$  dH<sub>2</sub>O vs Minocycline within each radiation dose; a,  $P<0.05$  vs 0 Gy within dH<sub>2</sub>O groups; b,  $P<0.05$  vs 0 Gy within Minocycline groups.

## Cytokines in Spleen Supernatants

Numerous changes occurred in cytokine levels that were dependent on minocycline, radiation, or both. Hence, for the sake of clarity in the discussion, as well as simplicity, each cytokine was assigned to one of two groups. Group I included cytokines that went up in the presence of the drug, while Group II included cytokines that went down in the presence of the drug.

### **Group I Cytokines**

This group included G-CSF, GM-CSF, IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-10, IL-13, IL-17 and RANTES (Figure.2.7). Two-way ANOVA showed that radiation had a significant main effect ( $P<0.05$ ) on the levels of all of these cytokines except IL-10, IL-17 and RANTES, while a main drug effect was noted for all except IL-5. A drug x radiation interaction ( $P<0.05$ ) was noted for all of the cytokines in this group except for IL-10 and RANTES.

Post-hoc Tukey analysis revealed that minocycline significantly enhanced the concentrations of IL-13 and RANTES in non-irradiated and all irradiated groups compared to their counterparts that did not receive the drug ( $P<0.05$ ). Significant drug-induced increases in G-CSF, GM-CSF and IL-1 $\beta$  occurred in all irradiated groups. With respect to the remaining cytokines, spleen supernatants from irradiated mice treated with minocycline had significantly higher levels of IL-1 $\alpha$ , IL-4, IL-5, IL-6, IL-10 and IL-17 compared to their counterparts exposed to 1, 2 and/or 3 Gy ( $P<0.05$ ).

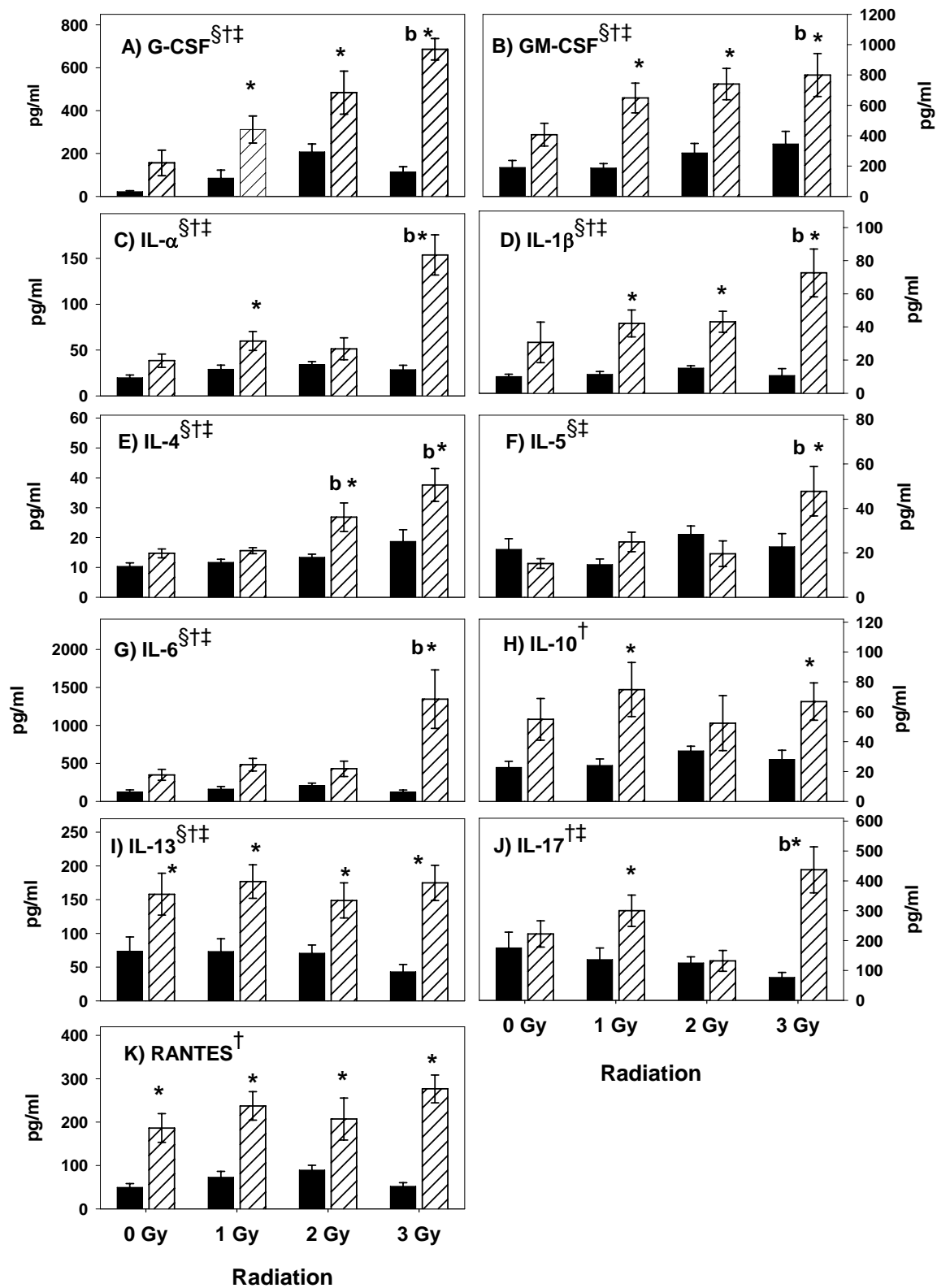


Figure 2.7. Quantitative Analysis of G-CSF, GM-CSF, IL-1 $\beta$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-10, IL-13 and IL-17 and RANTES. Each bar represents the mean  $\pm$  SEM for n=8-10 mice/group. Two-way ANOVA: §, P<0.05 for main effect of radiation; †, P<0.05 for main effect of drug; ‡, P<0.05 for interaction between radiation and minocycline. Tukey test: \*, P<0.05 dH<sub>2</sub>O vs Minocycline within each radiation dose; a, P<0.05 vs 0 Gy within dH<sub>2</sub>O groups; b, P<0.05 vs 0 Gy within Minocycline groups.

## **Group II Cytokines**

This group consisted of IFN- $\gamma$ , TNF- $\alpha$ , MCP-1, MIP-1 $\alpha$ , KC, IP-10, IL-7, IL-12(p70), IL-2, VEGF and IL-15 (Figure 2.8). Two-way ANOVA showed that radiation had a significant main effect on the levels of all of these cytokines except IL-12(p70) and that minocycline had a main effect on all of them ( $P < 0.05$ ). A significant drug x radiation interaction was noted for IFN- $\gamma$ , TNF- $\alpha$ , MCP-1, KC, IL-7, IL-12(p70) and IL-2 ( $P < 0.05$ ).

The post-hoc Tukey test revealed that radiation, especially at the higher doses, significantly enhanced the levels of IFN- $\gamma$ , TNF- $\alpha$ , MCP-1, MIP-1 $\alpha$ , KC, IP-10 and VEGF and decreased the levels of IL-2 and IL-7 compared to 0 Gy ( $P < 0.05$ ). However, addition of minocycline frequently minimized these radiation-induced changes. Mice treated with the drug had significantly lower IFN- $\gamma$ , MIP-1 $\alpha$ , IL-7 and VEGF concentrations in spleen supernatants ( $P < 0.05$ ) compared to their irradiated counterparts, regardless of dose; low levels were also noted compared to 0 Gy ( $P < 0.05$ ). In addition, the drug reduced the enhancing effects of radiation on MCP-1, KC, IP-10 and IL-15, although statistical significance was not obtained at all radiation doses.

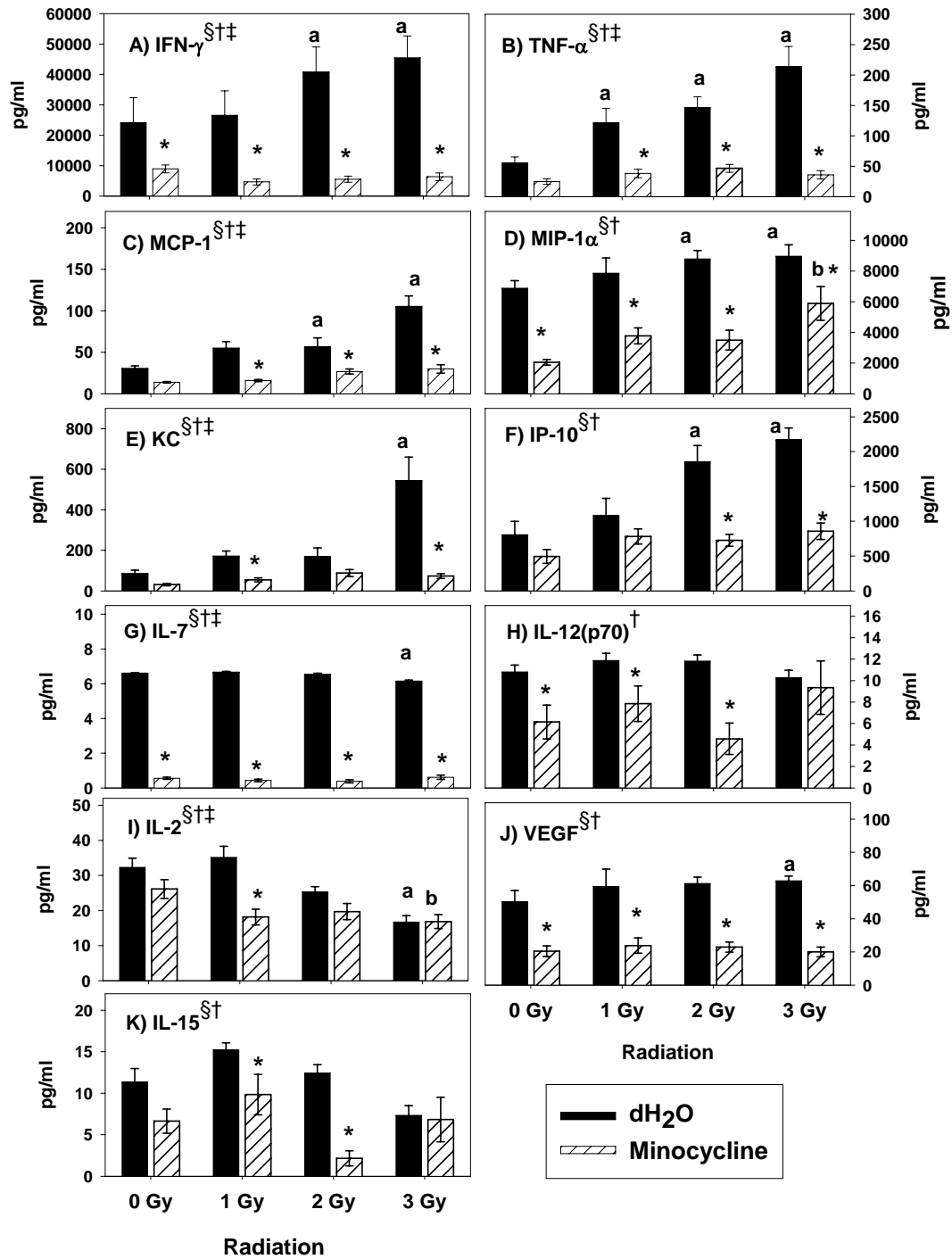


Figure 2.8. Quantitative Analysis of IFN- $\gamma$ , TNF- $\alpha$ , MCP-1, MIP-1 $\alpha$ , KC, IP-10, IL-7, IL-12(p70), IL-2, VEGF and IL-15. Each bar represents the mean  $\pm$  SEM for n=8-10 mice/group. Two-way ANOVA: §, P<0.05 for main effect of radiation; †, P<0.05 for main effect of drug; ‡, P<0.05 for interaction between radiation and minocycline. Tukey test: \*, P<0.05 dH<sub>2</sub>O vs Minocycline within each radiation dose; a, P< 0.05 vs 0 Gy within dH<sub>2</sub>O groups; b, P<0.05 vs 0 Gy within Minocycline groups.

## Discussion

Our study is unique in that it characterized the impact of minocycline on important immune parameters relevant to ARS in an irradiated mammalian model. In essence, the results indicate that the drug has potential to promote hematopoietic recovery after radiation exposure. The significantly increased spleen mass relative to body mass, i.e. the RSM, is a broad indicator that supports minocycline as an enhancer of hematopoiesis. Indeed, spleen weight has long been used as an index for determining hematopoietic recovery after irradiation (Popp, Congdon et al. 1965).

A serious consequence of radiation injury is bone marrow failure, resulting in leukopenia that can influence morbidity and mortality (Gale 1987). Agents that act as radiation protectants/mitigators when used near the time of radiation exposure are urgently needed. Many of the compounds under investigation stimulate the differentiation of stem cells in bone marrow (Citrin, Cotrim et al. 2010). In the present study, there were numerous differences among groups in cytokine concentrations and cell distribution patterns. Since the major goal was to quantify the efficacy of minocycline as a radioprotectant, as well as for the sake clarity, most of the remainder of the discussion is divided into two sections based on the modulatory effects of the drug. Group I cytokines were generally increased by minocycline, whereas the Group II cytokines were decreased.

### Group I: Cytokines Up-Regulated by Minocycline and Related Cell Populations

Treatment with minocycline increased G-CSF, GM-CSF and IL-1 $\beta$  in spleen supernatants from irradiated mice. Furthermore, these increases were incrementally



enhanced with increasing radiation dose. Similar, although less pronounced, radiation-dependent drug effects were noted in IL-1 $\alpha$ , IL-4, IL-5 and IL-6.

Although T cells in the spleen samples were activated with anti-CD3 mAb, other cell types (e.g., fibroblasts), may have contributed to the levels of G-CSF, GM-CSF, IL-5 and IL-6, particularly in the groups treated with minocycline (Baiocchi, Scambia et al. 1993). However, the pattern for all of these cytokines is remarkably similar to the granulocyte percentages in the spleen (as well as in blood). This is important because the assay involved a constant number of leukocytes per well and proportional differences likely played a dominant role in the cytokine response. Neutrophils tend to persist when microenvironmental signals that involve infection are present (Colotta, Re et al. 1992) and proportional increase in this population may be due to similar signals generated by radiation-induced cell debris. This increased survival could lead to increases in the presence of cytokines produced by this population.

Indeed, neutrophils, the major type of granulocyte, and monocytes can secrete GM-CSF, IL-1 $\alpha$  and IL-1 $\beta$  (Cassatella 1995). In addition, GM-CSF induces production of IL-1, IL-6 and G-CSF by neutrophils (Cicco, Lindemann et al. 1990). Although IL-1 $\alpha$  and IL-1 $\beta$  are two distinct cytokines, they bind to the same receptors (Luheshi, Rothwell et al. 2009). Regardless of the source, a minocycline-induced increase in these cytokines is important because they can be radioprotective or aid in recovery post-exposure (Neta 1997). For instance, lethality due to total-body irradiation is decreased substantially by G-CSF treatment (Sureda, Valls et al. 1993). G-CSF and GM-CSF hasten myeloid progenitor cell proliferation (Okano, Suzuki et al. 1989); GM-CSF also inhibits radiation-induced apoptosis (Collins, Marvel et al. 1992) and promotes eosinophil growth and

function(Owen, Rothenberg et al. 1987). Administration of IL-1 has improved survival in animals (Peterson, Adamovicz et al. 1994). Radioprotective mechanisms of IL-1 include: a) induction of bone marrow cells into the relatively radioresistant S phase of the cell cycle (Neta, Sztein et al. 1987); b) induction of manganese superoxide dismutase (MnSOD) (Eastgate, Moreb et al. 1993) which protects against oxidative stress (Borrelli, Schiattarella et al. 2009); and c) facilitation of granulopoiesis (Gershanovich, Filatova et al. 2001). Interestingly, some researchers also mention that IL-1 can be cytotoxic to tumors (Neta, Douches et al. 1986).

Although the drug generally increased IL-4, IL-5 and IL-6, its impact was most prominent in the mice that also received 3 Gy. While a common factor among these cytokines is activation of T or B cells (Hirano, Yasukawa et al. 1986), they also have other activities. IL-4 and IL-5 are Th2 cell-derived cytokines (Kopf, Le Gros et al. 1993), IL-4 stimulates hematopoietic progenitor cell proliferation (Peschel, Paul et al. 1987) and IL-5 promotes proliferation and differentiation of eosinophils (Lampinen, Carlson et al. 2004). The up-regulation of IL-5 in the presence of minocycline may explain the correlative increase in eosinophils. Similar patterns were observed for IL-6, a cytokine produced by dendritic cells, macrophages and B cells (Diehl and Rincon 2002) that promotes differentiation of the Th2 lineage while inhibiting Th1 cell differentiation (Diehl and Rincon 2002). IL-6 enhances hematopoietic recovery by accelerating multilineage hematopoiesis and increasing myeloid progenitors (Okano, Suzuki et al. 1989; Neta 1997); its administration after lethal radiation promotes survival in animals (Peterson, Adamovicz et al. 1994).

For IL-10 and IL-17, the drug response was biphasic, where significant drug-induced increases occurred only in the 1 Gy and 3 Gy treated mice. Values for the 0 Gy and 2 Gy groups, however, were consistently higher when minocycline was included. IL-10 is produced by macrophages, Th2 cells and especially Treg cells (Asadullah, Sterry et al. 2003), while IL-17 is produced by the CD4<sup>+</sup> (Fossiez, Djossou et al. 1996) and CD8<sup>+</sup> T cell subsets (Hinrichs, Kaiser et al. 2009). However, the pattern of secretion was not reflected in the proportions of the mentioned cells. IL-10 inhibits the suppressive activity of IFN on hematopoiesis (Geissler, Kabrna et al. 2002). The minocycline-induced increase in IL-10 is in accordance with previous observations (Lee, Yune et al. 2003). An interesting additional point is that IL-10 may decrease risk for fibrosis (Nelson, Tu et al. 2003; Zhang, Zheng et al. 2007), a serious late consequence of radiation treatment. IL-17, similarly to IL-10, induces the production of hematopoietic cytokines, e.g. IL-6 and G-CSF (Fossiez, Djossou et al. 1996).

IL-13 and RANTES, were increased by minocycline, but radiation had no detectable influence. In contrast to other cytokines in Group I, IL-13 is produced by Th2 (McKenzie, Emson et al. 1998), NK (Hoshino, Winkler-Pickett et al. 1999) and dendritic cells (Bellinghausen, Brand et al. 2003), as well as eosinophils (Gessner, Mohrs et al. 2005; Spencer, Szela et al. 2009). The minocycline-induced increases found here are consistent with reports that the drug preferentially stimulates Th2 cells over Th1 (Chen, Ma et al. 2011), thus enhancing IL-13 production. Furthermore, the high eosinophil percentages observed in the presence of the drug could also contribute to this response. Since IL-13 stimulates the growth of hematopoietic progenitor cells (Jacobsen, Okkenhaug et al. 1994), minocycline could help speed up recovery. RANTES is secreted

by CD8<sup>+</sup> T cells (Walzer, Marcais et al. 2003; Catalfamo, Karpova et al. 2004), including memory T cells that tend to be relatively radioresistant (Yao, Jones et al. 2011). This could explain the lack of a radiation response on the level of this chemokine despite an overall decrease in total lymphocyte proportions. NK cells also produce RANTES (Zeng, Chen et al. 2006) and this correlated with increased NK cell percentages. RANTES attracts monocytes, eosinophils and T cells to sites of tissue damage (Alam, Stafford et al. 1993; Gao, Kuhns et al. 1993), is required for normal T cell function (Makino, Cook et al. 2002), and increases CD8<sup>+</sup> T cell counts (Crawford, Angelosanto et al. 2011).

#### Group II: Cytokines/Chemokines Down-Regulated by the Drug and Related Cell Populations

While radiation increased production capacity for IFN- $\gamma$ , TNF- $\alpha$ , MCP-1, MIP-1 $\alpha$ , KC, and IP-10, minocycline dramatically reduced their expression. Since many of these cytokines inhibit hematopoietic activity, the minocycline-induced decreases further support a role for this drug in hematopoietic recovery. The low level of IFN- $\gamma$  and TNF- $\alpha$  noted here is consistent with the literature (Kloppenburger, Verweij et al. 1995). Both of these cytokines can inhibit hematopoiesis, but also have potent pro-inflammatory effects (Peetre, Gullberg et al. 1986; Murase, Hotta et al. 1987; Eng, Car et al. 1995). Activation of the p38 mitogen-activated protein kinase (p38 MAPK) pathway can lead to suppression of hematopoiesis. This pathway also activates TNF- $\alpha$  and IFN- $\gamma$  which are myelosuppressive cytokines (Navas, Mohindru et al. 2006). Minocycline has been shown to inhibit the p38 MAPK pathway (Tikka, Fiebich et al. 2001). The drug may be helpful in restoration of hematopoiesis because it inhibits this pathway.

MCP-1 is produced by keratinocytes and attracts monocytes, basophils and T cells (Nakamura, Williams et al. 1995). Like IFN- $\gamma$  and TNF- $\alpha$ , MIP-1 $\alpha$  down-regulates the proliferation of hematopoietic progenitor cells (Moore 1991). Karpus and Kennedy found that anti-MIP-1 $\alpha$  treatment prevented the occurrence of acute experimental autoimmune encephalitis (Karpus and Kennedy 1997). Since minocycline decreased both MIP-1 $\alpha$  and MCP-1 production, it may aid in repression of inflammation that occurs after radiation insult.

KC, a chemokine that attracts neutrophils and monocytes, has been reported to increase in response to radiation (Ao, Zhao et al. 2009). Recent studies have shown that it can be produced by pulmonary fibroblasts in response to radiation and thus may be utilized as a marker of radiation-induced lung injury (Ao, Zhao et al. 2009). Monocytes, lymphocytes, keratinocytes and endothelial cells produce IP-10 in response to IFN- $\gamma$  (Cassatella, Gasperini et al. 1997). As with many of the other cytokines in this group, IP-10 inhibits early hematopoietic progenitors (Sarris, Broxmeyer et al. 1993). It can also inhibit the actions of GM-CSF (Aronica, Mantel et al. 1995). Finally, IP-10 has been implicated in radiation-induced lung fibrosis (Keane, Arenberg et al. 1997).

In contrast to the first 6 cytokines in Group II, the impact of radiation on IL-7, IL-12(p70) and VEGF was minimal (albeit significant for both IL-7 and VEGF), while minocycline caused significant decreases in most groups of mice. IL-7 is produced by stromal cells in the bone marrow, spleen, thymus and gut (van Roon, Glaudemans et al. 2003) and is important for development of B and T cells (Akashi, Kondo et al. 1998). Administration of IL-7 to patients with metastatic melanoma or sarcoma has decreased the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells (Rosenberg, Sportes et al. 2006). The Treg

cells contain inflammation and prevent carcinogenesis by reducing cellular damage and cell proliferation (Zamarron and Chen 2011). It has also been reported that naturally occurring Treg cells secrete high amounts of thioredoxin which confers increased tolerance to oxidative stress (Mougiakakos, Johansson et al. 2011). However, IL-7 has also been reported to promote development of T-cell lymphoma and acute lymphoblastic leukemia (Or, Abdul-Hai et al. 1998). Thus, the very low level of IL-7 in the minocycline groups suggests that risk for these types of malignancies may be decreased by the drug.

