Clemson University
TigerPrints

All Theses | Theses

5-2010

The Role of IL-1beta, IL-6, and TNF-alpha in Radiation-Induced Bone Loss

Stephanie Riffle
Clemson University, sriffle@g.clemson.edu

Follow this and additional works at: https://tigerprints.clemson.edu/all_theses

 Part of the Biology Commons

Recommended Citation
Riffle, Stephanie, "The Role of IL-1beta, IL-6, and TNF-alpha in Radiation-Induced Bone Loss" (2010). *All Theses*. 778.
https://tigerprints.clemson.edu/all_theses/778

This Thesis is brought to you for free and open access by the Theses at TigerPrints. It has been accepted for inclusion in All Theses by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.

THE ROLE OF IL-1β, IL-6, AND TNFα IN RADIATION-INDUCED BONE LOSS

Presented to

The Graduate School of

Clemson University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Biological Sciences

---

by

Stephanie Erin Riffle

May 2010

Advisor: Dr. Ted A. Bateman

ABSTRACT

Bone fractures greatly decrease an individual's quality of life, as well as increase an individual's risk for further complications, including death. Ionizing radiation causes bone loss, leaving bones at increased risk of fracture. This exposure, particularly in the context of cancer patients receiving radiotherapy, results in damage to normal (non-tumor) tissue. Inflammation is a common response to radiation-induced tissue damage, characterized by increased presence of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNFα. However, little is known about the roles of these cytokines in radiation-induced bone loss. This thesis hypothesized that the up regulation of pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) after irradiation would lead to rapid activation of osteoclasts and subsequent bone loss. Three approaches were used to investigate the roles of these cytokines in radiation-induced bone loss: 1) Rodent models deficient in IL-1β receptor, IL-6, TNFα, and TNFα/IL-1β receptor combined; 2) Administration of TNF-binding protein (Enbrel), IL-1 receptor antagonist (Kineret), or a combination of the two; 3) Administration of a p38 blocking molecule (AR-447). Irradiation did result in a decline of bone volume and overall deterioration of micro-architecture within the first few weeks after exposure. Additionally, pro-inflammatory cytokine presence and expression were elevated at early time points after exposure. However, using IL-1β, IL-6, and TNFα knockout mouse strains or applying agents that block the activity of these cytokines did not prevent bone loss after radiation exposure. Providing an inhibitor of p38 activity, an important upstream and downstream mediator of pro-inflammatory

cytokine production, likewise did not prevent radiation-induced osteoporosis. Therefore, within the confines of these animal studies, pro-inflammatory cytokines did not play a significant role, if any role at all, in radiation-induced bone loss, suggesting the possibility that these cytokines are not responsible for the radiation-induced activation of osteoclasts.

DEDICATION

I dedicate this work to my father, mother, and sister. This thesis exists because of their love and support.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Bateman, for all of his assistance and guidance. I would also like to thank my committee members: Dr. Blob, Dr. Scott, and Dr. Willey. Their support and advice has helped me in the completion of this thesis. In addition, I would like to thank the members of the Bateman lab: Hope Smith-Sielicki, Eric Livingston, Michael Lemus, and Laura Bowman. Their continued support and assistance has made the completion of this thesis possible. I am also extremely grateful to the staff members of the Godley Snell Animal Research building. Their assistance has also made the completion of this thesis possible.

TABLE OF CONTENTS

Page

Table of Contents (Continued) Page

Table of Contents (Continued) Page

LIST OF TABLES

List of Tables (Continued)

Table Page

LIST OF FIGURES

List of Figures (Continued)

Figure Page

List of Figures (Continued)

Figure Page

CHAPTER 1 – INTRODUCTION

Bone fractures greatly decrease an individual's quality of life, as well as increase an individual's risk for further complications, including death. An estimated 24% of patients older than fifty, who suffer a hip fracture, will die within the first year following their fracture [1]. Radiation exposure causes bone loss, increasing the risk of fracture [2, 3]. Exposure to ionizing radiation, particularly in the context of cancer patients receiving radiotherapy, results in damage to normal (non-tumor) tissue, including bone. Inflammation after irradiation could account for this osteoporosis. Inflammation is a common response to radiation-induced tissue damage, characterized by increased presence of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF) α [4, 5]. IL-1, IL-6, and TNFα are all active stimulators of bone resorption. In order to better develop therapies to decrease the bone loss experienced by patients following radiotherapy, the mechanistic roles of these cytokines in the activation of osteoclasts needs to be determined.

The long term goal of this research is to understand the molecular mechanisms that cause radiation-induced bone loss, with the goal of blocking these factors in order to prevent osteoporosis after exposure to radiation. The objective of this thesis is to determine the individual and combined effects of IL-1, IL-6, and TNFα as they relate to bone loss after radiation exposure. The hypothesis behind this thesis is that the up regulation of pro-inflammatory cytokines (IL-1, IL-6, and TNFα), after irradiation leads to the rapid activation of osteoclasts and subsequent bone loss. By determining the roles

of IL-1, IL-6, and TNFα in radiation-induced bone loss, effective therapies involved in the blocking of these cytokines could be utilized to prevent bone loss and reduce fracture risk in cancer patients receiving treatment with ionizing radiation.