IL-12 is produced by dendritic cells, macrophages and B cells (Heufler, Koch et al. 1996). Golab *et al.* found that this cytokine has myelosuppressive properties and slows down hematopoiesis in the bone marrow (Golab, Stoklosa et al. 1998). Although IL-12 promotes hematopoiesis in the spleen, this does not fully compensate for its depressive effect on cells in the bone marrow (Sarris, Broxmeyer et al. 1993). Also, since IL-12 induces the production of IFN- $\gamma$  by Th1 cells (Robertson and Ritz 1996), the low IL-12 levels correlate well with the reduced IFN- $\gamma$  levels seen in our study.

VEGF is typically produced by endothelial cells, platelets and macrophages (Helotera and Alitalo 2007; Duffy A.M., Bouchier-Hayes D. J. et al. 2012). Previous researchers have shown that levels of this cytokine tend to increase after radiation exposure (Park, Qiao et al. 2001). VEGF is a potent promoter of angiogenesis and helps to facilitate tumor growth. Thus, the minocycline-induced reduction in the high VEGF levels seen in the irradiated groups adds to the potential of this drug for utilization during cancer radiotherapy.

Like other cytokines in Group II, levels of IL-2 and IL-15 induced by anti-CD3 mAb were generally decreased by minocycline treatment. These findings are consistent

with reports that the drug inhibits T cell activation (Giuliani, Hader et al. 2005). IL-2 is produced by T lymphocytes while activated splenic dendritic cells can produce IL-15 in response to IFN- $\gamma$  (Mattei, Schiavoni et al. 2001), the latter being a product of activated Th1 cells. Venkataraman *et al.* have reported that radiation causes increased production and decreased utilization of IL-2 by mononuclear cells (Venkataraman and Westerman 1990). Thus, suppression of T cell activation by minocycline is a likely explanation for reduced IL-2 and IL-15 levels. However, unlike all of the other cytokines in Group II, IL-2 and IL-15 levels generally decreased with increasing radiation dose. This is consistent with the low T cell proportions in the irradiated groups. These two cytokines share many biological properties such as promotion of T cell proliferation and NK cell development. However, while IL-15 is primarily implicated in inflammatory diseases, IL-2 can both promote and control inflammation (Hoyer, Doms et al. 2008).

### **Conclusions**

In summary, our data demonstrate that treatment with minocycline modulates capacity to produce cytokines related to hematopoiesis in irradiated and non-irradiated mice. The drug also counteracted radiation-induced declines in certain cell populations, especially monocytes/macrophages, granulocytes, NK cells, T cells and CD8<sup>+</sup> T cells in the spleen. Protection of these cell populations can be attributed to the cytokine pattern obtained, with special emphasis on increased G-CSF, GM-CSF, IL-1 $\alpha$ , IL-1 $\beta$  levels and decreased IFN- $\gamma$ , TNF- $\alpha$ , MIP-1 $\alpha$ , IL-12(p70) and IL-15 levels. Minocycline up-regulated both pro- and anti-inflammatory cytokines/chemokines and cell populations that could promote, as well as contain, inflammation. This implies that the drug may be

balancing the radiation-induced inflammatory response from getting out of control, while simultaneously enhancing the body's capacity to maintain innate and adaptive immune cell types. Overall, the results do indicate that minocycline up-regulates production capacity for several cytokines that have been strongly implicated in myelorestitution. The findings also suggest that the drug may be utilized to speed up hematopoietic recovery post-irradiation. Further testing of minocycline should be carried out to confirm its potential as a countermeasure against ARS and as an addition during cancer radiotherapy.

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CHAPTER THREE  
EFFECTS OF MINOCYCLINE ON HEMATOPOIETIC RECOVERY  
AFTER WHOLE-BODY IRRADIATION

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*Running title:* Minocycline, radioprotection and Immune System.

## Abstract

This study was undertaken to determine if minocycline treatment close to the time of whole-body irradiation has a prolonged effect on immune parameters. C57BL/6 mice were treated with minocycline intraperitoneally for 5 days beginning immediately before irradiation with 1, 2 and 3 Gy  $^{60}\text{Co}$  gamma-rays in a single fraction. Spleen, blood and plasma were collected on day 32 post-exposure. Cell populations were determined in the blood and spleen. Splenic T cells were activated with anti-CD3 monoclonal antibody and supernatants were collected after 48 h. Cytokines were quantified in spleen supernatants and plasma. A number of parameters were significantly ( $P < 0.05$ ) affected at this relatively late time point of assessment post-irradiation. 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 significantly increased the levels of cytokines including interleukin-1 $\alpha$  (IL-1 $\alpha$ ) 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 certain cytokines that could prevent hematopoiesis, e.g. interferon- $\gamma$ . Overall, the data suggest that minocycline exerts a relatively long-term effect on certain cell populations and cytokine production capacity. Further testing of this drug as a countermeasure for Acute Radiation Syndrome is necessary to realize its full potential.

## Introduction

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 due to 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. There is increasing concern also in occupational settings. For example, astronauts on space missions can get exposed to radiation doses within the range of 1-3 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, there is an urgent need for safe and effective normal tissue radioprotectants and radiomitigators.

Radiation can cause a variety of physiological changes collectively referred to as Acute Radiation Syndrome (ARS). The hematopoietic system is most sensitive to radiation (Dorr and Meineke 2011) and one of the early manifestations of ARS is the hematopoietic syndrome. Indeed, declines in circulating white blood cell (WBC), lymphocyte, platelet 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 Gray (Gy). Management of hematopoietic syndrome includes administration of colony-stimulating factors that promote granulocyte and macrophage regeneration (G-CSF, GM-CSF), blood products like erythrocyte concentrate and hematopoietic stem cell transplantation (HSCT), depending upon the severity of the case (Dainiak 2010). Clinical management of radiation injury also includes administration of antibiotics, e.g.,

fluoroquinolones with broad-spectrum activity, to minimize risk for systemic infections and facilitate recovery of tissues such as bone marrow and gastrointestinal tract (Thomas, Storb et al. 1976; Vriesendorp, Chu et al. 1994). However, administration of antimicrobials, as well as antiemetics, antidiarrheal agents and analgesics, is considered primarily as supportive care.

Evidence in the literature suggests that tetracyclines can be robust radioprotectors of the hematopoietic system with potential utility in radiation emergencies and anticancer radiotherapy. Minocycline 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, anti-apoptotic, neuroprotective (Stirling, Koochesfahani et al. 2005), and free radical scavenging effects (Kraus, Pasieczny et al. 2005) and also possesses anti-tumorigenic potential (Teicher, Dupuis et al. 1995). Given the properties of minocycline, we hypothesized that it would facilitate regeneration of immune cell populations following whole-body irradiation and thus have potential to minimize serious complications associated with ARS. Our previous results obtained at day 4 post-irradiation were very promising (manuscript accepted for publication). 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 a long-term effect, as opposed to a transient/short-lived effect, on the assessed parameters.

## Materials and Methods

### Animals and Experimental Design

Female C57BL/6 mice (n = 80; 8-9 weeks of age; Charles River Breeding Laboratories, Inc. Hollister, CA) were acclimatized 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 (dH<sub>2</sub>O) + 0 Gy; b) dH<sub>2</sub>O + 1 Gy; c) dH<sub>2</sub>O + 2 Gy; d) dH<sub>2</sub>O + 3 Gy; e) Minocycline + 0 Gy; f) Minocycline + 1 Gy; g) Minocycline + 2 Gy; and h) Minocycline + 3 Gy. Animals were rapidly sacrificed on day 32 post-irradiation using 100% CO<sub>2</sub> in compliance with the recommendations of the National Institutes of Health and the Panel of Euthanasia of the American Veterinary Medical Association. The study was approved by the Loma Linda University Institutional Animal Care and Use Committee prior to initiation.

### Drug Treatment and Irradiation

Minocycline hydrochloride was purchased from Triax Pharmaceuticals, LLC, Cranford, NJ. Animals in the respective treatment groups received an injection of the drug (45mg/kg in a volume of 0.1 ml) or an equivalent volume of dH<sub>2</sub>O, intraperitoneally (i.p.) immediately before irradiation. A retired Co-60 therapy unit was used to administer whole-body  $\gamma$ -radiation at a dose rate of 1.58 Gy/min for a total dose of 1, 2 or 3 Gy. Rectangular plastic aerated cubicles (30 x 30 x 85 mm<sup>3</sup>) were used to immobilize each non-anesthetized mouse during exposure. A second injection of minocycline (45mg/kg) or dH<sub>2</sub>O was administered to the appropriate groups immediately after irradiation,

followed by three injections of minocycline (22.5 mg/kg) or dH<sub>2</sub>O on three consecutive days post-irradiation. The sham-irradiated groups were given similar treatment.

### Blood and Spleen Collection

Cardiac puncture was performed immediately after euthanasia to collect blood in 1 ml tuberculin syringes containing [K<sub>2</sub>]EDTA. Spleens were harvested and processed into single-celled suspensions in complete RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) using sterile applicator sticks. Washing and centrifugation of the spleens was carried out to remove the debris, followed by lysis of RBC using 2 ml lysis buffer for 4 min at 4°C. Splenic leukocytes were suspended in 2 ml of RPMI 1640 medium and whole blood samples were then analyzed for various cell populations as described below.

### Body and Relative Spleen Mass (RSM)

Each mouse was weighed at the time of euthanasia and the spleens were weighed immediately after excision. The following formula was used to calculate relative spleen mass:  $RSM = \text{spleen mass (mg)}/\text{body mass (g)}$ .

### Analysis of White Blood Cells (WBC) in Blood and Spleen

An automated analyzer (HESKA<sup>TM</sup> Vet ABC- Diff Hematology Analyzer, HESKA Corp., Waukesha, WI) was used to quantify WBC and major leukocyte populations in blood and spleen. Total WBC counts, as well as numbers and percentages of lymphocytes, granulocytes, and monocytes/macrophages were obtained. Although the spleen contains a reservoir of monocytes, in addition to macrophages (Swirski,

Nahrendorf et al. 2009), 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: platelet (PLT) counts, mean platelet volume (MPV), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular HGB concentration (MCHC), mean corpuscular volume (MCV), RBC counts, RBC distribution width (RDW), and hemoglobin (HGB).

### Flow Cytometry 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 purchased from Pharmingen, San Diego, CA as previously described (Kajioka, Gheorghe et al. 1999). T regulatory (Treg) cell analysis was carried out with a staining kit which included FJK-16s\*PE (anti-Foxp3), CD4\*FITC, and CD25\*APC (eBioscience, Inc., San Diego, CA). The CD4<sup>+</sup>CD25<sup>-</sup> T cells, both with and without Foxp3, were quantified by gating on side scatter and the CD4<sup>+</sup> cells, followed by analysis of the CD25<sup>-</sup> versus FJK-16s<sup>-</sup> (Foxp3<sup>-</sup>) subset.

### Splenocyte Activation Using Anti-CD3 mAb

The splenocytes were quantified after lysis of RBC using an automated hematology analyzer (HESKA Corp.). After concentration was adjusted to  $2 \times 10^6$  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). Each well contained  $4 \times 10^5$  cells in 0.2 ml of medium.

After a 48 h incubation at 37°C in a humidified chamber, the supernatants were harvested and stored in -80°C until further analysis.

### Cytokine Analysis

Immediately after thawing, spleen supernatants were analyzed using the Mouse Cytokine/Chemokine Milliplex MAP Kit purchased from Millipore, Billerica, MA. The 22 cytokines/chemokines were: interleukin-1 $\alpha$  (IL-1 $\alpha$ ), IL-1 $\beta$ , 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- $\gamma$  (IFN- $\gamma$ ), IFN- $\gamma$ -induced protein 10 (IP-10), keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), regulated upon activation, normal T-cell expressed and secreted (RANTES) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). In order to quantify vascular endothelial growth factor (VEGF), an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) was used.

### Statistical Analysis

The data were analyzed using SigmaStat<sup>TM</sup> software, version 2.03 (SPSS Inc., Chicago, IL) Two-way analysis of variance (ANOVA) and, when indicated, Tukey's test was done 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 Relative Spleen Mass (RSM)

Overall, the values for body mass ranged from  $22.34 \pm 0.25$  g (Minocycline + 0 Gy) to  $21.2 \pm 0.2$  g (dH<sub>2</sub>O + 3 Gy). No statistical difference in RSM was observed regardless of minocycline treatment or irradiation. RSM ranged from  $3.6 \pm 0.2$  (dH<sub>2</sub>O + 3 Gy) to  $4.2 \pm 0.2$  (Minocycline + 0 Gy).

### WBC and Leukocyte Populations in Blood

As shown in Figure 3.1, the WBC count ranged from  $3.3 \times 10^3/\text{mm}^3$  to  $4.9 \times 10^3/\text{mm}^3$ . Radiation had a main effect on the WBC counts due to decreasing counts with increasing dose. In post-hoc analysis, significantly low 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 addition of the drug did not have a significant impact (Figure. 3.1).

Although radiation had a main effect on both monocytes 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 in lymphocytes. Addition of minocycline brought the lymphocyte percentages close to normal in the 3 Gy 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 x radiation interaction for granulocytes ( $P < 0.05$ ).

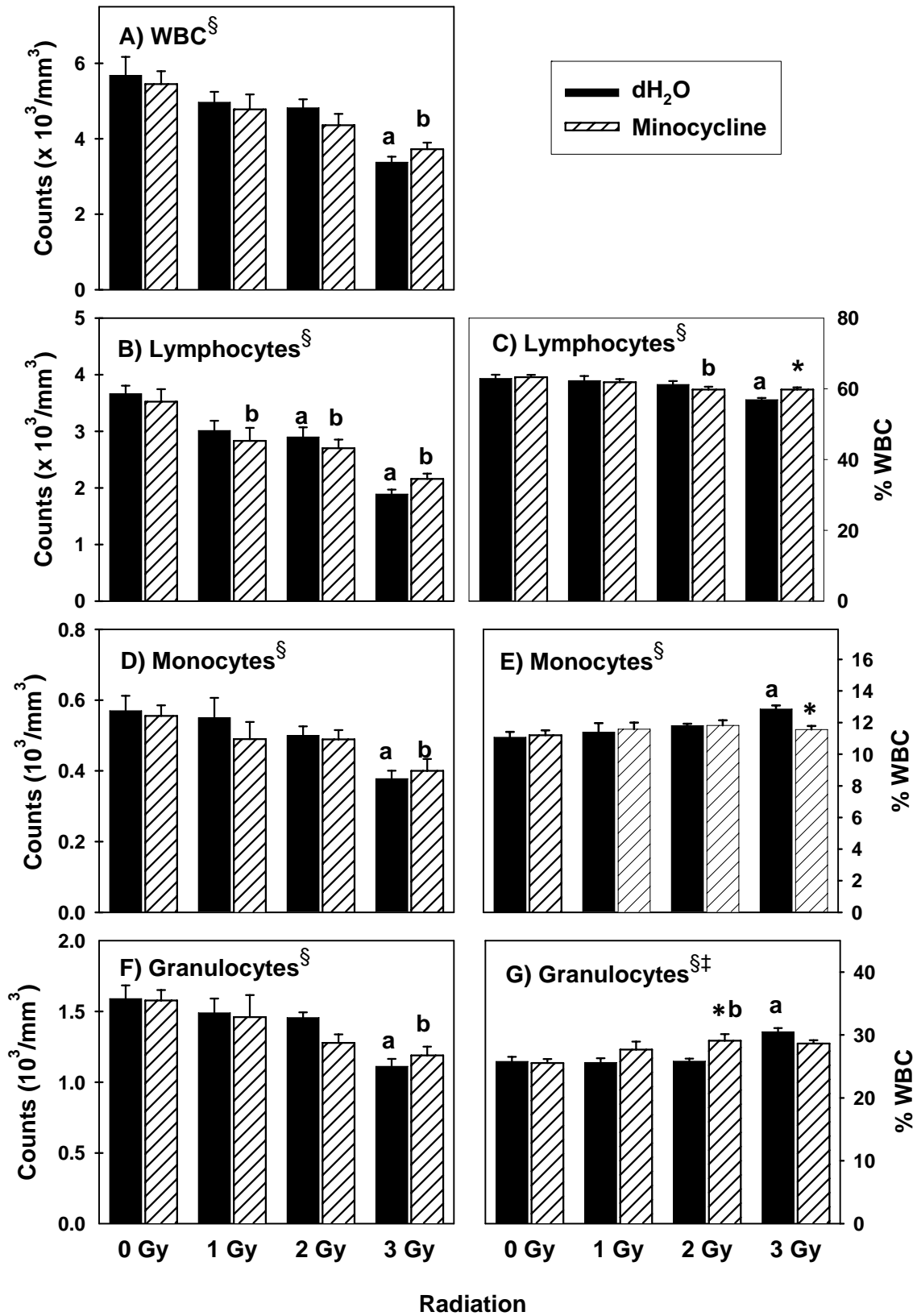


FIGURE. 3.1. Major Leukocyte Populations in Blood. An automated hematology analyzer was used to obtain data for  $n = 9-10$  mice/group. Each bar represents the mean  $\pm$  SEM. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; ‡,  $P < 0.05$  for drug x radiation interaction, Tukey test: \*,  $P < 0.05$  for dH<sub>2</sub>O vs Minocycline within each radiation dose; a,  $P < 0.05$  vs 0 Gy within dH<sub>2</sub>O groups; b,  $P < 0.05$  vs 0 Gy within Minocycline groups.

## PLT and RBC Characteristics in Blood

The results of thrombocyte and erythrocyte characteristics are listed in Table 3.1. Radiation generally decreased the PLT counts and increased MPV ( $P<0.05$ ). Although the changes primarily occurred in the 2 Gy and 3 Gy 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 counts ( $P<0.05$ ), this effect only reached significance in the 3 Gy groups ( $P<0.05$ ) and there were no significant drug x radiation interactions.

Radiation had main effects on RBC counts and HCT, generally decreasing both of these 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 increased MCV ( $P<0.05$ ) post-hoc analysis showed no differences between groups. The drug-treated groups generally had lower MCV with significant differences at 3 Gy. However, this is likely due to the artificially low significant error in the 3 Gy minocycline treated group. The low error is because all the data for that group was equivalent to 46 because the machine only gives out integers. Although there was no radiation or drug effect on MCH, a drug x 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 groups (without drug) had higher values compared to 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.

Table 3.1. Summary of Platelet and Erythrocyte Characteristics in Blood. The data were obtained using an automated hematology analyzer (n = 8-10 mice/group). Mino: Minocycline. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; †,  $P < 0.05$  for main effect of drug; ‡,  $P < 0.05$  for drug x radiation interaction. Tukey test (within dH<sub>2</sub>O or Minocycline): \*,  $P < 0.05$  dH<sub>2</sub>O vs. Minocycline within each radiation dose; a,  $P < 0.05$  vs. 0 Gy within dH<sub>2</sub>O groups.

|   |                   | Dose       |                       |            |                       |
|---|-------------------|------------|-----------------------|------------|-----------------------|
|   |                   | 0 Gy       | 1 Gy                  | 2 Gy       | 3 Gy                  |
| <b>PLT<sup>§†</sup></b><br><b>(10<sup>5</sup>/mm<sup>3</sup>)</b> | dH <sub>2</sub> O | 845.4±11.8 | 612.0±36.7            | 615.9±20.8 | 626.6±15.6            |
|   | Mino              | 799.8±18.0 | 639.9±19.6            | 570.6±17.0 | 546.5±14.7*           |
| <b>MPV<sup>§</sup></b> (μm <sup>3</sup> )                         | dH <sub>2</sub> O | 5.2±0.05   | 5.1±0.02              | 5.2±0.03   | 5.4±0.1               |
|   | Mino              | 5.3±0.03   | 5.2±0.04              | 5.4±0.05*  | 5.4±0.02              |
| <b>RBC<sup>§</sup></b><br><b>(10<sup>6</sup>/mm<sup>3</sup>)</b>  | dH <sub>2</sub> O | 9.0±0.1    | 8.2±0.2 <sup>a</sup>  | 8.5±0.2    | 8.3±0.1 <sup>a</sup>  |
|   | Mino              | 8.7±0.2    | 8.5±0.2               | 8.6±0.2    | 8.3±0.2               |
| <b>HGB (g/dl)</b>   | dH <sub>2</sub> O | 13.6±0.1   | 12.6±0.4              | 13.0±0.1   | 13.0±0.2              |
|   | Mino              | 13.0±0.2   | 13.0±0.3              | 13.0±0.3   | 13.0±0.2              |
| <b>HCT<sup>§</sup> (%)</b>  | dH <sub>2</sub> O | 41.1±0.3   | 37.2±0.5              | 33.8±0.7   | 30.8±1.3              |
|   | Mino              | 40.0±0.3   | 37.2±0.6              | 33.0±1.1   | 31.8±1.1              |
| <b>MCV<sup>§</sup></b> (μm <sup>3</sup> )                         | dH <sub>2</sub> O | 45.60±0.16 | 46.30±0.30            | 45.89±0.20 | 46.70±0.21            |
|   | Mino              | 46.00±0.21 | 46.00±0.00            | 46.30±0.15 | 46.00±0.00*           |
| <b>MCH<sup>‡</sup></b> (pg)                                       | dH <sub>2</sub> O | 15.1±0.1   | 15.3±0.1              | 15.2±0.1   | 15.4±0.2              |
|   | Mino              | 15.3±0.1   | 15.3±0.1              | 15.5±0.1   | 15.1±0.1              |
| <b>MCHC (g/dl)</b>  | dH <sub>2</sub> O | 33.1±0.2   | 33.0±0.3              | 33.0±0.2   | 33.0±0.2              |
|   | Mino              | 33.0±0.2   | 33.0±0.01             | 33.0±0.2   | 33.0±0.01             |
| <b>RDW<sup>§†</sup> (%)</b>                                       | dH <sub>2</sub> O | 14.4±0.1   | 14.8±0.1 <sup>a</sup> | 14.7±0.1   | 15.0±0.1 <sup>a</sup> |
|   | Mino              | 14.4±0.1   | 14.7±0.1              | 14.6±0.1   | 14.8±0.1*             |

### WBC and Leukocyte Populations in Spleen

Figure 3.2 shows that the WBC counts in the spleen ranged from  $38.3 \times 10^3/\text{mm}^3$  to  $49.9 \times 10^3/\text{mm}^3$ . 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 dH<sub>2</sub>O + 3 Gy group compared to the dH<sub>2</sub>O + 1 Gy group ( $P < 0.05$ ) in post-hoc analysis. The distribution of lymphocytes, macrophages and granulocytes is also shown in Figure 3.2. 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 the lymphocyte percentages decreased significantly at 0 Gy in the presence of minocycline ( $P < 0.05$ ), this effect did not result in significant main effects or interactions. 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.

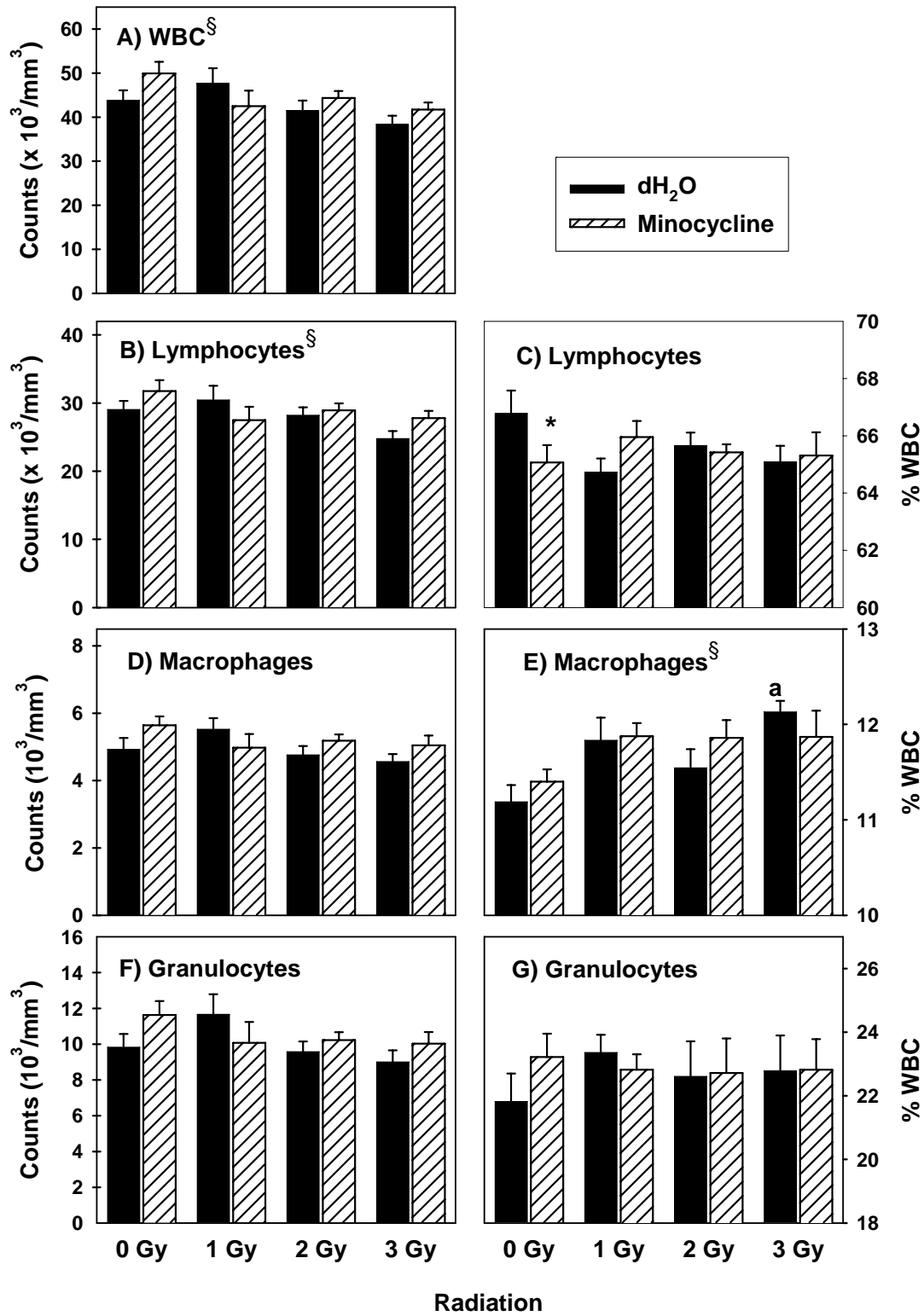


FIGURE. 3.2. Major Leukocyte Populations in Spleen. An automated hematology analyzer was used to obtain data for  $n = 9-10$  mice/group. Each bar represents the mean  $\pm$  SEM. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; Tukey test: \*,  $P < 0.05$  for dH<sub>2</sub>O vs Minocycline within each radiation dose; a,  $P < 0.05$  vs 0 Gy within dH<sub>2</sub>O groups.



## Flow Cytometric Analysis of Lymphocyte Populations in Blood

Radiation generally caused a decline in the circulating T, B and CD8<sup>+</sup> Tc cell counts (Figure. 3.3), 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 group that received minocycline. Although radiation generally decreased the CD4<sup>+</sup> Th cell counts (trend,  $P<0.1$ ), no significance was obtained in post-hoc analysis. The CD4:CD8 ratio generally increased with increasing radiation doses ( $P<0.05$ ). The NK cell counts were not significantly affected by either drug or radiation. In terms of percentages (Figure. 3.4), radiation alone decreased 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 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 drug.

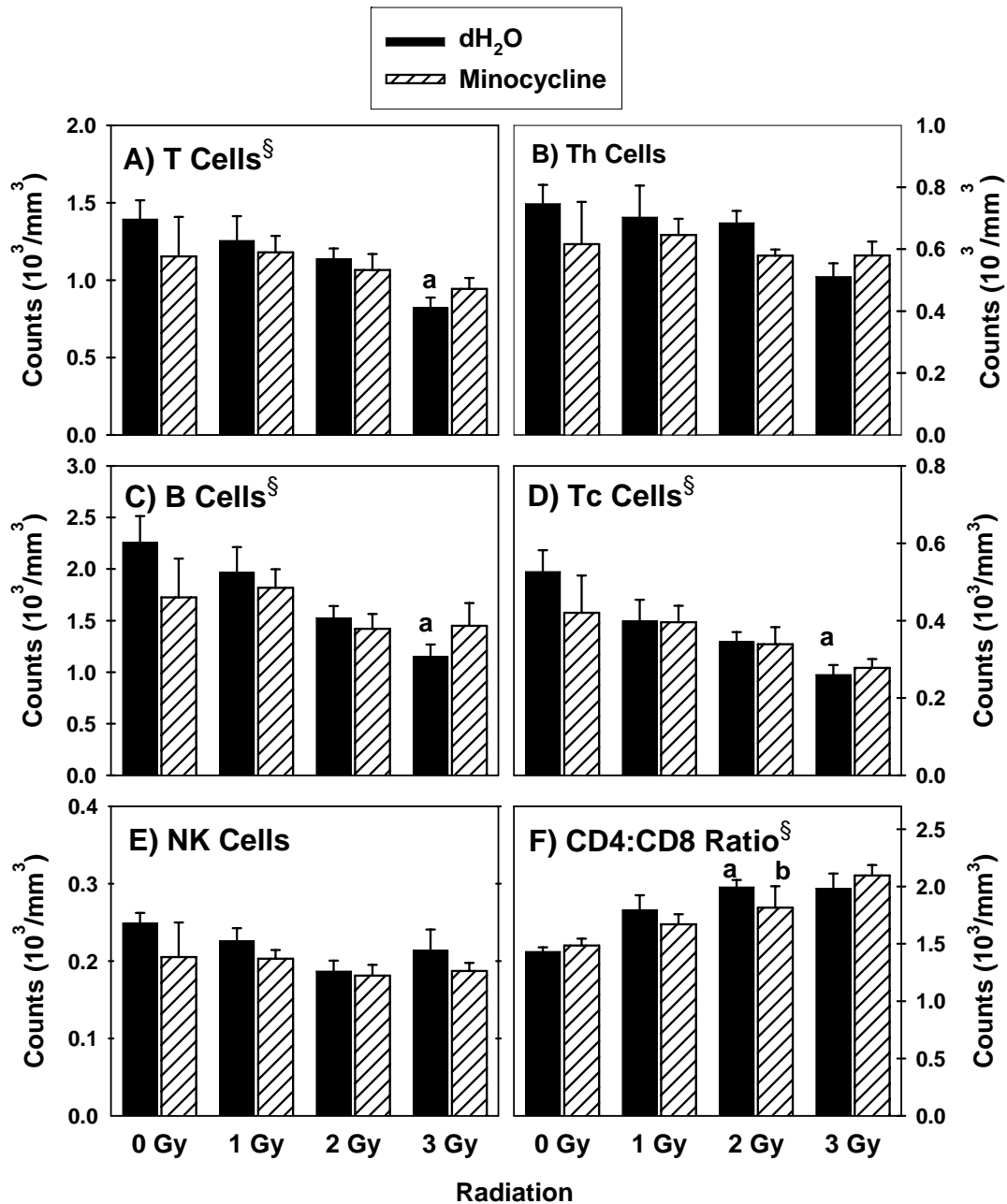


Figure. 3.3. Lymphocyte Population Counts in Blood. The data for  $n = 8-10$  mice/group are based on fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean  $\pm$  SEM. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation. Tukey test: a,  $P < 0.05$  vs 0 Gy within dH<sub>2</sub>O groups; b,  $P < 0.05$  vs 0 Gy within Minocycline groups.

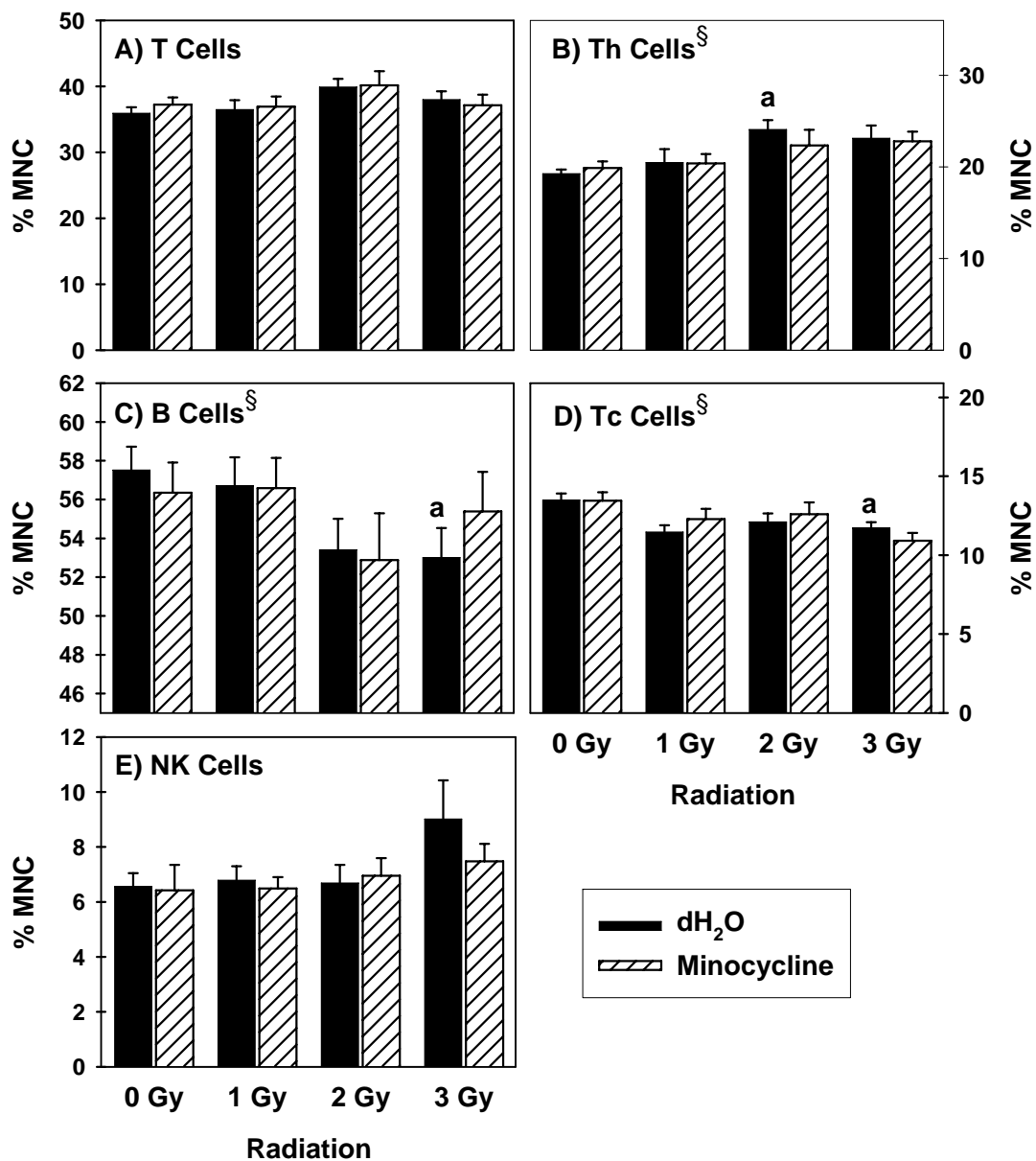


Figure. 3.4. Percentages of Lymphocyte Population in Blood. The data for  $n = 8-10$  mice/group are based on fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean  $\pm$  SEM. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation. Tukey test: a,  $P < 0.05$  vs 0 Gy within dH<sub>2</sub>O groups; b,  $P < 0.05$  vs 0 Gy within Minocycline groups.

## Flow Cytometric Analysis of Lymphocyte Populations in Spleen

Radiation had no significant impact on T, B, NK cell counts (Figure. 3.5). While there was no significant main effect of drug on T cell counts, they were significantly reduced in the 1 Gy minocycline-treated group compared to 1 Gy alone ( $P<0.05$ ) in post-hoc analysis. A significant drug x radiation interaction was present for B cells ( $P<0.05$ ). This was due to the fact that radiation significantly decreased the B cell counts at 3 Gy ( $P<0.05$ ), but not when minocycline treatment was included. While radiation had no main effect on NK cell count, the cells were significantly reduced at 2 Gy in the drug-treated group compared to 2 Gy alone ( $P<0.05$ ). Radiation also did not have a significant effect on the CD4<sup>+</sup> Th or CD8<sup>+</sup> Tc cell counts (Figure. 3.5). However, minocycline significantly decreased the counts for both of these subsets at 1 Gy ( $P<0.05$  vs. the counterpart groups without drug). A main effect of radiation was seen on the CD4:CD8 ratio which significantly increased with increasing radiation dose ( $P<0.05$ ). Addition of minocycline did not alter this increase.

When looking at the percentages (Figure. 3.6), radiation generally increased the level of T cells. Addition of minocycline reversed the radiation effect at 2 and 3 Gy, resulting in a significant drug x radiation interaction ( $P<0.05$ ). Minocycline caused a significant proportional increase in B cells at 2 Gy and 3 Gy ( $P<0.05$  vs. the respective irradiated groups that did not receive drug), resulting in a significant main effect of the drug ( $P<0.05$ ) and a drug x radiation interaction ( $P<0.05$ ). The NK cell percentages were significantly reduced in the presence of minocycline at 2 Gy ( $P<0.05$ ). Radiation caused an increase in Th cell and a decrease in Tc cell percentages ( $P<0.05$ ). Addition of minocycline reversed the radiation response in Th cell percentages at 3 Gy, resulting in a

main effect of drug and a drug x radiation interaction ( $P<0.05$ ). Tc percentages were significantly reduced at both 2 Gy and 3 Gy alone compared to both 0 Gy control groups and to their drug-treated counterparts, resulting in a main effect of drug ( $P<0.05$ ).

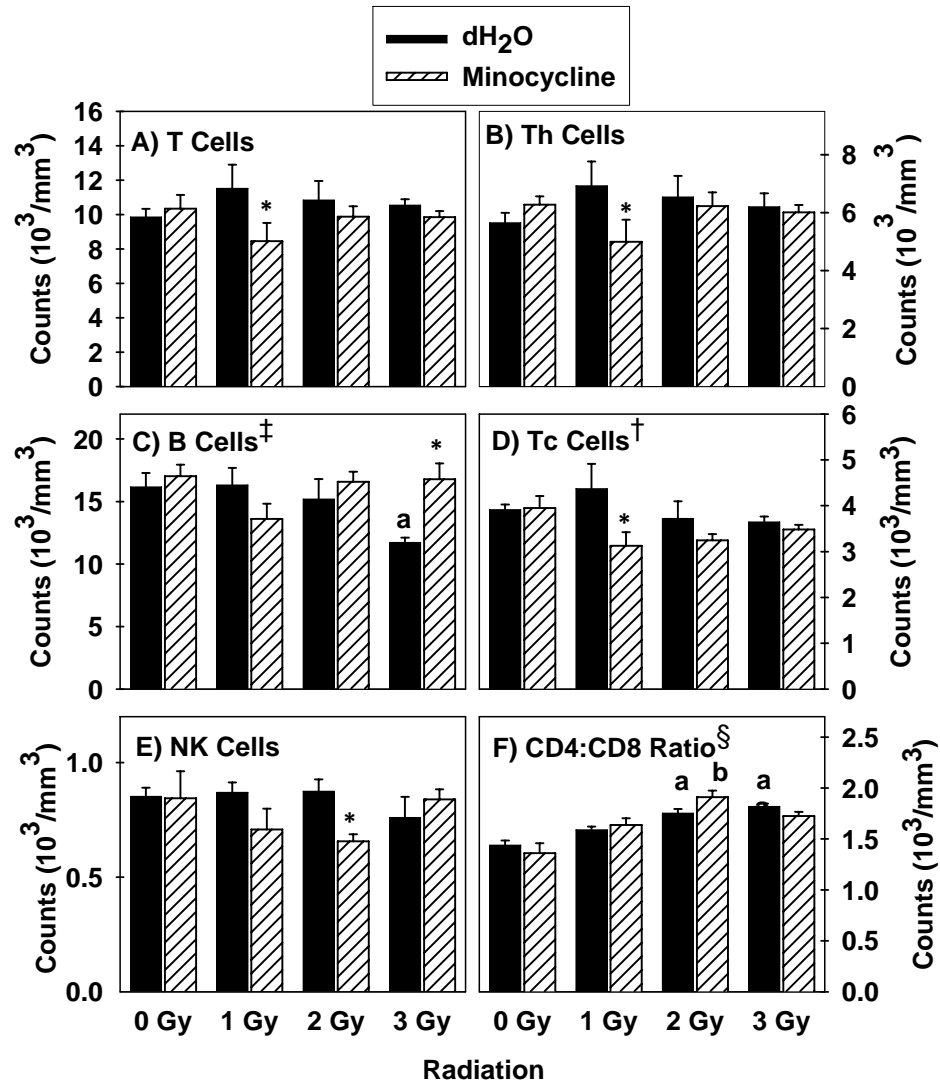


Figure. 3.5. Lymphocyte Population Counts in Spleen. The data for 5-10 mice/group are based on fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean  $\pm$  SEM. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; †,  $P < 0.05$  for main effect of drug; ‡,  $P < 0.05$  for drug x radiation interaction. Tukey test: \*,  $P < 0.05$  for dH<sub>2</sub>O vs Minocycline within each radiation dose; a,  $P < 0.05$  vs 0 Gy within dH<sub>2</sub>O groups; b,  $P < 0.05$  vs 0 Gy within Minocycline groups.

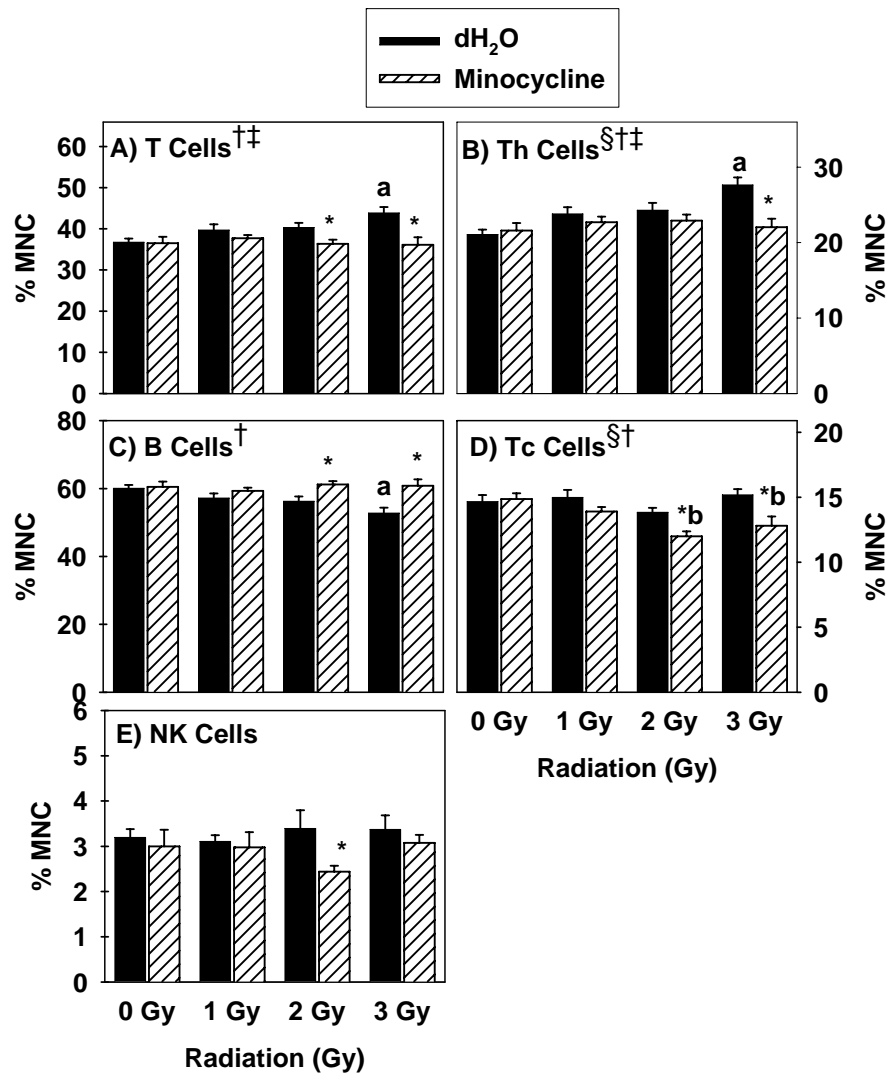


Figure. 3.6. Percentages of Lymphocyte Populations in Spleen. The data for 5-10 mice/group are based on fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean  $\pm$  SEM. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; †,  $P < 0.05$  for main effect of drug; ‡,  $P < 0.05$  for drug x radiation interaction. Tukey test: \*,  $P < 0.05$  dH<sub>2</sub>O vs Minocycline within each radiation dose; a,  $P < 0.05$  vs 0 Gy within dH<sub>2</sub>O groups; b,  $P < 0.05$  vs 0 Gy within Minocycline groups.

### Flow Cytometric Analysis of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T Cells in Spleen

There was no significant impact of drug or radiation on the counts of either CD4<sup>+</sup>CD25<sup>+</sup> or CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells (Figure. 3.7). In post-hoc analysis, addition of minocycline caused a significant increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup> cells, but only in the 0 Gy group that did not receive radiation ( $P > 0.05$  vs. the 0 Gy group without drug). This resulted in a significant main effect of drug and a drug x radiation interaction on CD4<sup>+</sup>CD25<sup>+</sup> T cell percentages ( $P < 0.05$ ). There was also a significant drug-induced increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells in the 3 Gy group compared to 3 Gy alone ( $P < 0.05$ ). However, this was not enough to result in any main effects or interactions.



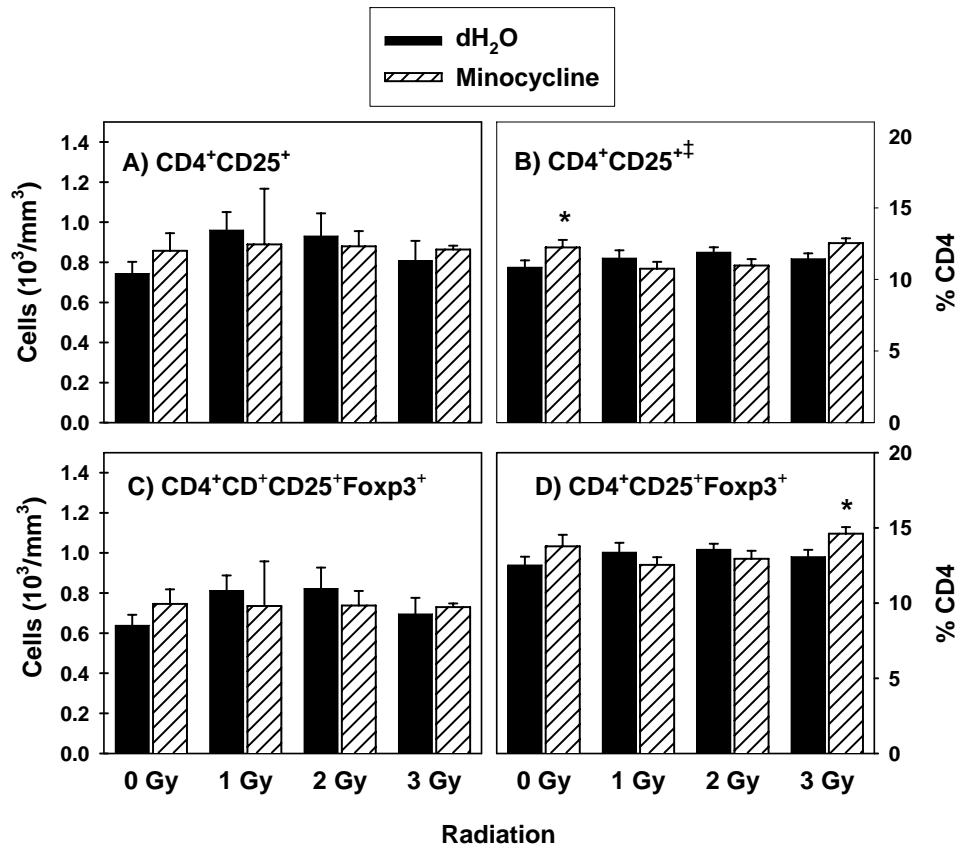


Figure. 3.7. T Regulatory Cells in Spleen. The data for 5-10 mice/group are based on fluorescence-labeled monoclonal antibodies and flow cytometry. Each bar represents the mean  $\pm$  SEM. Two-way ANOVA: †,  $P < 0.05$  for drug x radiation interaction. Tukey test: \*,  $P < 0.05$  for dH<sub>2</sub>O vs Minocycline within each radiation dose.

## Cytokine Analysis in Spleen Supernatants

Concentrations of cytokines that were generally increased in the groups that received minocycline are presented in Figure 3.8. Minocycline had a main 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 x radiation interaction was noted for G-CSF, IL-1 $\alpha$ , IL-4, IL-6 and IL-10. In post-hoc analysis, minocycline increased the levels of G-CSF, GM-CSF, IL-1 $\alpha$ , IL-4, IL-6, RANTES and IL-13 in only the non-irradiated groups ( $P < 0.05$ ). The drug-induced enhancement in these seven cytokines was no longer present when combined with radiation, regardless of dose. Among the drug-treated groups, the levels of IL-4 and IL-6 were significantly lower in all irradiated groups compared to the 0 Gy 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 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 irradiated groups.

The cytokines that generally were low in the groups treated with the drug or in which there was no effect of either drug or radiation are shown in Figure 3.9. Minocycline alone had a main effect on concentrations of IFN- $\gamma$ , IL-7, and VEGF, whereas a main effect of radiation was noted for IL-7, VEGF and MIP-1 $\alpha$  ( $P < 0.05$ ). Post-hoc analysis showed that minocycline treatment resulted in low IFN- $\gamma$  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 ( $P < 0.05$  vs. respective groups without drug).

The post-hoc analysis showed no significant differences among groups in the levels of MIP-1 $\alpha$ , IL-2, IL-12(p70), KC and TNF- $\alpha$ .

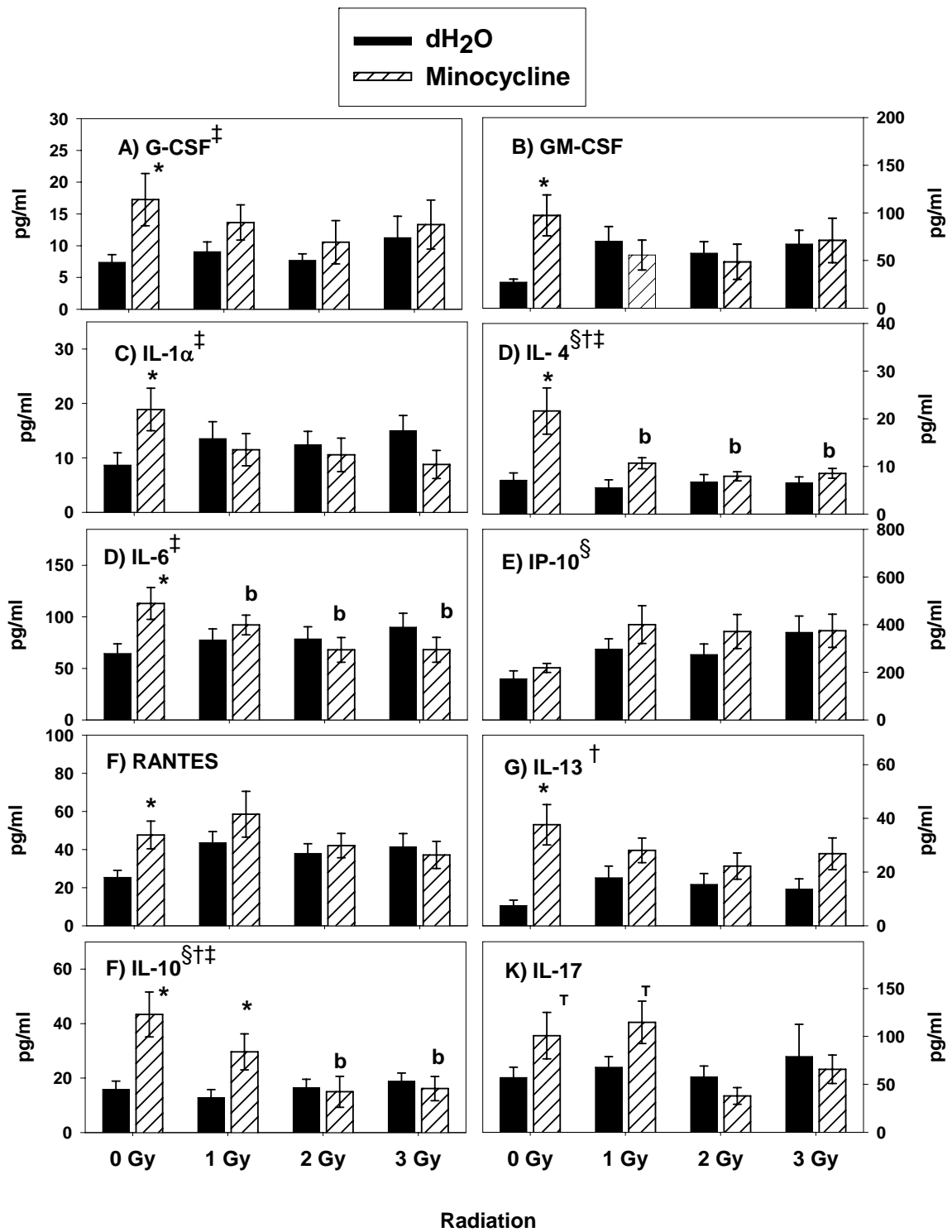


Figure. 3.8. Quantitative Analysis of G-CSF, GM-CSF, IL-1 $\alpha$ , IL-4, IL-6, IP-10, RANTES, IL-13, IL-10, and IL-17. Data were obtained from supernatants after activation of splenocytes with anti-CD3 monoclonal antibody. The mean  $\pm$  SEM is presented for n = 8-10 mice/group. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; †,  $P < 0.05$  for main effect of drug; ‡,  $P < 0.05$  for drug x radiation interaction. Tukey test: \*,  $P < 0.05$  for dH<sub>2</sub>O vs Minocycline within each radiation dose; b,  $P < 0.05$  vs 0 Gy within Minocycline groups; T:  $P < 0.1$  for dH<sub>2</sub>O vs Minocycline within each radiation dose.

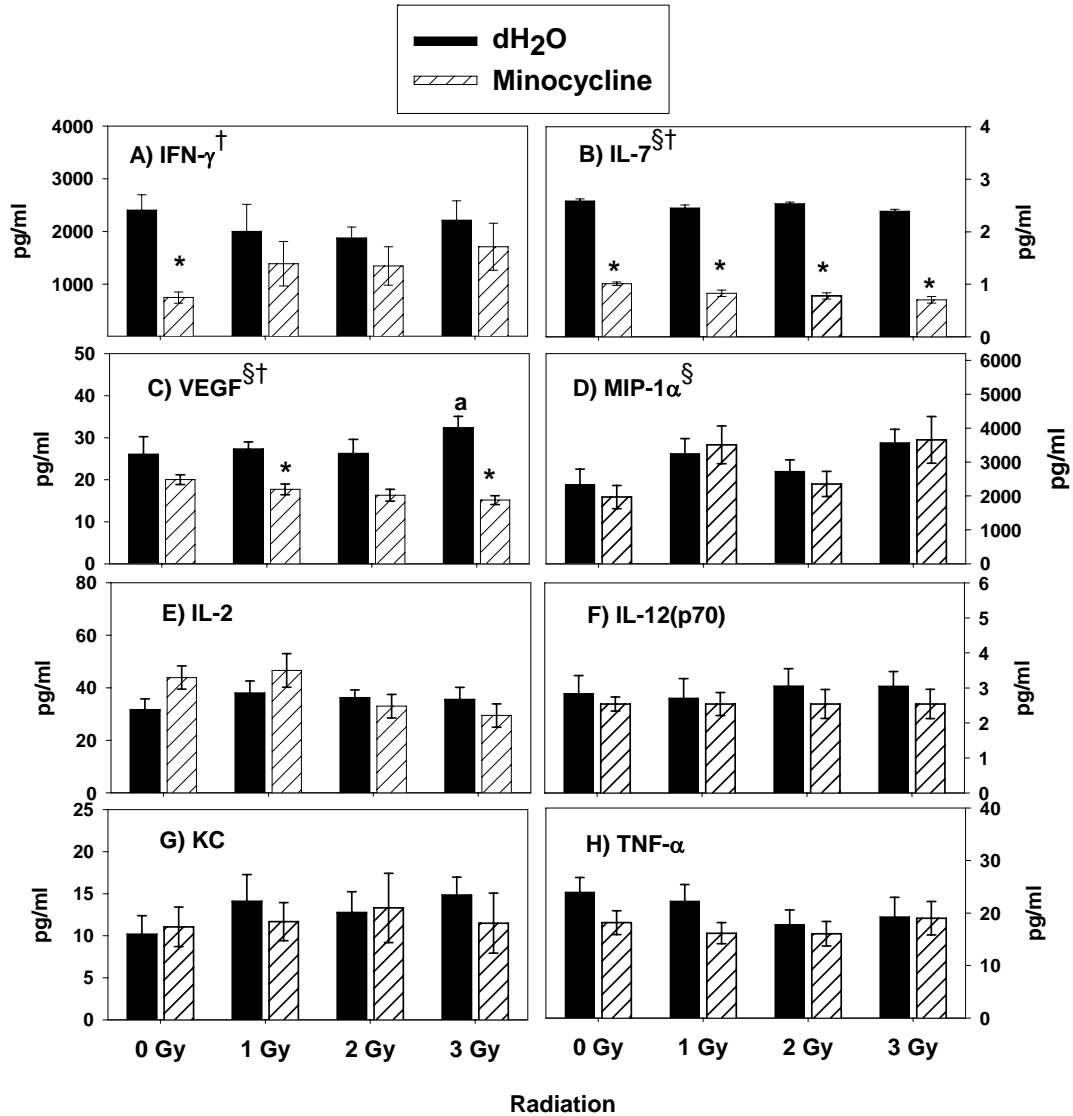


Figure. 3.9. Quantitative Analysis of IFN- $\gamma$ , IL-7, VEGF, MIP-1 $\alpha$ , IL-2, IL-12(p70), KC, and TNF- $\alpha$ . The mean  $\pm$  SEM is presented for n = 8-10 mice/group. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; †,  $P < 0.05$  for main effect of drug. Tukey test: \*,  $P < 0.05$  for dH<sub>2</sub>O vs Minocycline within each radiation dose; a,  $P < 0.05$  vs 0 Gy within dH<sub>2</sub>O groups

## 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 (Bodey, Buckley et al. 1966). The present study focused on the relatively late effects of minocycline on hematopoietic and immune parameters in an irradiated mouse model, i.e., on day 32 after exposure. Another goal was to determine if early (day 4) changes in cell profiles and cytokine production patterns that were noted in our previous study (manuscript in press) could be used as biomarkers for late changes due to radiation and/or drug treatment. All aspects of study design in the previous and present studies were identical except for time point for analyses. Major findings on day 4 post-irradiation 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.

In the present study, 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 decreased in both the blood and spleens from the 3 Gy group (without drug) compared to the non-irradiated group. This contradicts Kajioaka et al. who found that B cell counts in blood after 3 Gy whole-body irradiation of same strain mice were no longer significantly depressed by day 29 after exposure (Kajioaka, Andres et al. 2000). However, it should be noted that the mean value for B cell counts was ~35% below 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 Kajioaka 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 after exposure to a relatively high radiation dose. Although splenic T cell counts were equivalent to normal, circulating T cells were still low in the 3 Gy groups that did not receive the drug. Similarly, although CD4<sup>+</sup> Th cells recovered in both compartments (blood and spleen), the CD8<sup>+</sup> Tc cell counts at 3 Gy in the blood were significantly lower compared to the dH<sub>2</sub>O + 0 Gy group ( $P < 0.05$ ). This is consistent with the literature where Tc cells are regarded to be more radiosensitive compared to the Th cell subset (Kajioka, Andres et al. 2000; Wilkins, Kutzner et al. 2002). Interestingly, it has been proposed that the level of radiation-induced apoptosis in the CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations has potential to identify patients who are hypersensitive to radiation, and thus may be used to help predict risk for development of late toxicities (Ozsahin, Crompton et al.).

Our previous study in which assessments were conducted on day 4 post-irradiation showed that treatment with minocycline increased splenic production capacity for radioprotective cytokines and growth factors, but suppressed production of cytokines that could limit hematopoiesis. Results of the present study show that the drug on its own significantly increased the levels of G-CSF, GM-CSF, IL-1 $\alpha$ , IL-4, IL-6, IL-10, IL-13, and RANTES even at day 32 after irradiation. Although, IL-1 $\alpha$  and IL-6 are radioprotective (Neta 1997) 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. In 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 level of IL-10, a Th2 cell-derived anti-inflammatory cytokine. There is also evidence that IL-10 can prevent the development of fibrosis (Zhang, Zheng et al. 2007), 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 (Nelson, Tu et al. 2003). 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 (Munford and Pugin 2001) and inhibits the suppression of hematopoiesis induced by IFN and TNF- $\alpha$  (Geissler, Kabrna et al. 2002). Other investigators have shown that minocycline increases the expression of IL-10 mRNA and reduces TNF- $\alpha$  mRNA (Lee, Yune et al. 2003).

There was a trend for drug-induced enhancement of IL-17 level in spleen supernatants from the 0 Gy and 1 Gy groups. IL-17, a cytokine secreted by activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, induces the production of hematopoietic cytokines (Fossiez, Djossou et al. 1996; Hinrichs, Kaiser et al. 2009). It has been shown to stimulate fibroblast production of IL-6 and G-CSF, both of which assist in hematopoiesis (Xiao, Leemhuis et al. 1992). 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 ( $P < 0.05$  vs 0 Gy), minocycline suppressed its production at both 1 and 3 Gy. Since VEGF is an angiogenic cytokine which helps primary tumors to grow and metastasize, 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 anti-tumor properties (Markovic, Vinnakota et al. 2011; Pourgholami, Mekkawy et al. 2012). 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. IL-7 is also known to promote the secretion of Th1 cell-derived pro-inflammatory cytokines such as IFN- $\gamma$  and TNF- $\alpha$  and thus perpetuate responses associated with this T cell subset (van Roon, Glaudemans et al. 2003). Reduced levels of IFN- $\gamma$  observed in the drug-treated group that received 0 Gy could be due to inadequate activation of Th1 cells. Minocycline is known to suppress T cell activation (Kloppenburger, Verweij et al. 1995; Szeto, Pomerantz et al. 2011).

### **Conclusions**

Although minocycline increased several anti-inflammatory, immunosuppressive cytokines such as IL-4, IL-6, and IL-10 and decreased IFN- $\gamma$ , it also up-regulated pro-inflammatory cytokines like G-CSF, GM-CSF, IL-1 $\alpha$ , IL-17, and RANTES. Whether it is beneficial that treatment with the drug increases production capacity in the spleen for immunosuppressive cytokines on its own even at day 32, 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 (Kielian, Esen et al. 2007). However, the drug also counteracted radiation-induced declines in the B cell population in the spleen, which would promote humoral immunity. 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 human subjects during cancer radiotherapy and for the management of ARS.

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CHAPTER FOUR  
EFFECTS OF MINOCYCLINE ON THE CENTRAL NERVOUS SYSTEM IN  
RESPONSE TO RADIATION INJURY

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*Key Words:* Ionizing radiation, brain, cytokines, microarrays

*Running title:* Minocycline, Radioprotection And Brain

## Abstract

This study was focused on the effects of minocycline, an antibiotic, on mouse brain and human glioblastoma versus non-tumor cell lines after radiation exposure. C57BL/6 mice were treated with minocycline intraperitoneally for 5 days beginning immediately before radiation with 1, 2 and 3 Gy  $^{60}\text{Co}$  gamma rays in a single fraction. Brains were collected on days 4 and 32 post-irradiation. Cytokines were quantified in supernatants from homogenized brain tissue. Minocycline significantly increased the levels of interleukin (IL)-10, IL-13, IL-15 and vascular endothelial growth factor (VEGF) on day 4 in one or more of the irradiated groups compared to radiation alone ( $P < 0.05$ ). IL-10 and IL-13 are anti-inflammatory, IL-15 can prevent apoptosis and VEGF has neuroprotective properties. On day 32, the drug decreased the level of IL-1 $\beta$  in the 2 Gy irradiated group ( $P < 0.05$  vs 2 Gy alone); this cytokine is implicated in several immune-related central nervous system (CNS) pathologies. Microarray analysis of brains on day 32 showed that while radiation increased several inflammatory genes like Il1f10, Il17, Tnfrsf11b, Tnfsf12, Il12b and Il1f8, these were no longer up-regulated in minocycline-treated groups. Similarly, pro-apoptotic gene Bik and nitric oxide synthase producer (Nostrin) were no longer up-regulated in the drug-treated groups. *In vitro* results showed that minocycline enhanced the viability of human astrocytes ( $P < 0.05$ ) without increasing or reducing the viability of human glioblastoma cells. Overall, these data suggest that the drug could be a useful addition during radiotherapy of CNS malignancies. Further testing of minocycline as a radioprotectant in an *in vivo* model of glioblastoma is necessary to determine its potential application.



## Introduction

Radiation is a major treatment option for tumors located close to or within the central nervous system (CNS). Eradication of the cancer, however, is accompanied by damage to normal brain tissue and may impact cognition (Raber, Rola et al. 2004; Butler, Rapp et al. 2006; Meyers and Brown 2006). It has been estimated that 50% of adult and 100% of pediatric cancer survivors who receive whole-brain irradiation for metastatic disease will develop cognitive dysfunction (Ramanan, Zhao et al. 2010). Patients who are subjected to cranial radiotherapy for management of primary and metastatic tumors form the most severe cases (~200,000/yr) (Abayomi 1996; Ramanan, Zhao et al. 2010). Neurocognitive deficits are an especially harmful side effect for children who undergo radiotherapy as a life-saving option. Whole-brain irradiation has also been shown to impact conditioned taste aversion in rodent models (Rabin and Hunt 1986).

Irradiation of brain tissue often results in inflammation (Moore, Olschowka et al. 2004; Moravan, Olschowka et al. 2011). It is believed that radiation induces a neuroinflammatory milieu of pro-inflammatory cytokines and other factors, resulting in prolongation of oxidative stress initiated by radiation. This, in turn, is responsible for the pathogenesis of radiation-induced brain injury (Rola, Fishman et al. 2008; Ramanan, Zhao et al. 2010). An acute response to radiation in the brain involves an increase in inflammatory cytokines and mediators such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), intercellular adhesion molecule 1 (ICAM-1) and cyclooxygenase-2 (COX-2), as well as activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and activator protein 1 (AP-1) (Hong, Chiang et al. 1995; Raju, Gumin et al. 1999; Raju, Gumin et al. 2000).

Irradiation of the brain can also lead to apoptosis in the neural stem cell pools in the hippocampus, a structure critical to memory and other behaviors (Reh 2002; Bálentová, Hajtmanová et al. 2011). Indeed, radiation-induced decrements in stem cell populations has been shown to impact behavior in animal models (Kempermann, Kuhn et al. 1997; van Praag, Christie et al. 1999).

Minocycline, a tetracycline derivative, has been reported to be neuroprotective, anti-apoptotic, anti-tumorigenic and anti-inflammatory by several researchers (Markovic, Vinnakota et al. 2011). Markovic et al. also reported its potential in preventing the spread of glioma cells (Markovic, Vinnakota et al. 2011). The purpose of this study was to determine the efficacy of minocycline as an anti-inflammatory radioprotectant by characterizing the brain cytokine microenvironment in an animal model. In addition, we also verified the effects of minocycline on the radiation response in five different cell lines: three human glioblastoma cell lines and two “normal” cell lines (human astrocytes and microglia).

## **Materials and Methods**

### **Animals and Experimental Design**

Female C57BL/6 mice (n = 80; 8-9 weeks of age; Charles River Breeding Laboratories, Inc. Hollister, CA) were acclimatized for 5-7 days in large plastic cages (n=10/cage) and under standard vivarium conditions. Animals were assigned to 8 groups (10 mice/group): (a) deionized water (dH<sub>2</sub>O) + 0 Gy (b) dH<sub>2</sub>O + 1Gy; (c) dH<sub>2</sub>O + 2 Gy; (d) dH<sub>2</sub>O + 3 Gy; (e) Minocycline + 0 Gy; (f) Minocycline + 1 Gy; (g) Minocycline + 2 Gy; and (h) Minocycline + 3 Gy. Animals were rapidly sacrificed on days 4 and 32 post-

irradiation using 100% CO<sub>2</sub> in compliance with the recommendations of the National Institutes of Health and the Panel of Euthanasia of the American Veterinary Medical Association. All procedures were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

### Drug Treatment and Irradiation

Minocycline was purchased from Triax Pharmaceuticals, LLC, Cranford, NJ. Animals in the respective treatment groups were administered an injection of minocycline hydrochloride (45mg/kg in a volume of 0.1 ml) intraperitoneally (i.p.) or dH<sub>2</sub>O immediately before irradiation. A Co-60 source was used to administer whole-body radiation at a dose rate of 1.58 Gy/min for a total dose of 1, 2 or 3 Gy. Rectangular plastic aerated boxes (30 x 30 x 60 mm<sup>3</sup>) were used to place non-anesthetized mice individually. A second injection of minocycline (45mg/kg) or dH<sub>2</sub>O was administered to the appropriate groups immediately after irradiation. Three consecutive injections of minocycline (22.5 mg/kg) or dH<sub>2</sub>O were administered on the following three days post-irradiation. The sham-irradiated groups were given similar treatment, but without the radiation.

### Brain Collection

Brains were collected following euthanasia and immediately snap frozen in liquid nitrogen. Brain homogenates were prepared using phosphate buffered saline (PBS without calcium and magnesium) containing a cocktail of protease inhibitor tablets from Hoffman-La Roche, Inc. (Pleasanton, CA) and 0.05% Tween-20. One ml of buffer

solution was used for every 0.2 gm tissue for homogenization followed by centrifugation. The supernatants were stored at -80°C until further analysis.

### Cytokine Analysis

Brain supernatants from -80°C were thawed and analyzed for 22 different cytokines and chemokines using the Mouse Cytokine/Chemokine Milliplex MAP Kit (Millipore, MA) and analyzed with the Luminex100 (Linco Research, Inc., St. Charles, MO) as per the manufacture's instructions. The cytokines/chemokines evaluated were as follows: IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, interferon- $\gamma$  (IFN- $\gamma$ ), IFN- $\gamma$ -induced protein 10 (IP-10), keratinocyte chemoattractant (KC), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), regulated upon activation, normal T-cell expressed and secreted (RANTES) and TNF- $\alpha$ . Vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) were analyzed via enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions.

### Microarray Analysis of Cytokine and Neurotoxicity Gene Expression in Brain

Expression of cytokine- and neurotoxicity-related genes was determined using quantitative real-time polymerase chain reaction (qRT-PCR). The PAMM-21 Mouse Cytokine and PAMM-96 Mouse Neurotoxicity gene arrays were purchased from SABiosciences, Frederick, MD. Standard procedures were used throughout.

## Cell Lines for *In Vitro* Study

Three human glioblastoma cells lines were purchased from American Type Culture Collection (ATCC) for the study: A172, T98G and U87MG. All three cell lines have a mutation in the phosphatase and tensin homolog (PTEN) gene while T98G also has a point mutation in exon 7 of the TP53 gene. Human astrocytes and human microglia were purchased from ScienCell Research Laboratories (Carlsbad, CA). The tumor cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) with 5% fetal bovine serum (FBS) and 5% Penicillin-Streptomycin. Cell line specific growth media from ScienCell were used for astrocytes and microglia. Cells were maintained at 37°C and 5% CO<sub>2</sub>. Tumor cells that were analyzed were within 12-15 passages and non-tumor cells were analyzed within 7-8 passages from the time of arrival.

## Tritiated Thymidine (<sup>3</sup>H-TdR) Incorporation Assay

Cells were harvested, washed, re-suspended and counted manually using trypan blue, to obtain viable cells, and the cell concentration was adjusted to 1x10<sup>5</sup> cells/ml. Aliquots (100 µl) of single-celled suspensions were dispensed into flat-bottom wells of 96-well plates and allowed to establish overnight. Minocycline at concentrations of 0, 1, 2 or 4 µM was added 2 h prior to irradiation in replicates of four. Equal amount of the appropriate medium was added to the wells that did not receive the drug. The cells were irradiated approximately 24 h after plating at radiation doses of 0, 2, 4, 6 and 8 Gy using Co-60 γ rays. Four hours prior to each time point of cell harvest, <sup>3</sup>H-TdR (specific activity = 46 Ci/mmol; ICN Radiochemicals, Irvine, CA) was added at 1 µCi/50µl/well. Cells were harvested 24, 48 and 72 h after irradiation with 0.25% trypsin solution and a

Tomtec multiple-sample harvester (Hamden, CT) onto a single filter paper subdivided into 96 sections. Each filter paper was sealed in a sample plastic bag with 5 ml of beta scintillation fluid. The incorporation of radioactivity into cell DNA (counts per minute; cpm) was then quantified in the Trilux 1450 MicroBeta liquid scintillation counter (Wallac, Turku, Finland). Three independent experiments were performed.

### Cell Viability Assay

To quantify proportional changes in viable cells, we used the Cell Titre Glo Luminescent Cell Viability assay kit (Promega, Madison, WI) as per the manufacturer's instructions. Briefly, cells were grown on 96-well plates in 4 replicates. One-hundred  $\mu$ l of cells were plated per well at a concentration of  $10^5$  cells/ml. Cells were allowed to grow for 24 h; 25  $\mu$ l minocycline solution at concentrations of 0, 1, 2 or 4  $\mu$ M was added 4 h prior to irradiation. Cells were irradiated 24 h after plating using Co-60  $\gamma$  rays and cell viability was determined. Assay reagent (125  $\mu$ l) was added to cells at room temperature, mixed on a plate shaker and incubated at RT for approximately 30 min. A multimode plate reader (Infinite M200, TECAN, Durham, NC) was used to read the luminescent signal. Three independent experiments were performed.

### Statistical Analysis

The data were analyzed by two-way analysis of variance (ANOVA). Tukey's test was performed for pair-wise multiple comparisons when indicated. Means and standard errors of means (SEM) are presented here. A *P* value of <0.05 indicated significance.

SigmaStat<sup>TM</sup> software, version 2.03 (SPSS Inc., Chicago, IL) was used to analyze the data.

## Results

### Cytokine Analysis

Twelve cytokines were detectable in the multiplex and ELISA analyses on day 4 (Figure. 4.1). There was no significant impact of drug or radiation on IFN- $\gamma$ , KC, IL-1 $\alpha$ , IL-4, IL-7 and IL-13. The cytokines that were up-regulated in the presence of minocycline were IL-10, IL-15 and VEGF. There were minocycline-induced increases in IL-10 in all irradiated groups ( $P < 0.05$  vs. counterparts that did not receive drug), causing a main effect of drug ( $P < 0.05$ ). While radiation generally resulted in lower IL-10 levels with increasing radiation dose ( $P < 0.05$ ), this response was eliminated or reversed in the drug-treated mice, resulting in a drug x radiation interaction ( $P < 0.05$ ). Radiation did not have an effect on IL-13 and IL-15. The drug-treated 1 Gy group showed a significant increase in IL-13 level ( $P < 0.05$  vs 1 Gy without drug). In contrast, minocycline generally increased the level of IL-15, resulting in a main effect ( $P < 0.05$ ). However, this drug-induced increase was only significant in post-hoc analysis compared to 1 Gy alone ( $P < 0.05$ ). Although there was a significant main effect of radiation on IP-10 ( $P < 0.05$ ), this was primarily due to the relatively high levels noted in both 3 Gy groups, but this did not reach significance in post-hoc analysis. Similarly, although the drug generally decreased IP-10 compared to their dH<sub>2</sub>O-treated counterparts, resulting in main effects of radiation ( $P < 0.05$ ), there were no drug-induced differences noted in post-hoc analysis. Radiation generally decreased IL-9 ( $P < 0.05$ ), although significance was found only in 2

and 3 Gy, dH<sub>2</sub>O groups that did not receive drug ( $P<0.05$ ). Addition of minocycline did not modify this response.

On day 32 after irradiation, 10 cytokines were detectable. However, only two of these, i.e. IL-1 $\beta$  and IP-10, were significantly affected by either radiation or drug. There was as a main effect of drug on IL-1 $\beta$  ( $P<0.05$ ), most likely due to the relatively low levels at 0 and 2 Gy. This drug effect on IL-1 $\beta$  reached significance in post-hoc analysis at 2 Gy ( $P<0.05$ ). Despite this radiation dose-dependent bi-phasic response, there were no significant main effects or interactions involving radiation for this cytokine. In contrast, radiation generally increased IP-10 levels, resulting in a main effect of radiation ( $P<0.05$ ). However, there were no significant differences among individual groups in post-hoc analysis. Minocycline did not affect or alter this response.



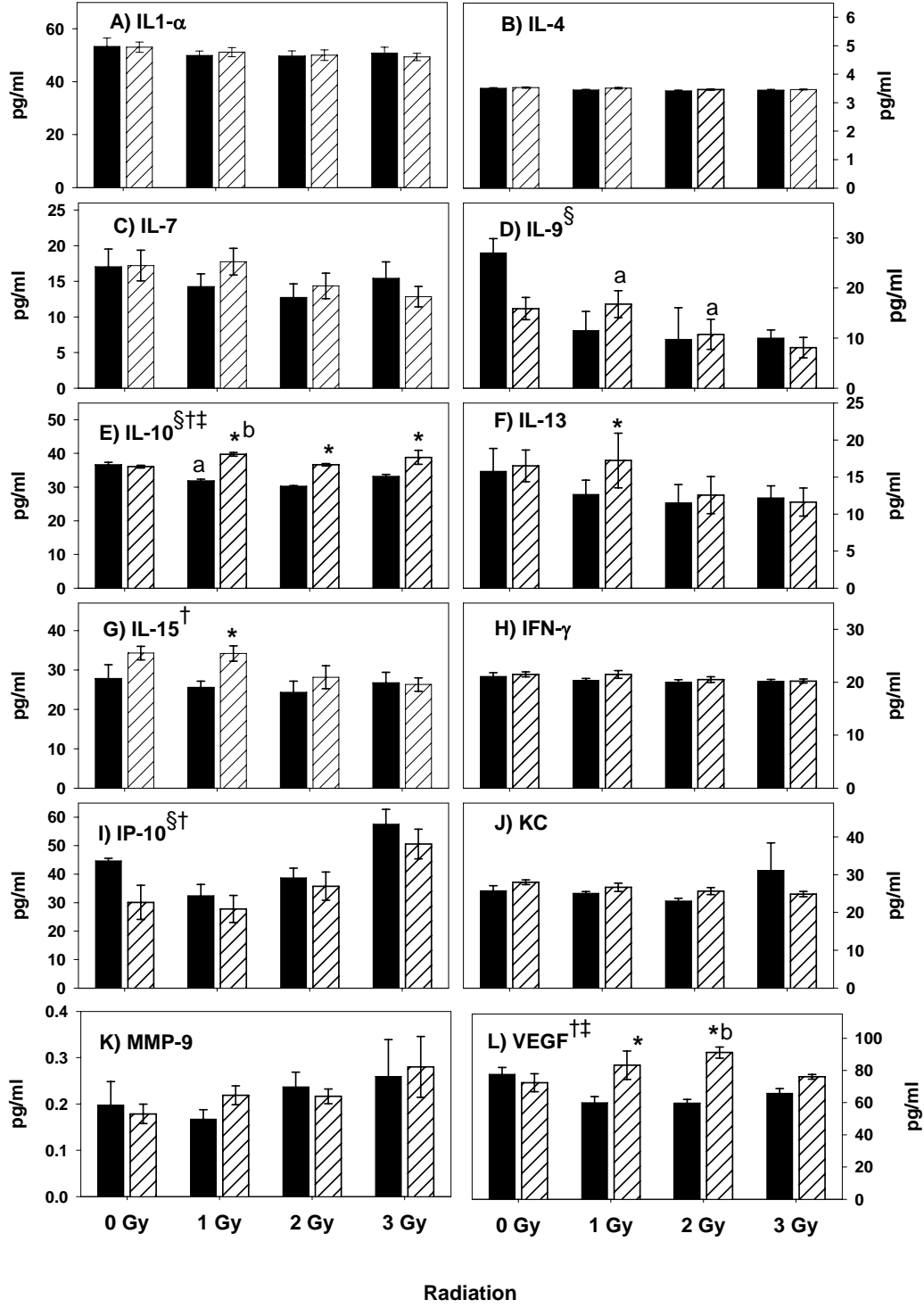
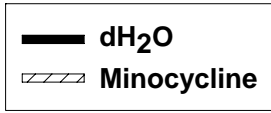


Figure. 4.1. Cytokine Analysis on Day 4. Each bar represents the mean  $\pm$  SEM for n= 4-5 mice/group. Two-way ANOVA: §,  $P<0.05$  for main effect of radiation; †,  $P<0.05$  for main effect of drug; ‡,  $P<0.05$  for interaction between radiation and minocycline, Tukey test: \*,  $P<0.05$  dH<sub>2</sub>O vs Minocycline within each radiation dose; a,  $P<0.05$  vs 0 Gy within dH<sub>2</sub>O groups; b,  $P<0.05$  vs 0 Gy within Minocycline groups.

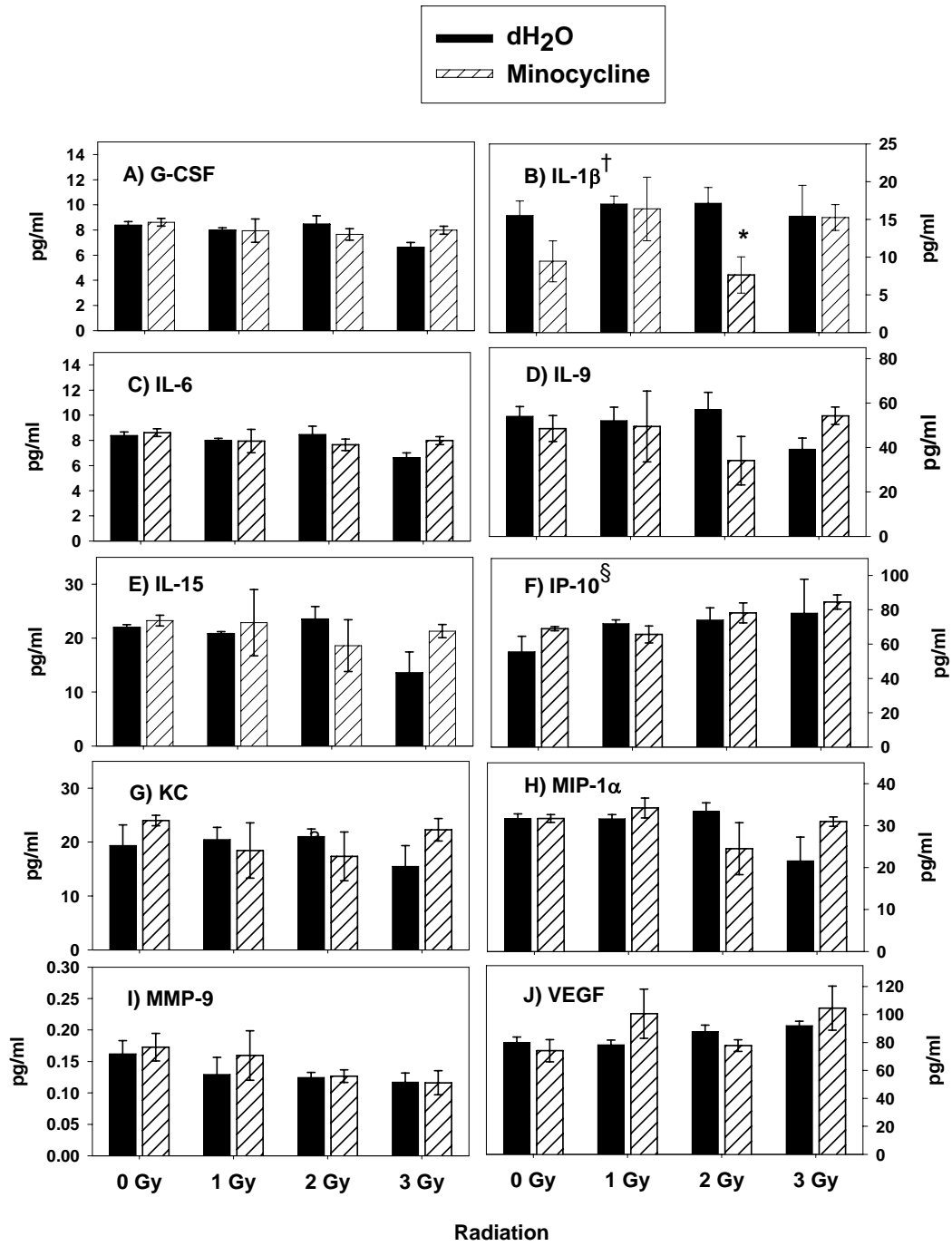


Figure. 4.2. Cytokine Analysis on Day 32. Each bar represents the mean  $\pm$  SEM for n = 4-5 mice/group. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; †,  $P < 0.05$  for main effect of drug, Tukey test: \*,  $P < 0.05$  dH<sub>2</sub>O vs Minocycline within each radiation dose.

## Microarray Analysis of Cytokine and Neurotoxicity Gene Expression in Brain

The results for cytokine and neurotoxicity gene expression are shown in Tables 4.1 and 4.2, respectively. These analyses were performed only on day 32 post-irradiation. To be considered significant, the changes in gene expression had to meet the criteria of P value  $<0.05$  and fold change  $>1.5$  vs 0 Gy dH<sub>2</sub>O.

Based on results from the cytokine array, radiation alone increased the expression of Csf2, Ctf1, Il15, Inha, Bmp10, Il11, Il17c, Il20, Tnfrsf11b, Tnfsf12, Gdf1, Ifnb1, Gdf15, Il12b, Il1f6, Il1f8, Il24, Il1f10, Gdf10, Gdf2, Cd70, Bmp5 and Bmp6 and the drug alone increased Cd70 and Bmp5 (See Table 3). The combined treatment resulted in a decrease in Gdf5 and increased Il1rn, Il3, Il1f9, Bmp7, Tnfsf10 and Tnfsf11, while either radiation or drug alone did not have a significant impact on any of these genes. Minocycline did not have any effect on radiation-induced increased expression of Tnfrsf12. In contrast, the drug slightly reduced the radiation-induced increases in Inha, Gdf1, Gdf15, Il1f6, Il1f8 and Bmp6 and the decrease was more enhanced in Il1f10 and Gdf2.

Data from the neurotoxicity array showed that radiation increased the expression of Ereg, Hspa5, Nostrin, Birc2, Txnip, Atf4, Fas, Bik, Cidea and Pappa, while the drug increased Tyrp1 (See Table 4). The combined treatment resulted in slight increases in Birc2 and Txnip expression, while slightly reducing Fas and Cidea. The radiation induced increases in Ereg, Hspa5, Nostrin, Bik and Pappa were no longer present when minocycline was added to irradiated groups.

Table 4.1 Fold Changes in Cytokine Gene Expression in Brain Tissue vs dH<sub>2</sub>O + 0 Gy on Day 32 Post-IR (P Value <0.05 and Fold Change >1.5). The data were obtained from n = 4 mice/group. Radiation alone increased the expression of Csf2, Ctf1, Il15, Inha, Bmp10, Il11, Il17c, Il20, Tnfrsf11b, Tnfsf12, Gdf1, Ifnb1, Gdf15, Il12b, Il1f6, Il1f8, Il24, Il1f10, Gdf10, Gdf2, Cd70, Bmp5 and Bmp6 and the drug alone increased Cd70 and Bmp5. The combined treatment resulted in a decrease in Gdf5 and increased Il1rn, Il3, Il1f9, Bmp7, Tnfsf10 and Tnfsf11 while radiation or drug did not have any impact on any of these genes. Minocycline did not have any effect on radiation induced increased expression of Tnfrsf12. In contrast, the drug slightly reduced the radiation induced increases in Inha, Gdf1, Gdf15, Il1f6, Il1f8 and Bmp6 and the decrease was more enhanced in Il1f10 and Gdf2.

| <b>Gene</b> | <b>dH<sub>2</sub>O +<br/>3 Gy</b> | <b>Mino +<br/>0 Gy</b> | <b>Mino +<br/>3Gy</b> | <b>Gene description</b>  |
|-------------|-----------------------------------|------------------------|-----------------------|--|
| Csf2        | 1.5                               |                        |                       | Colony stimulating factor 2 (granulocyte-macrophage)                     |
| Ctf1        | 1.6                               |                        |                       | Cardiotrophin 1  |
| Il15        | 1.6                               |                        |                       | Interleukin 15   |
| Inha        | 1.6                               |                        | 1.5                   | Inhibin alpha  |
| Bmp10       | 1.7                               |                        |                       | Bone morphogenetic protein 10  |
| Il11        | 1.7                               |                        |                       | Interleukin 11   |
| Il17c       | 1.7                               |                        |                       | Interleukin 17C  |
| Il20        | 1.7                               |                        |                       | Interleukin 20   |
| Tnfrsf11b   | 1.7                               |                        |                       | Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin) |
| Tnfsf12     | 1.7                               |                        | 1.7                   | Tumor necrosis factor (ligand) superfamily, member 12                    |
| Gdf1        | 1.8                               |                        | 1.7                   | Growth differentiation factor 1  |
| Ifnb1       | 1.8                               |                        |                       | Interferon beta 1, fibroblast  |
| Gdf15       | 1.9                               |                        | 1.6                   | Growth differentiation factor 15   |
| Il12b       | 1.9                               |                        |                       | Interleukin 12B  |
| Il1f6       | 1.9                               |                        | 1.6                   | Interleukin 1 family, member 6   |
| Il1f8       | 1.9                               |                        | 1.8                   | Interleukin 1 family, member 8   |
| Il24        | 1.9                               |                        |                       | Interleukin 24   |
| Il1f10      | 2.1                               |                        | 1.6                   | Interleukin 1 family, member 10  |
| Gdf10       | 2.7                               |                        |                       | Growth differentiation factor 10   |
| Gdf2        | 2.7                               |                        | 1.6                   | Growth differentiation factor 2  |
| Cd70        | 3.3                               | 2.1                    |                       | CD70 antigen   |
| Bmp5        | 3.6                               | 2.7                    | 3.9                   | Bone morphogenetic protein 5   |
| Bmp6        | 8.5                               |                        | 8.4                   | Bone morphogenetic protein 6   |
| Gdf5        |                                   |                        | -2.4                  | Growth differentiation factor 5  |
| Il1rn       |                                   |                        | 1.6                   | Interleukin 1 receptor antagonist  |
| Il3         |                                   |                        | 1.6                   | Interleukin 3  |
| Il1f9       |                                   |                        | 1.7                   | Interleukin 1 family, member 9   |
| Bmp7        |                                   |                        | 2.4                   | Bone morphogenetic protein 7   |
| Tnfsf10     |                                   |                        | 2.4                   | Tumor necrosis factor (ligand) superfamily, member 10                    |
| Tnfsf11     |                                   |                        | 2.7                   | Tumor necrosis factor (ligand) superfamily, member 11                    |

Table 4.2 Fold Changes in Neurotoxicity Gene Expression in Brain Tissue vs dH<sub>2</sub>O + 0 Gy on Day 32 Post-IR (P Value <0.05 and Fold Change >1.5). The data were obtained from n = 4 mice/group. Radiation increased the expression of Ereg, Hspa5, Nostrin, Birc2, Txnip, Atf4, Fas, Bik, Cidea and Pappa while the drug increased Tyrp1. The combined treatment resulted in slight increase in Birc2 and Txnip while slightly reducing Fas and Cidea.

| Gene    | dH <sub>2</sub> O+<br>3 Gy | Mino<br>+0 Gy | Mino<br>+ 3 Gy | Gene description  |
|---------|----------------------------|---------------|----------------|---|
| Ereg    | 1.5                        |               |                | Epiregulin  |
| Hspa5   | 1.5                        |               |                | Heat shock protein 5  |
| Nostrin | 1.5                        |               |                | Nitric oxide synthase trafficker  |
| Birc2   | 1.6                        |               | 1.7            | Baculoviral IAP repeat-containing 2   |
| Txnip   | 1.7                        |               | 1.8            | Thioredoxin interacting protein   |
| Atf4    | 1.8                        |               | 1.8            | Activating transcription factor 4   |
| Fas     | 1.8                        |               | 1.7            | Fas (TNF receptor superfamily member 6)                                     |
| Bik     | 1.9                        |               |                | Bcl2-interacting killer   |
| Cidea   | 1.9                        |               | 1.6            | Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A |
| Pappa   | 2.0                        |               |                | Pregnancy-associated plasma protein A                                       |
| Tyrp1   |                            | 2.2           |                | Tyrosinase-related protein 1  |
| Tacr1   |                            |               | 1.6            | Tachykinin receptor 1   |

## DNA Synthesis

There were radiation-induced decreases in tritiated thymidine incorporation in A172 glioma cells (Figure 4.3) at all three time points of assessment ( $P<0.05$ ). However, at 72 h the radiation effect was primarily due to differences between the 2 Gy and 8 Gy exposures; no individual radiation dose resulted in significant difference when compared to the 0 Gy control in post-hoc analysis. There were no effects or interactions involving the drug on this cell line at any time point.

For T98G glioma cells (Figure 4.4), radiation generally reduced DNA synthesis at all time points ( $P<0.05$ ). At 24 and 48 h, radiation typically decreased DNA synthesis with increasing dose of radiation when compared to 0 Gy ( $P<0.05$ ). However, at 72 h the decrease occurred only at 8 Gy. There was also a main effect of drug at 24 h ( $P<0.05$ ), primarily due to relatively low DNA synthesis in the presence of 4  $\mu\text{M}$  minocycline ( $P<0.05$ ).

For U87MG glioma cells (Figure 4.5), both radiation and drug consistently resulted in reductions in DNA synthesis at each time point ( $P<0.05$ ). A drug x radiation interaction was also present at both 24 and 72 h post-exposure. At 24 h, the interaction was likely due to the anomalous decrease in the cells that were cultured in presence of 1  $\mu\text{M}$  drug and irradiated with 4 Gy. At 72 h, the interaction was likely due to similarly anomalous increases when the drug was present at 1  $\mu\text{M}$  and 2  $\mu\text{M}$  and radiation was at 0 Gy and 4 Gy, respectively.

The data for the normal human cell lines, HA (astrocytes) and HM (microglia) are shown in Figures 4.6 and 4.7, respectively. For both of these cell types, radiation and drug significantly impacted DNA synthesis at each time point ( $P<0.05$ ). Radiation



drastically reduced synthesis at all doses, typically plateauing between 2 and 4 Gy for all time points and in both cell lines. For HA cells, the drug effect at 24 h was due to decreases regardless of drug concentration compared to 0  $\mu\text{M}$ , although this reached significance only at the lower radiation doses in post-hoc analysis. At 48 and 72 h, the drug effect was due to low values when it was present at 1 and 4  $\mu\text{M}$  versus the 0  $\mu\text{M}$  control. At 72 h, there was no response at 2 Gy and 6 Gy, resulting in a drug x radiation interaction.

For HM cells, the drug effect at 24 h ( $P < 0.05$ ) was due to decreased DNA synthesis when the medium contained 2 and 4  $\mu\text{M}$  minocycline compared to 0  $\mu\text{M}$  at 0 Gy. Although the increase noted with 4  $\mu\text{M}$  at 8 Gy was only a trend ( $P < 0.1$ ), this increase, combined with the decreases noted at 0 Gy, was enough to result in a significant radiation x drug interaction ( $P < 0.05$ ). At 48 and 72 h, this was due to low values in medium with 1 and 4  $\mu\text{M}$  compared to the 0  $\mu\text{M}$  control. Again, post-hoc analysis showed significant differences only at 0 Gy at 48 h and at 0 and 4 Gy at 72 h ( $P < 0.05$ ). The response at 72 h also resulted in a significant drug x radiation interaction.

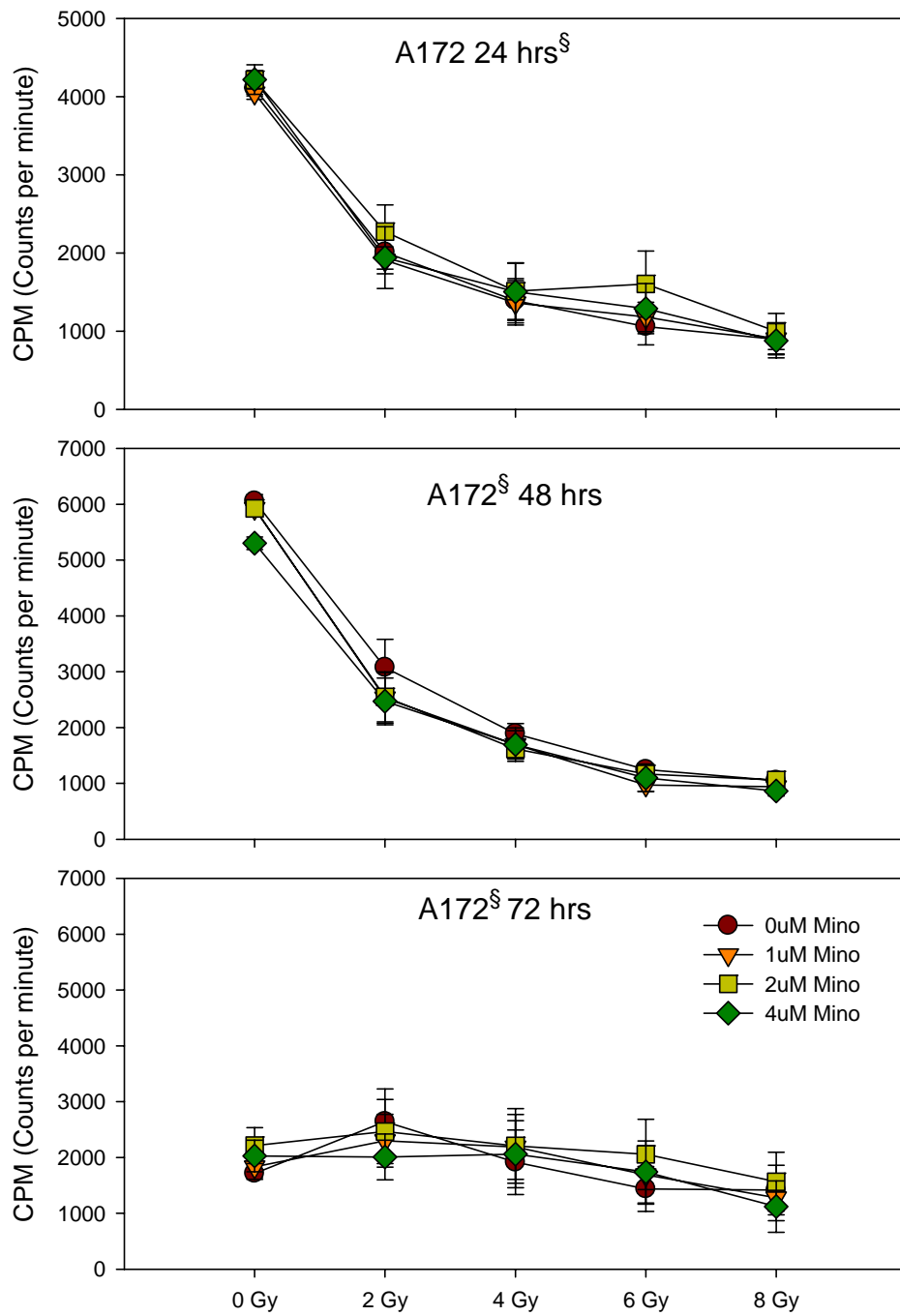


Figure. 4.3. Tritiated Thymidine Incorporation Assay for Glioblastoma Cell Line A172. Each bar represents the mean  $\pm$  SEM for n=7-8 samples/group. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation.

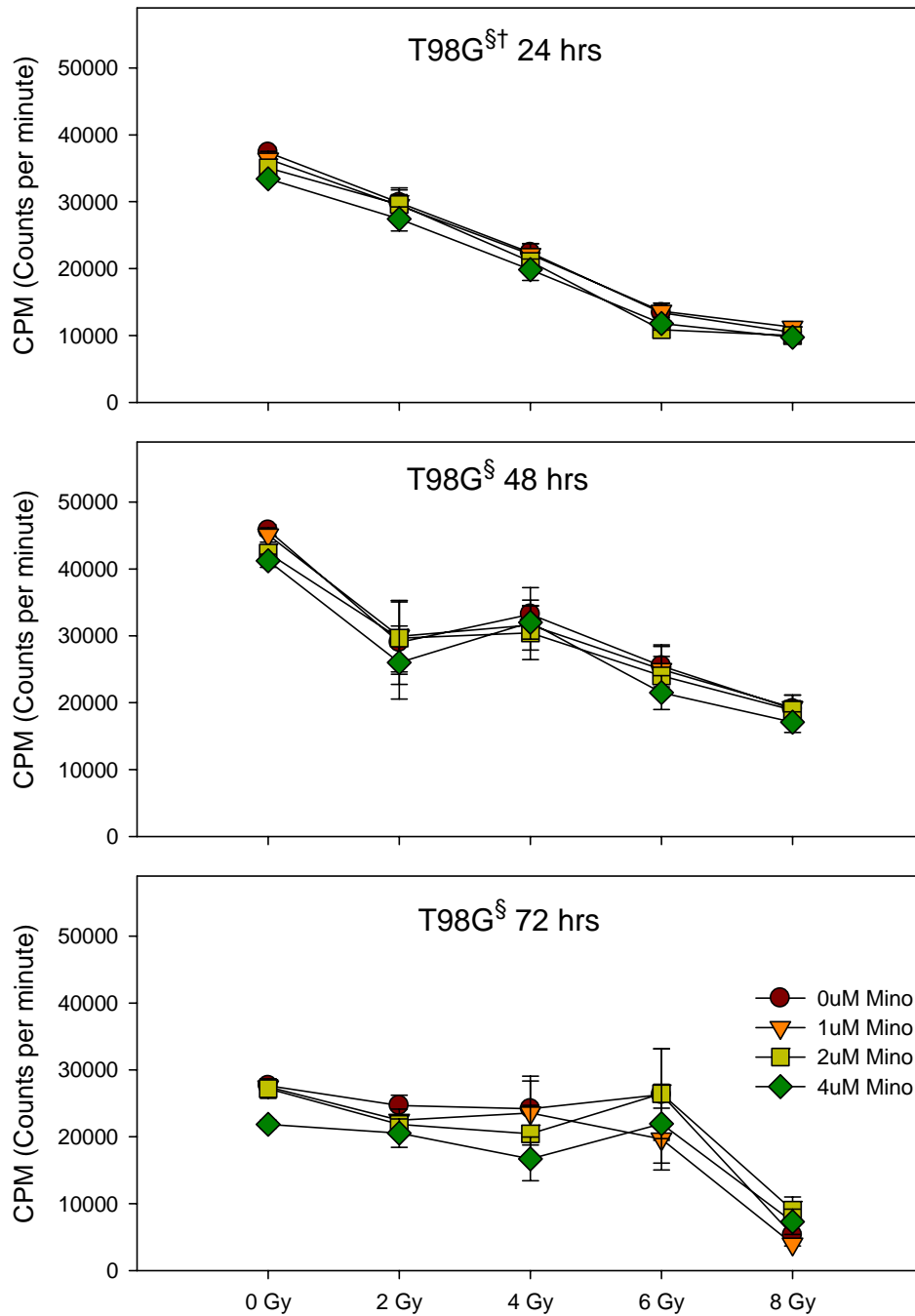


Figure. 4.4. Tritiated Thymidine Incorporation Assay for Glioblastoma Cell Line T98G. Each bar represents the mean  $\pm$  SEM for n=7-8 samples/group. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; †,  $P < 0.05$  for main effect of drug.

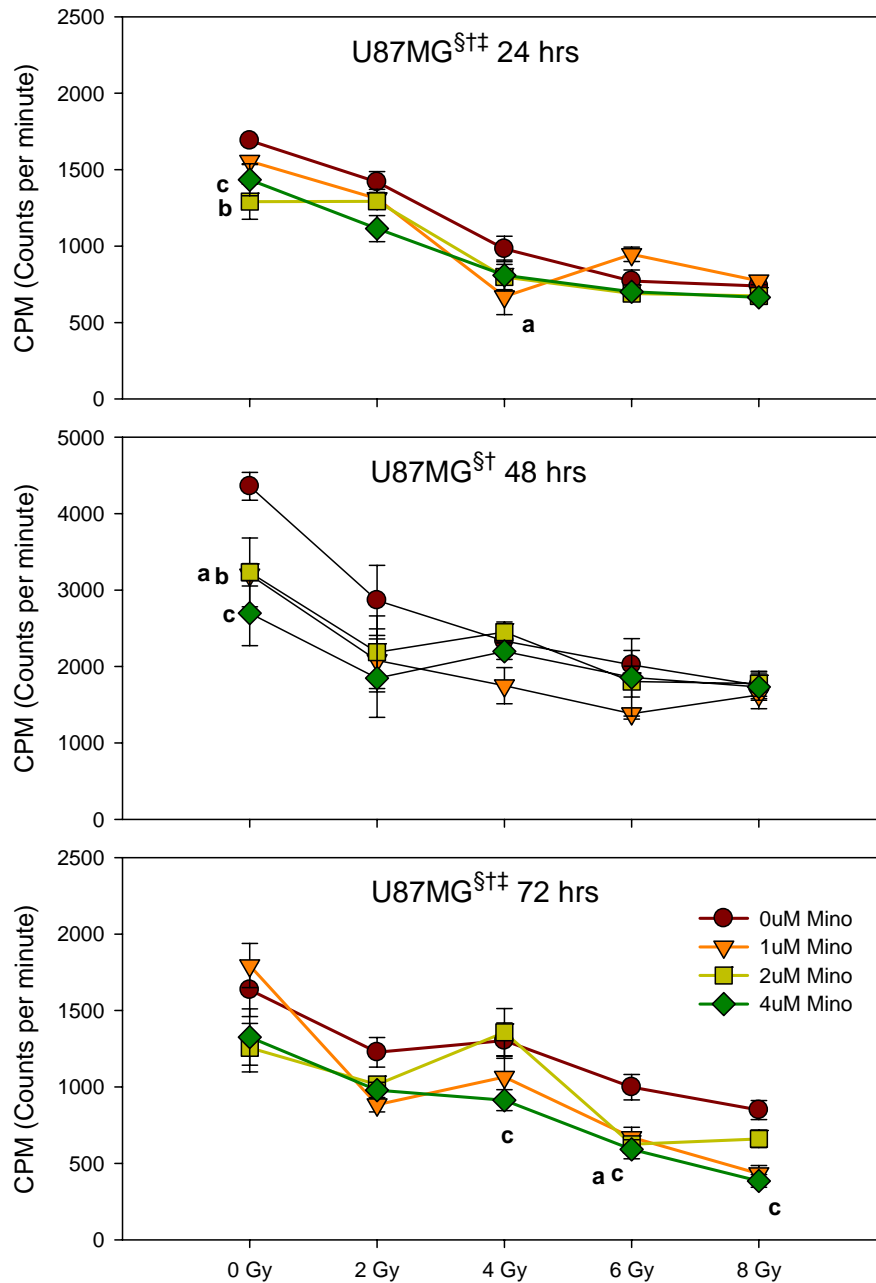


Figure. 4.5. Tritiated Thymidine Incorporation Assay for Glioblastoma Cell Line U87MG. Each bar represents the mean  $\pm$  SEM for n=7-8 samples/group. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; Tukey test: a,  $P < 0.05$  1 $\mu$ M vs 0 $\mu$ M Mino within radiation group, b,  $P < 0.05$  2 $\mu$ M vs 0 $\mu$ M Mino within radiation group, c,  $P < 0.05$  4 $\mu$ M vs 0 $\mu$ M Mino within radiation group.

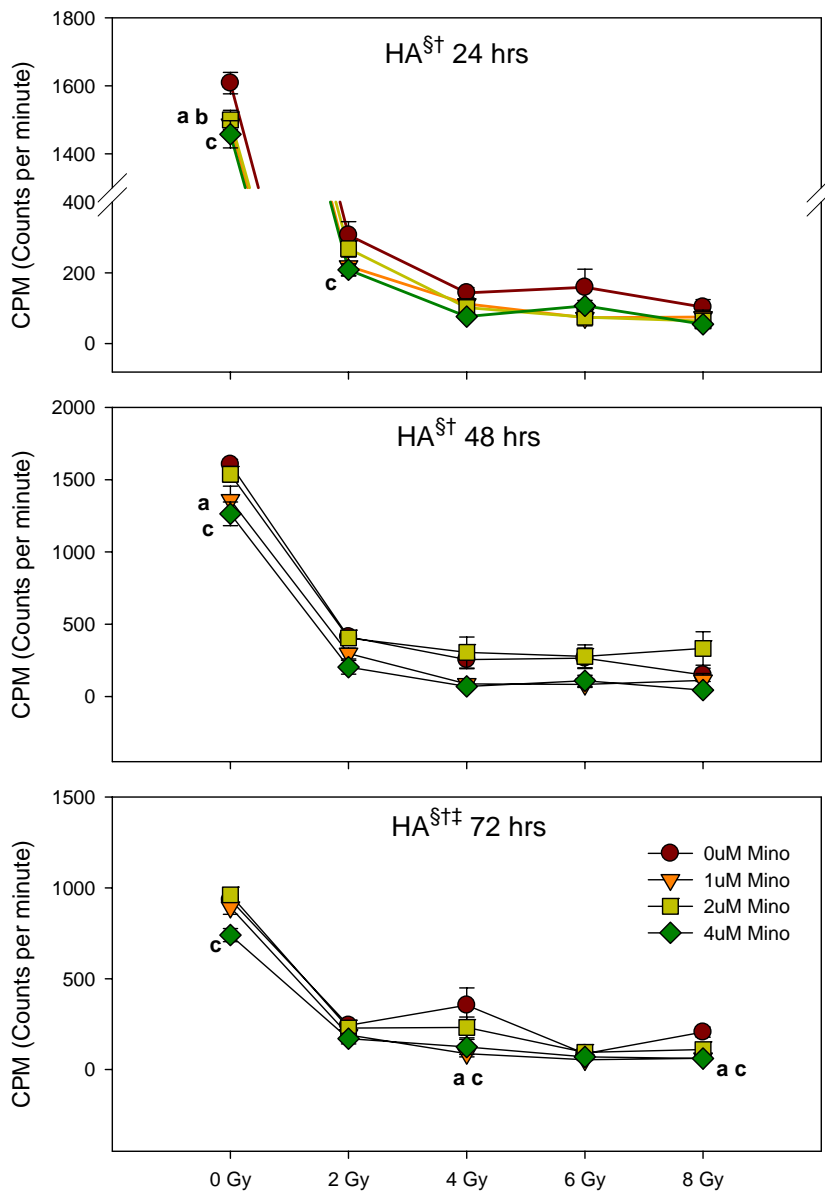


Figure. 4.6. Tritiated Thymidine Incorporation Assay for Human Astrocyte (HA) Cell Line. Each bar represents the mean  $\pm$  SEM for  $n=7-8$  samples/group. Two-way ANOVA: §,  $P<0.05$  for main effect of radiation; ‡,  $P<0.05$  for interaction between radiation and minocycline. Tukey test: a,  $P<0.05$  1 $\mu$ M vs 0 $\mu$ M Mino within radiation group, b,  $P<0.05$  2 $\mu$ M vs 0 $\mu$ M Mino within radiation group, c,  $P<0.05$  4 $\mu$ M vs 0 $\mu$ M Mino within radiation group.

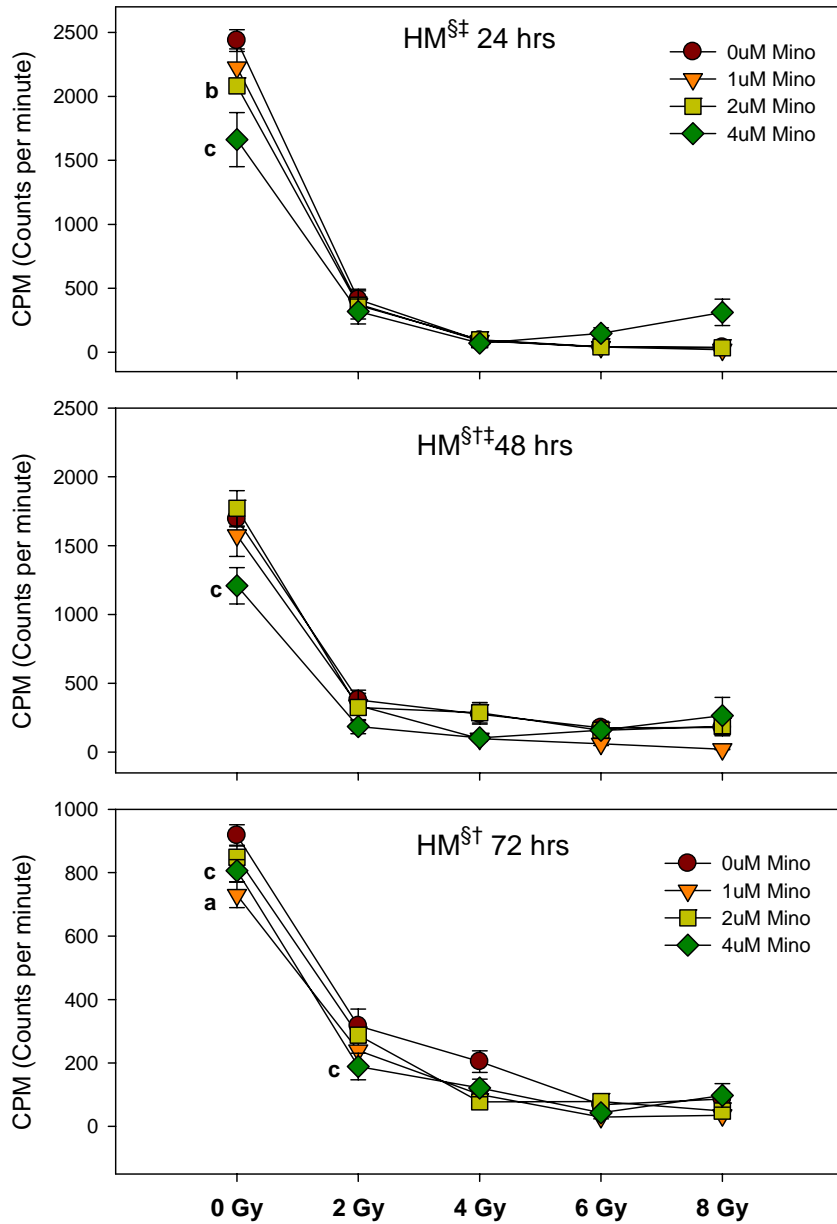


Figure. 4.7. Tritiated Thymidine Incorporation Assay for Human Microglia (HM) Cell Line. Each bar represents the mean  $\pm$  SEM for  $n=7-8$  samples/group. Two-way ANOVA: §,  $P<0.05$  for main effect of radiation; ‡,  $P<0.05$  for interaction between radiation and minocycline. Tukey test: a,  $P<0.05$  1 $\mu$ M vs 0 $\mu$ M Mino within radiation group, b,  $P<0.05$  2 $\mu$ M vs 0 $\mu$ M Mino within radiation group, c,  $P<0.05$  4 $\mu$ M vs 0 $\mu$ M Mino within radiation group.

## Cell Viability Assay

Figure 4.8 shows that there was a main effect of radiation on A172 cells at each time point due to an overall decrease in the percentage of viable cells with increasing dose ( $P < 0.05$ ). There were no drug effects or interactions on this cell type.

A main effect of radiation also existed for T98G cells (Figure 4.9) at each time point ( $P < 0.05$ ; Figure 4.9) due to reduction in percent viability mostly at the higher two doses of 6 Gy and 8 Gy that was only apparent when the effects of the drug were taken into account. There were no differences among individual groups in post-hoc analysis compared to 0 Gy. There was also a main effect of drug at 48 h ( $P < 0.05$ ). When the effects of radiation were taken into account, the 2  $\mu\text{M}$  drug concentration generally resulted in greater % viability compared to the 0  $\mu\text{M}$ . However, this response was not significant in post-hoc analysis.

For U87MG cells (Figure 4.10), there were main effects of radiation noted at every time point ( $P < 0.05$ ). At 24 h, this was due to slight increases in viable cells compared to 0 Gy controls. However, at 48 and 72 h radiation tended to decrease viability, although statistical significance was not obtained in post-hoc analysis. There was also a main effect of drug at 48 h, presumably due to overall differences between the 2  $\mu\text{M}$  and 4  $\mu\text{M}$  concentrations ( $P < 0.05$  when the effects of radiation were taken into account), but post-hoc analysis showed no significant differences between the various levels of the drug compared to 0  $\mu\text{M}$ .

For the non-tumor cell line HA (Figure 4.11), both radiation and the drug had significant main effects at each time point ( $P < 0.05$ ). While radiation generally decreased the percentage of viable cells, particularly at the later time points, there were consistent

(though not always statistically significant in post-hoc analysis) drug-induced increases with 1  $\mu\text{M}$  compared to 0  $\mu\text{M}$  and 4  $\mu\text{M}$ .

There was significant impact of radiation on the HM cells at each time point ( $P < 0.05$ ), as shown in Figure 4.12. Radiation generally decreased the percentage of viable cells, again particularly at the later time points. The drug at 4  $\mu\text{M}$  concentration at 24 hrs, generally resulted in a significantly lower percent viability from the 0  $\mu\text{M}$  control ( $P < 0.05$ , when the effects of radiation were taken into account), thus resulting in main effect of drug ( $P < 0.05$ ). However, this difference between 0 and 4  $\mu\text{M}$  was only significant at 6 Gy ( $P < 0.05$ ).



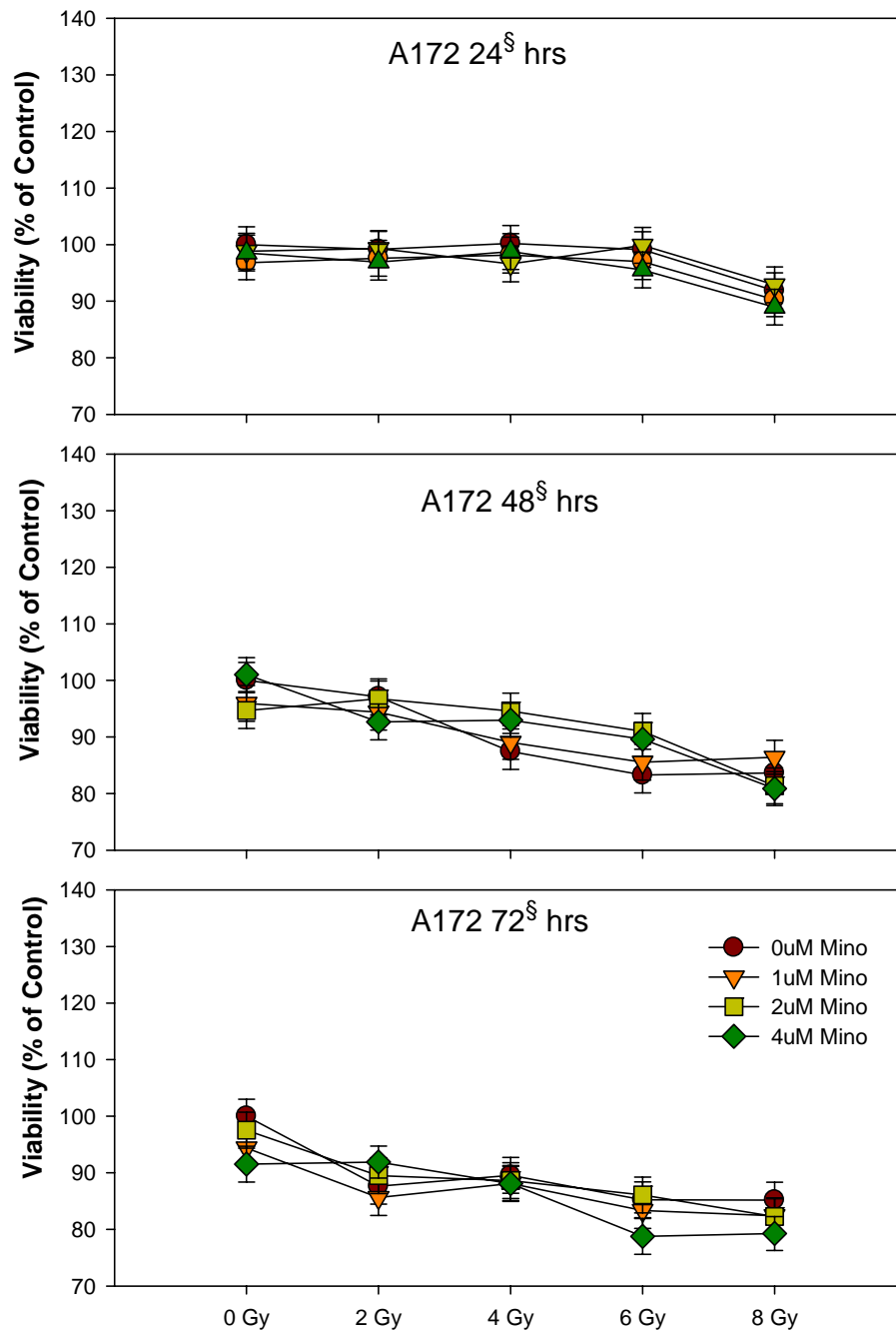


Figure. 4.8. Cell Viability Assay for Glioblastoma Cell Line A172. Each bar represents the mean  $\pm$  SEM for n=8-10 samples/group. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation.

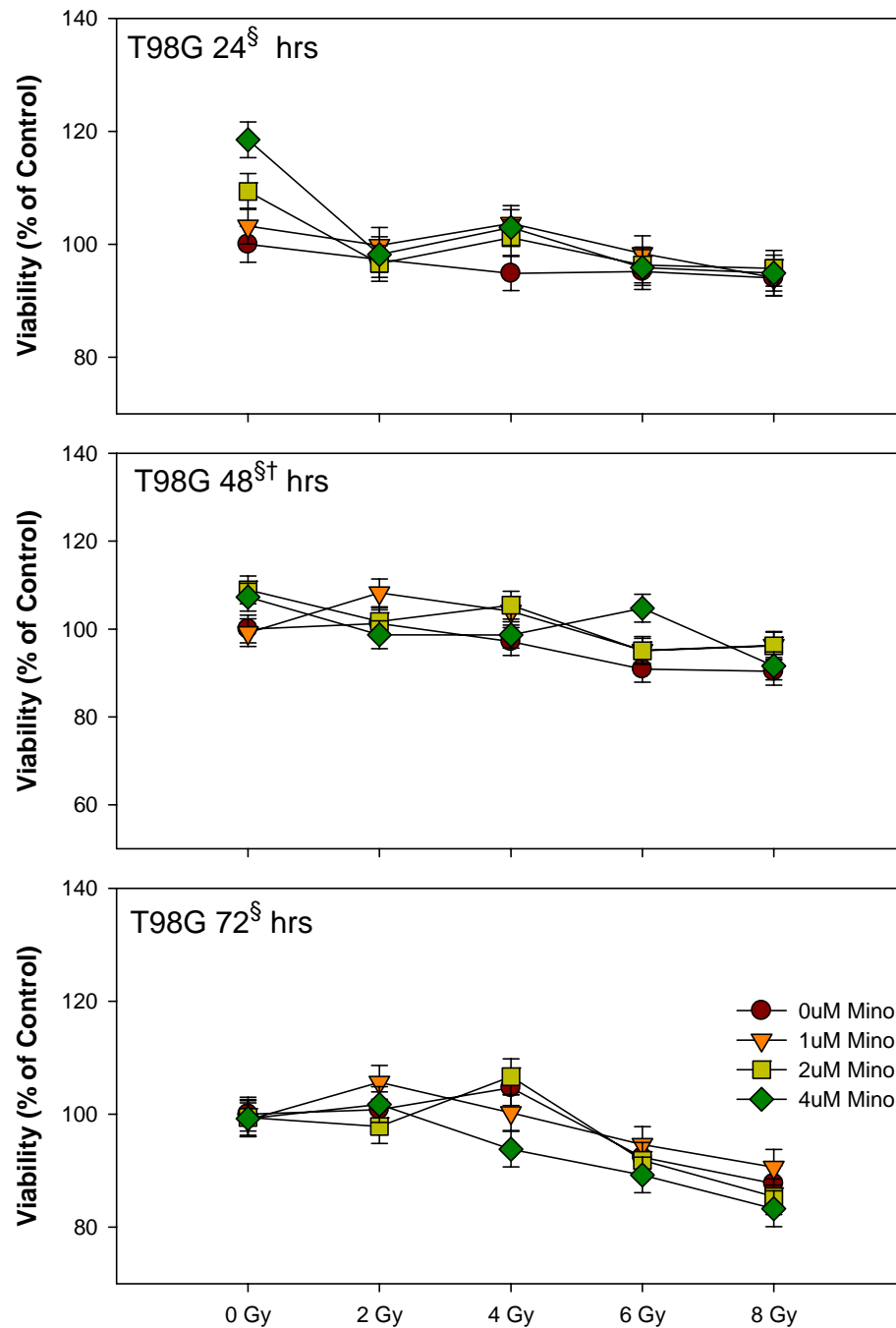


Figure. 4.9. Cell Viability Assay for Glioblastoma Cell Line T98G. Each bar represents the mean  $\pm$  SEM for  $n=8-10$  samples/group. Two-way ANOVA:  $\S$ ,  $P<0.05$  for main effect of radiation;  $\dagger$ ,  $P<0.05$  for main effect of drug. Tukey test:  $c$ ,  $P<0.05$   $4\mu\text{M}$  vs  $0\mu\text{M}$  Mino within radiation group.

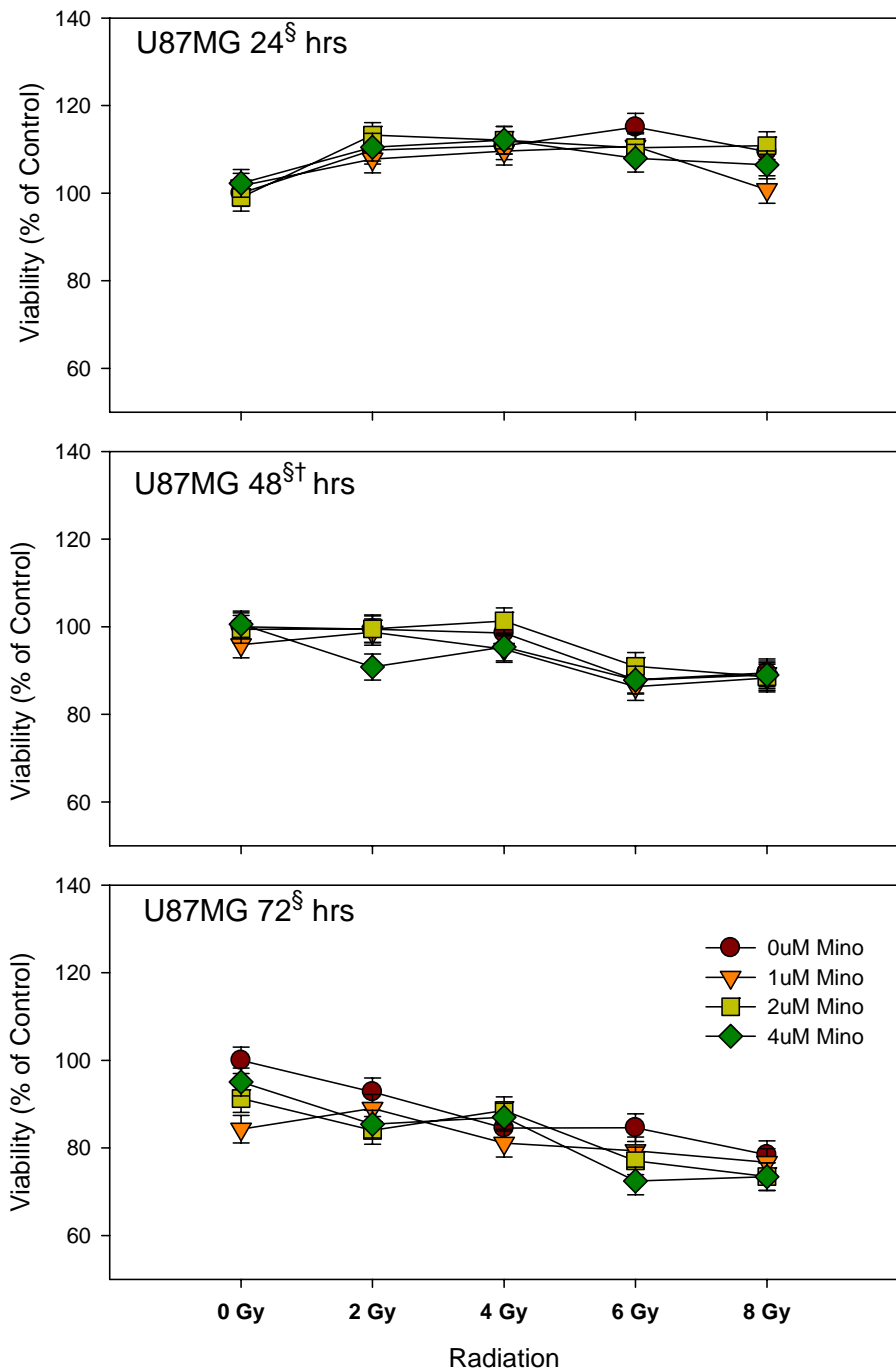


Figure. 4.10. Cell Viability Assay for Glioblastoma Cell Line U87MG. Each bar represents the mean  $\pm$  SEM for n=8-10 samples/group. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation. †,  $P < 0.05$  for main effect of drug.

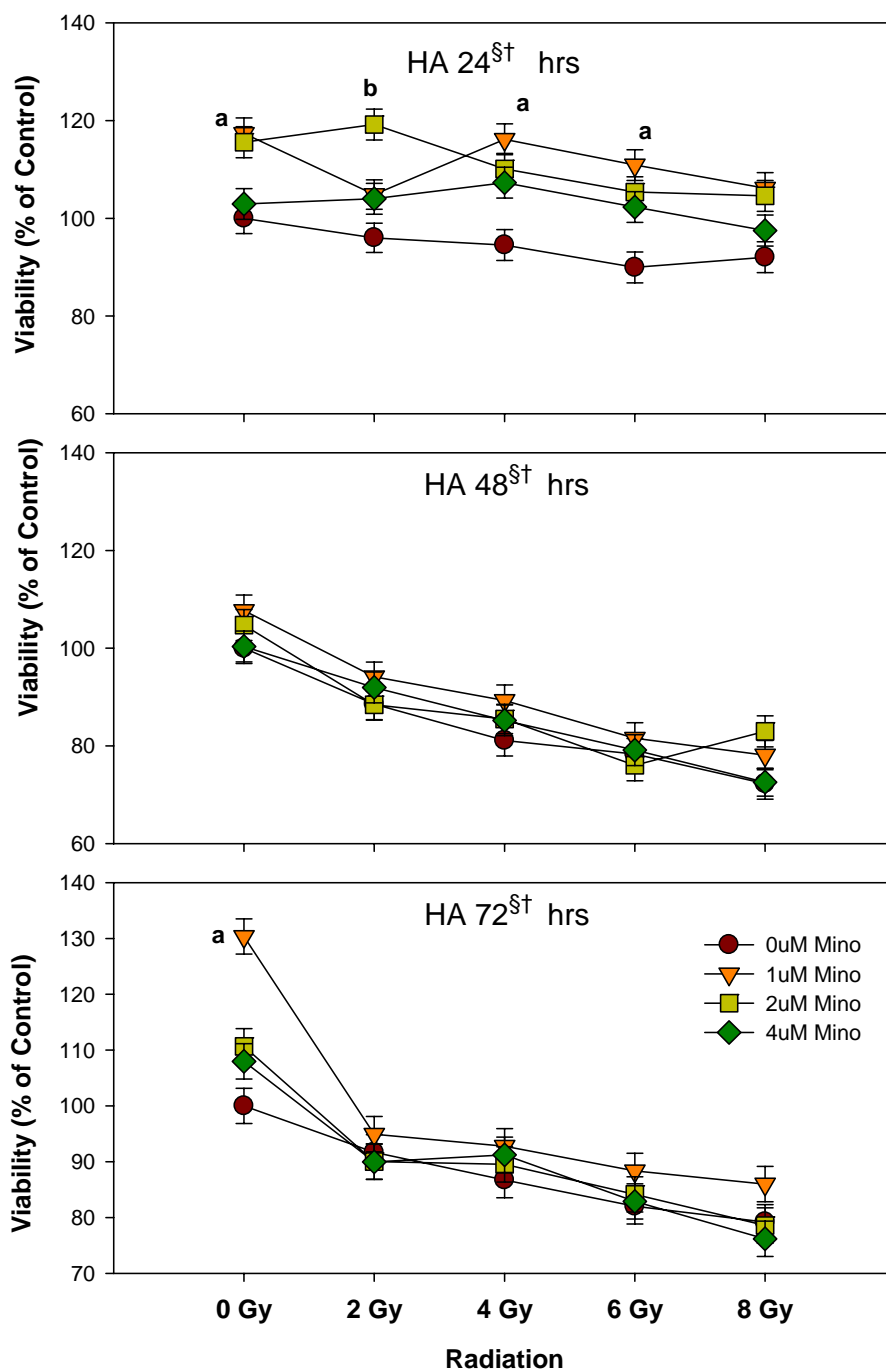


Figure. 4.11. Cell Viability Assay for Human Astrocyte (HA) Cell Line. Each bar represents the mean  $\pm$  SEM for n=8-10 samples/group. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation. Tukey test: a,  $P < 0.05$  1 $\mu$ M vs 0 $\mu$ M Mino within radiation group, b,  $P < 0.05$  2 $\mu$ M vs 0 $\mu$ M Mino within radiation group.

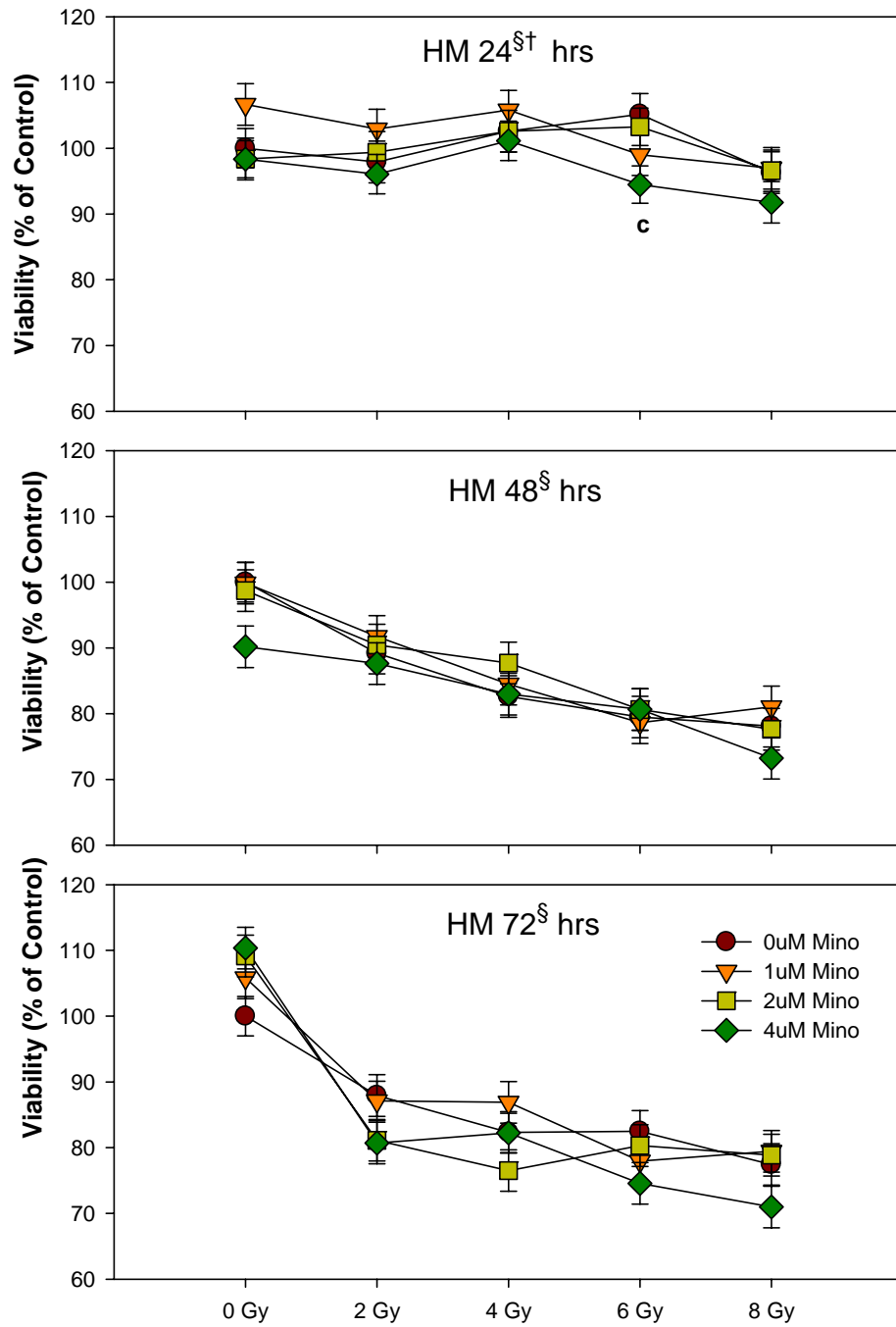


Figure. 4.12. Cell Viability Assay for Human Microglia (HM) Cell Line. Each bar represents the mean  $\pm$  SEM for n=8-10 samples/group. Two-way ANOVA: §,  $P < 0.05$  for main effect of radiation; †,  $P < 0.05$  for main effect of drug. Tukey test: c,  $P < 0.05$  4 $\mu$ M vs 0 $\mu$ M Mino within radiation group.

## Discussion

Some researchers believe that apoptosis is the most significant response of the adult brain to damage caused by ionizing radiation (Báilentová, Hajtmanová et al. 2011). Although the CNS is relatively radioresistant and significant damage occurs at higher doses, several studies have reported that exposure to relatively low doses of radiation can significantly alter the proliferation of neural stem cells in the adult neurogenic regions of the brain and induce apoptosis of the neural precursor stem cell pool (Monje, Mizumatsu et al. 2002; Mizumatsu, Monje et al. 2003; Monje, Toda et al. 2003; Raber, Rola et al. 2004; Andres-Mach, Rola et al. 2008; Báilentová, Hajtmanová et al. 2011). Adult neurogenesis occurs in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Temple and Alvarez-Buylla 1999; Gage 2000). Minocycline suppresses the microglial production of IL-1 $\beta$ , TNF- $\alpha$  and IL-6 (Kielian, Esen et al. 2007). These anti-inflammatory effects of minocycline on the brain are thought to be responsible for its neuroprotective properties. In our study, minocycline alone increased the levels of IL-10 significantly from the non-drug treated irradiated counterpart and modified the radiation response for the cytokine on day 4. Increase in IL-10 upon treatment with minocycline is consistent with the literature (Lee, Yune et al. 2003; Shi, Diez-Freire et al. 2010). IL-10 is an anti-inflammatory cytokine which also possesses neuroprotective qualities (Brewer, Bethea et al. 1999; Bachis, Colangelo et al. 2001; Burkovetskaya, Levin et al. 2007). Neuroprotective properties of IL-10 have been attributed to its ability to suppress pro-apoptotic proteins (Bachis, Colangelo et al. 2001). IL-10 is currently being evaluated in clinical trials for suppressing inflammation (Asadullah, Sterry et al. 2003).

The drug significantly increased VEGF levels in the 1 and 2 Gy irradiated groups on day 4, but had no effect on day 32. Increase in VEGF levels in response to minocycline has also been reported by others (Hollborn, Wiedemann et al. 2010; Sakata, Niizuma et al. 2012). VEGF has been reported to be neuroprotective (Jin, Mao et al. 2000). It promotes neurogenesis in ischaemic brain (Sun, Jin et al. 2003) and prevents neuronal loss in traumatic brain injury (TBI) (Ma, Liu et al. 2011). A protective role of VEGF against radiation injury has been reported by Warner- Schmidt et al., who showed that low doses of radiation can lead to lowered hippocampal neurogenesis and this can be reversed by mechanisms involving VEGF increase (Warner-Schmidt, Madsen et al. 2008). Although VEGF is indicated in late radiation-induced blood brain barrier (BBB) disruption, no changes in early levels of VEGF have been reported following radiation (Tsao, Li et al. 1999; Li, Ballinger et al. 2001). Although we did not determine the source of this cytokine, astrocytes are a likely source for VEGF in response to stress. In the *in vitro* studies presented here, we found that radiation drastically reduced viability, even at 2 Gy, suggesting that a similar decrease could occur *in vivo*. However, the drug-treated HA cells (astrocytes), showed increased viability at 2 Gy at 1  $\mu$ M concentration ( $P < 0.05$ ). This might explain the increase in VEGF levels.

Although the drug increased levels of IL-13 and IL-15 at 1 Gy, and generally decreased radiation-induced increases in IP-10, it did not modify the radiation response for any of these cytokines, which would otherwise have supported our hypothesis. There is an indication for the neuroprotective role of IL-13 (Rossi, Mancino et al. 2011) which is also an anti-inflammatory cytokine. Although IL-15 has been reported to be a pro-inflammatory cytokine, several studies have suggested an anti-inflammatory role for this

cytokine and its receptor, i.e., IL-15R $\alpha$  (Pan, Wu et al. 2011). Beneficial effects of IL-15 have been indicated by several researchers. It increases proliferation of adult neural precursor cells and IL-15 deficient mice have lowered neurogenesis (Gomez-Nicola, Valle-Argos et al. 2011). IP-10, also known as C-X-C motif chemokine 10 (CXCL10), is a protein implicated in several neuronal pathologies (Vikolinsky, Siggins et al. 2004).

Minocycline alone reduced the expression of IL-1 $\beta$  in the brain on day 32.

Reduction in IL-1 $\beta$  levels in the brain with minocycline treatment has also been observed by other researchers (Tikka, Fiebich et al. 2001; Choi, Kim et al. 2007). IL-1 $\beta$  has been implicated in numerous neurodegenerative diseases of the CNS and is reported to be neurotoxic (Rothwell 1998; Lucas, Rothwell et al. 2006; Pott Godoy, Tarelli et al. 2008).

As mentioned earlier, minocycline increased the viability of human astrocytes at the lowest dose of 1 $\mu$ M used in our study. Some reports have indicated that minocycline did not exert any toxicity on astrocytes between 1-75 $\mu$ M concentrations and actually increased viability and proliferation between 20-40  $\mu$ M (Kernt, Neubauer et al. 2010), whereas others have reported no such beneficial effects (Matsukawa, Yasuhara et al. 2009). The reason for this discrepancy could be due to the fact that the drug was tested in two different experimental models, i.e., optic nerve head astrocytes *in vitro* and a mouse model of ischemic stroke, respectively.. The drug may simply be inhibiting the division of the cells while simultaneously increasing astrocyte viability at the low dose of 1  $\mu$ M.

Several studies have indicated new functions of astrocytes, different from their role in neuronal inflammation (Barreto, White et al. 2011). They can play roles in increasing neuronal survival. Astrocytes have been shown to facilitate adult neurogenesis (Song, Stevens et al. 2002), secretion of neurotrophic factors, elimination of excess



glutamate to promote neuronal survival (Swanson, Ying et al. 2004) and rebuilding the BBB (Kaur and Ling 2008; del Zoppo 2009). Astrocytes also release glutathione and superoxide dismutase (SOD) to promote neuronal survival from free radicals following stroke (Dringen 2000; Dringen, Gutterer et al. 2000; Bambrick, Kristian et al. 2004). They could be performing similar functions following radiation-induced generation of free radicals.

Minocycline reduced the proliferation of microglial cells without affecting their viability at the lower drug doses used in our study. However, viability was only reduced at 6 Gy in the 4  $\mu$ M drug-treated cells at 24 h. Over-expression of IL-1 $\alpha$  and  $\beta$  from microglia, contributes to neurodegeneration (Griffin 2006). A significant decrease in neurons was associated with a simultaneous increase in activated microglia (Fike, Rosi et al. 2009). Rapidly proliferating microglia can contribute to increased inflammation resulting in neuronal apoptosis. Markovic et al. indicated the potential of minocycline in suppressing glioma spread (Markovic, Vinnakota et al. 2011).

With respect to the tumor cell lines, minocycline did not increase their level of DNA synthesis. In fact, it reduced the incorporation of  $^3\text{H}$ -TdR by U87MG cells at all three time points. Furthermore, T98G cells had lower DNA synthesis at 24 h at the highest concentration of the drug. When looking at the viability profile of the cancer cell lines, it was clear that the drug did not increase the percentage of viable A172, T98G and U87MG cells.

In our microarray analysis of brain samples for cytokine- and neurotoxicity-related gene profiles on day 32, radiation exposure up-regulated gene expression of the IL family (Il11, Il12b, Il15, Il17c, Il20 and Il24), IL-1 family (Il1f6, Il1f8 & Il1f10), TNF

superfamily (Tnfrsf11b & Tnfsf12), growth differentiating factors (Gdf1, 2, 10 & 15) and bone morphogenetic proteins (Bmp, 5, 6, 7 & 10). With the combined treatment (minocycline + radiation), the expression of many of these genes no longer met the criterion for being significantly different from 0 Gy controls (i.e.  $P < 0.05$  and fold change  $> 1.5$ ). Generally this means that the expression level was essentially equivalent to normal. However, in some cases fold changes in the combined treatment groups did not necessarily change substantially compared to radiation only, but significance was no longer found due to increased variability. For example, Bmp6 was significantly up-regulated by more than 8-fold in both irradiated groups (with and without the drug). However, expression in the drug-treated group was elevated (fold change = 6.9), but statistical significance was not achieved ( $P > 0.1$ ). However, in the drug-treated group, the fold change was elevated (fold change = 6.9) but significance was not achieved ( $P > 0.1$ ). This inconsistency may be addressed by increasing the sample size.

Radiation increased the expression of Gdf, but when the drug was added the expression was lowered or no longer significantly different from control. Although some Gdfs are reported to be neuroprotective (Sullivan, Opacka-Juffry et al. 1999), the reason why they were not increased in the presence of minocycline could be due to decreased damage to neurons, hence the lessened need to promote recovery. This can be verified through histological examination of brain tissue. Another factor to consider is the time point of analysis, which was day 32 and neurogenic factors might not have been essential at that time point. The drug increased the expression of Bmp5 and its expression went up even further in the combined treatment group. Bmp5 has been indicated in ameliorating Parkinson's disease, a condition that includes brain inflammation (Brederlau, Faigle et al.

2002). Some reports however, indicate that Bmp signaling leads to reduced neurogenesis(Groppe, Greenwald et al. 2002; Gobeske, Das et al. 2009) and inhibition of Bmp signaling which lead to increased neurogenesis in adult SVZ(Lim, Tramontin et al. 2000). In contrasting evidence, Colak et al indicated that Bmp4 signaling was crucial for neurogenesis in stem cells(Colak, Mori et al. 2008). Increase in Bmp7 was observed only in the combined treatment group. However the drug alone had no impact on Bmp 10.

IL-1 family members contribute to brain inflammation (Wang, Meinhart et al. 2005; Goldbach-Mansky and Kastner 2009) and several of these members were no longer up-regulated in minocycline-treated groups. Il12b (up-regulated by radiation alone, but not in either of the minocycline groups) encodes a segment of the pro-inflammatory IL-12 cytokine, which is secreted mostly by microglia and not astrocytes (Becher, Dodelet et al. 1996; Aloisi, Penna et al. 1997). IL-17, another potent inflammatory cytokine important in brain inflammation, was no longer up-regulated with the addition of the drug (Huppert, Closhen et al. 2010). Although we observed an increase in IL-15 protein with the drug on day 4, this was not evident on day 32. This is consistent with our day 32 microarray results for this cytokine.

Some of the inflammatory cytokines that are members of the Tnf family were up-regulated by radiation. Many neurodegenerative diseases are related with the increased expression of TNF(Tansey and Szymkowski 2009). Tnfsf11 encodes TNF-11, also known as receptor activator of nuclear factor kappa-B ligand or (RANKL) which is implicated in osteoclastogenesis. Anti-RANKL treatments are being developed for inflammatory diseases(Anandarajah and Schwarz 2006; Ferrari-Lacraz and Ferrari 2011). The reduction in TNF- $\alpha$  in the brain with the use of minocycline has previously been

reported (Park, Shin et al. 2011). This suggests that minocycline may be useful as a neuroprotective agent and may reduce the risk for neurocognitive deficits. It is to be noted, however, that the expression of Tnfsf10 and Tnfsf11 went up in the combined treatment group. Cd70, which is secreted by glioblastomas and promotes inflammation (Held-Feindt and Mentlein 2002; Manocha, Rietdijk et al. 2009) was among the genes that was no longer significantly different in the combined treatment group compared dH<sub>2</sub>O + 0 Gy. The interaction of the drug with radiation and how it modifies the radiation response is essential to further understand the implications of utilizing this drug in a clinical setting.

While radiation up-regulated the gene expression for Bcl-2 interacting killer (Bik), nitric oxide synthase trafficker (Nostrin) and heat shock 70kDa protein 5 (Hspa5), addition of minocycline resulted in the differences to be no longer significantly different from 0 Gy. Bik encodes a pro-apoptotic protein while the protein derived from Nostrin induces the synthesis of nitric oxide. Reduction in the expression of Nostrin is consistent with reports showing that minocycline inhibits nitric oxide (Sadowski and Steinmeyer 2001; Lee, Yune et al. 2004).

Overall, our data increase knowledge on the potential of minocycline as a neuroprotective agent in a radiation setting. Major promising findings of this study are that the drug did not protect at least two of the human tumor cell lines (A172 and U87MG) and increased production of cytokines that are neuroprotective and neurogenic in an intact mammalian model. Further studies with this drug are needed to confirm its full potential as a radioprotective agent of the CNS and whether it can reduce radiation-induced cognitive decline.

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## CHAPTER FIVE

### DISCUSSION

There are many salient features of our research. This is the first study that tested the radioprotective potential of minocycline after whole-body irradiation. The study was undertaken primarily to evaluate the prospective utilization of minocycline as a radioprotective agent for ARS, with main emphasis towards hematopoietic recovery and alleviation of radiation-induced damage to the CNS. The overall findings are very promising and support further testing of minocycline for clinical use.

It is well established that ionizing radiation impairs hematopoiesis through numerous mechanisms. This includes direct destruction of the hematopoietic stem cells, as well as alteration in the capacity of bone marrow stromal elements to support or maintain hematopoiesis {Dainiak, 2002 #678}. Evidence in the literature suggests that tetracyclines can be robust radioprotectors of the hematopoietic system with potential utility in radiation emergencies and anticancer radiotherapy. For instance, tetracyclines have been reported to have radioprotective properties for hematopoietic cells by Kim et al. (Kim, Pollard et al. 2009).

In Chapters 2 and 3, we presented evidence that minocycline up-regulated several cytokines that are potentially useful for speeding up hematopoietic recovery. This was manifested as a reduction in radiation-induced changes in the splenic and circulating leukocyte profiles. As expected, radiation caused depression of the blood and spleen WBC populations. The drug counteracted the radiation-induced declines in

monocytes/macrophages, granulocytes, NK cells, T cells and CD8<sup>+</sup> T cells in the spleen on day 4 post-IR. This correlates with the increased splenic production capacity for cytokines such as G-CSF, GM-CSF, IL-1 $\alpha$  and IL-1 $\beta$  at this early time point. Even on day 32, a depressive effect of radiation on B cell population in the spleen was still evident. Since the major function of the B lymphocytes is to produce antibodies against foreign materials (including bacteria and other microbes), the radiation-induced depression in these cells could increase risk for infection. However, the drug-treated groups showed a reversal the B cell population at 2 and 3 Gy, i.e., rendering the level essentially equivalent to normal.

Our hypotheses included the premise that minocycline would increase anti-inflammatory cytokines in the spleen and brain. Although we expected the drug to selectively up-regulate only anti-inflammatory cytokines, the results of our study showed that both pro- and anti-inflammatory cytokines were increased. There are some reports suggesting that minocycline can stimulate the production of pro-inflammatory cytokines such as TNF- $\alpha$  and IFN- $\gamma$  in addition to the anti-inflammatory cytokine IL-10 (Kloppenburg, Brinkman et al. 1996; Hourri-Haddad, Halabi et al. 2008). However, the up-regulation may be a result of monocyte stimulation with lipopolysaccharide prior to treatment with minocycline as postulated in the report by Kloppenburg et al. Also, it cannot be ruled out that minocycline may trigger different pathways leading to anti-inflammatory or pro-inflammatory cytokine/chemokine production depending on the source of cell stimulation and the effects of tissue microenvironment. This may at least partly explain why a mixed response in cytokine analysis was observed in the present study. In addition, with respect to the spleen, it should also be pointed out that cytokine

levels were quantified in supernatants after activation of the TCR/CD3 complex.

Therefore, the generated cytokines were representative of the organ's ability to respond to a stimulus that was initially focused on activation of essentially all mature T lymphocytes.

The use of an antibiotic has several advantages because a serious consequence of hematological syndrome is a period of 30-60 days during which the subject is highly susceptible to infections. Infection accounts for a substantial proportion of patient mortality rate. In one study, administration of antibiotics to irradiated dogs lowered the mortality rate (Jackson, Sorensen et al. 1959). Minocycline, is particularly useful as an antibiotic since it is a broad-spectrum tetracycline derivative with widespread accessibility and low cost and is available as an oral formulation. The oral route would be ideal when treatment is needed for a large population exposed to radiation. Furthermore, the drug is FDA approved and is commonly used in the treatment of infections related to *S. aureus* and *N. meningitides* and acne. The safety profile, clinical features and side effects of minocycline are well characterized. It is relatively well tolerated by patients. However, an effective dose of minocycline needs to be determined if it is ever to be used as a radioprotectant for radiation victims or in occupational settings such as space travel.

Several aspects need to be taken into account for utilizing a compound as a radioprotectant. In order to be an effective radioprotectant, the compound in question should not protect the tumor. The agent may block a cell in a non-proliferative state, i.e., force it to remain quiescent, thus reducing the efficacy of radiation on the destruction of rapidly proliferating cells (Greenberger 2009). Another major and often overlooked concern is that the agent may provide initial protection against irradiation of normal cells, but allow delayed effects to be expressed in their progeny (Dziegielewski, Goetz et al.

2010). These delayed effects could include cell transformation and subsequent progression to malignancy.

With respect to the brain, minocycline was tested for its ability to protect normal cells that reside in the CNS (astrocytes and microglia) versus glioblastoma cells. A promising finding of our study is that the drug did not protect tumor cells. Although we saw a drug effect for T98G cells at 48 hours (i.e. viability was consistently higher at 2  $\mu\text{M}$  compared to 0  $\mu\text{M}$  when the effects of radiation were taken into account), this was not found at any other time points and doses of drug or radiation, suggesting that the drug does not protect glioblastoma cells. Furthermore, the drug increased the viability of astrocytes when used at 1  $\mu\text{M}$ . There was no effect on viability of the microglia. The difference in responses observed between microglia and astrocytes might be attributed to the fact that they are different cell types. Microglia (cells of macrophage lineage that reside in the brain) are more efficient than astrocytes at antigen processing and stimulation of Th1 lymphocyte lymphocytes that promote cell-mediated immune responses that can lead to excessive and prolonged inflammation if not properly controlled. There is also some evidence that astrocytes restimulate responses of the Th2 subset during chronic inflammation (Aloisi, Ria et al. 1998) and possess a homeostatic role, assisting in recovery from Th1 cell-mediated inflammation in the CNS. It has been reported that minocycline and astrocytes may both be factors in the reduction of T cell activation and proliferation. Research on motor neurons in a model of amyotrophic lateral sclerosis (ALS) revealed that the neurons were being protected by glutathione secretion from astrocytes in a model of ALS (Vargas, Johnson et al. 2008). Another study demonstrated the protection of neurons from apoptosis by minocycline during ionizing

radiation (Tikka, Usenius et al. 2001). The differential effect of the drug on tumor versus non-tumor cells gives further support for testing minocycline as a neuronal radioprotectant.

The impact of minocycline on the brain could have implications beyond inflammation. Cytokines can influence cognition through various mechanisms resulting in both neurogenesis and neurodegeneration (Wilson, Finch et al. 2002). Research indicates that inflammatory mechanisms in the brain may promote cognitive decline and neurodegeneration through cytokine-mediated interactions (Wilson, Finch et al. 2002). Minocycline influenced the levels of several key cytokines in the brain that support our hypothesis. Information on the roles of IL-1 $\beta$ , IL-10 and VEGF in CNS inflammation and neuroprotection is widely recognized. Several researchers have reported that increased IL-1 $\beta$  during brain inflammation can lead to various pathologies (Belikova, Glumac et al. 2009; Plane, Shen et al. 2010). The literature supports the reduction in IL-1 $\beta$  levels in the brain by minocycline (Belikova, Glumac et al. 2009).

The increased expression of VEGF in the brain that was observed after treatment with minocycline is consistent with findings from other researchers (Sakata, Niizuma et al. 2012). Some researchers have also reported VEGF increase in retinal pigment epithelial cells (RPE) by minocycline (Hollborn, Wiedemann et al. 2010). While the drug increased the VEGF levels in the brain, it decreased VEGF levels in the spleen supernatants after anti-CD3 stimulation of T cells, but only on day 4. The reason(s) for the difference in VEGF production by the splenocytes and CNS is(are) not known. However, there are reports that minocycline decreases VEGF levels in experimental



models with tumors, suggesting that the drug has anti-angiogenic potential (Kakeji and Teicher 1997). Several speculations can be made in this regard.

IL-10 is a potent anti-inflammatory cytokine thought to help prevent cognitive decline (Richwine, Sparkman et al. 2009). This cytokine was increased in both spleen supernatants and brain from at least some of the irradiated groups that were treated with minocycline. These findings support the premise that the drug has potential to counteract radiation damage in both organs.

Overall, the anti-inflammatory effects of minocycline were more evident in the brain compared to the spleen with respect to the cytokine analysis. Although up-regulation of important pro-inflammatory cytokines in the spleen was also noted, this may be essential to recovery of the radiation-induced damage to the hematopoietic system. Pro-inflammatory cytokines can protect hematopoietic stem cells against radiation damage (Neta 1997). It remains to be determined whether minocycline will be able to restore hematopoietic populations and protect the CNS in populations (including radiotherapy patients) exposed to radiation doses higher than the maximum 3 Gy used in the present study. Higher doses of radiation are generally administered to the CNS during radiotherapy, owing to the relative radioresistance of the brain. This, however, is the first study of its kind highlighting the potential of minocycline as a radioprotective agent for this purpose.

### **Conclusions and Future Directions**

Several broad conclusions can be drawn from this study:

(i) Minocycline treatment up-regulated several hematopoietic recovery-related cytokines on days 4 and 32 post-irradiation. This was evident in the splenocyte population profiles mainly on day 4 with increases in macrophages, granulocytes, NK and CD8<sup>+</sup> T cells. On day 32, drug-induced enhancement was noted in B cells.

(ii) Minocycline alone up-regulated IL-1 $\alpha$ , which is a known radioprotective cytokine in spleen supernatants, as well as GM-CSF and G-CSF that accelerate neutrophil recovery at both time points.

(iii) Drug treatment reduced the levels of TNF- $\alpha$ , IFN- $\gamma$  and MIP-1 $\alpha$  on day 4 and IFN- $\gamma$  on day 32 in spleen supernatants i.e., cytokines that inhibit hematopoiesis.

(iv) The drug reversed the radiation-induced decrease in IL-10 in the brain on day 4, while increasing the level of VEGF. It also lowered IL-1 $\beta$  on day 32.

(v) Finally, *in vitro* work indicates that the drug protected astrocytes from radiation damage, as evidenced by the increase in post-radiation viability at lower drug doses. However, it did not protect tumor cells from radiation. There were no corresponding increases in post-irradiation viability of tumor cells. In addition, the drug reduced DNA synthesis by U87MG cells at all time points and on T98G cells at 24 hours after irradiation, but there was no drug effect on the A172 cells. Relatively low DNA synthesis was also noted for the normal astrocytes and microglia in presence of the drug.

Future studies should include a range of minocycline concentrations and routes in an *in vivo* model system to determine optimal treatment conditions for radioprotection. Analysis at time points between day 4 and day 32 are needed to confirm the speed at which the drug helps with recovery of hematopoietic cell populations. Identification of specific cellular and molecular mechanisms by which minocycline facilitates

hematopoietic recovery should also proceed. Studies involving the effect of the drug on hematopoietic stem cells in the bone marrow would reveal whether the drug actually protects them (and/or the more mature progenitor populations) from radiation-induced death. Other studies involving measurement of ROS levels in the cells can determine if scavenging free radicals is an important mechanism through which the drug acts. Effect of minocycline on neural stem cells would help determine its potential in promoting neurogenesis. Analysis of pathways affected through minocycline involving differential effects on tumor versus non-tumor cells is critical for further testing of this drug as a normal tissue protectant in the context of radiotherapy.

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APPENDIX

(A) FOLD CHANGES IN CYTOKINE GENE EXPRESSION IN  
LIVER TISSUE VS DH<sub>2</sub>O + 0 GY ON DAY 32 POST-IR (P VALUE  
<0.05 AND FOLD CHANGE >1.5)

| <b>Symbol</b>  | <b>dH<sub>2</sub>O<br/>+3 Gy</b> | <b>Mino<br/>+ 0 Gy</b> | <b>Mino<br/>+3 Gy</b> | <b>Gene Description</b>                               |
|----------------|----------------------------------|------------------------|-----------------------|---|
| <b>Il17f</b>   | -2.48                            |                        | -2.70                 | Interleukin 17F                                       |
| <b>Gdf10</b>   | -1.70                            |                        |                       | Growth Differentiation factor 10                      |
| <b>Tnfsf10</b> | -1.62                            | -1.55                  | -2.47                 | Tumor necrosis factor (ligand) superfamily, member 11 |
| <b>Il20</b>    | 1.55                             |                        |                       | Interleukin 20  |
| <b>Cd70</b>    | 1.57                             |                        | 1.52                  | CD70 antigen  |
| <b>Il1f8</b>   | 1.57                             |                        |                       | Interleukin 1 family, member 8                        |
| <b>Il19</b>    | 1.80                             |                        | 1.65                  | Interleukin 1 family, member 9                        |
| <b>Gdf15</b>   |                                  | -3.16                  |                       | Growth differentiation factor 15                      |
| <b>Tnfsf9</b>  |                                  | -2.23                  |                       | Tumor necrosis factor(ligand) superfamily, member 9   |
| <b>Il7</b>     |                                  | -1.78                  |                       | Interleukin 7   |
| <b>Il1b</b>    |                                  | -1.61                  |                       | Interleukin 1 beta                                    |
| <b>Tnfsf14</b> |                                  | 3.24                   |                       | Tumor necrosis factor(ligand) superfamily member 14   |
| <b>Il27</b>    |                                  |                        | -1.99                 | Interleukin 27  |
| <b>Tnfsf4</b>  |                                  |                        | 1.72                  | Tumor necrosis factor(ligand) superfamily member 4    |

The data were obtained from n = 4 mice/group. Radiation reduced the expression of Il17, Gdf10, Tnfsf10 while increasing Il20, Cd70, Ilf8 and Ilf9. When minocycline was added to these groups, this difference was no longer significant from 0 Gy dH<sub>2</sub>O. In contrast, adding minocycline increased the radiation response for Tnfsf10 (i.e. further reduced the expression). The drug decreased the expression of Gdf15, Tnfsf9, Il7, Il1b,

while increasing Tnfsf14 and the radiation did not have an impact on any of them. The combined treatment decreased Il27 and increased Tnfsf4 while radiation alone or drug alone did not have any effect on the expression of either gene.