Three different approaches will be used to test this hypothesis:

1. The bone loss response after radiation exposure will be examined in mice that have been manipulated by knocking out: IL-1β receptor; IL-6; TNFα; or TNFα and IL-1β receptor combined. By knocking out these cytokines or receptors individually or in combination, it will be possible to determine if each cytokine plays a critical role in radiation-induced bone loss. With this information, future studies can focus on radiation-induced osteoporosis treatments that target individual or multiple cytokines.
2. The inflammatory response after exposure to radiation will be interrupted by treatment with etanercept (Enbrel), anakinra (Kineret), or a combination of these. Enbrel is a TNF-binding protein. TNF-binding proteins bind to TNFα and inhibit TNFα from binding to its receptor, thereby blocking the action of TNFα in the body. Kineret is an IL-1 receptor antagonist (IL-1ra), which binds to IL-1 receptor, blocking IL-1 from binding and acting within the body. Therapeutically blocking these cytokines will help to further assess the extent to which each of these cytokines contributes to radiation-induced bone loss.
3. The increased production of pro-inflammatory cytokines after radiation exposure will be inhibited by treatment with the p38 blocking molecule AR-447. AR-447

blocks the action of p38 mitogen-activated protein kinase (MAPK), which is important in the expression and production of pro-inflammatory cytokines, including IL-1, IL-6, and TNFα [6-8]. Therapeutically blocking this signal transduction pathway, thereby inhibiting the production of pro-inflammatory cytokines, will result in further indications of the roles that each of these cytokines contributes to radiation-induced bone loss.

With the use of transgenic mice and various applied pharmacologic therapies, it will be possible to directly compare the findings of these three studies, in order to determine if there are differences between permanent genetic knockout, temporary protein blocking, and reduced transcription of proteins. By interrupting the action of these cytokines immediately before radiation exposure, we will be able to better assess therapeutic options for decreasing radiation-induced bone loss. It is expected that there will be a decrease in radiation-induced bone loss in all studies, due to the inactivity of these cytokines.

CHAPTER 2 – BACKGROUND

2.1 Radiation Effects On Normal Tissues

Ionizing radiation, including X-rays, releases energy at the quantum level and can cause the ejection of electrons from an atom or molecule [9]. When tissues or cells are exposed to ionizing radiation, the energy that is released during exposure is great enough to directly break a chemical bond, including the bonds holding DNA together. The majority of the biological effects observed after exposure to ionizing radiation are a result of direct or indirect damage to DNA [9, 10]. Ionizing radiation is able to damage DNA through the production of individual base damage, single/double-strand breaks, and DNA-DNA/DNA-protein cross links [11].

After exposure to ionizing radiation, cells usually undergo mitotic cell death or apoptosis [12]. Mitotic cell death occurs when damaged DNA has been unrepaired, resulting in the death of the cell during mitotic division [11, 12]. If damaged DNA is mis-repaired, mutations may result, some of which may be lethal to the cell. Lethal mutations usually result in apoptosis, which is a genetically regulated process in order to eliminate and remove cells that are not needed or are damaged [11]. Besides damaging DNA, ionizing radiation can also damage cells through membrane damage (particularly mitochondrial membranes), which results in apoptosis [11].

Ultimately, changes at the macromolecule level lead to damaged cells and tissues. In normal tissues, radiation exposure results in vascular damage, the expression and

activation of pro-inflammatory cytokines, as well as the activation of macrophages and the infiltration of neutrophils into the irradiated volume. These consequences result in an inflammatory response after radiation exposure [12, 13].

2.2 Inflammation: Mediated By Pro-inflammatory Cytokines

Inflammation is a non-specific response of the immune system to tissue injury. The purpose of the inflammatory response includes: preventing the spread of damage to nearby tissues; removing cell debris from the injured area; preparing the injured area for repair. Inflammation is characterized by four cardinal signs in the affected area: redness, heat, swelling, and pain. During an inflammatory response, there is an influx of leukocytes, phagocytes, and natural killer cells to the area of damage in order to begin removing cell debris and foreign material, as well as preparing the injured area for repair [14].

Inflammation is characterized by the production of pro-inflammatory cytokines, including IL-1, IL-6, and TNFα. These pro-inflammatory cytokines are important in stimulating inflammatory processes in response to tissue injury [15]. Even though there are a number of cytokines involved in regulating inflammation, particular attention will be given to IL-1, IL-6, and TNFα, all of which are biologically similar and have comparable roles in inflammation [16].

Interleukin – 1 is important in the initiation and maintenance of the inflammatory response [17]. It is present in two forms, IL-1α and IL-1β, collectively referred to as IL-1

[15-17]. Both forms bind to the same receptors, as well as elicit the same biological responses [16]. Interleukin – 1 is produced by a number of cell types, which include: antigen-presenting cells (e.g. monocytes, macrophages, B lymphocytes, Langerhans cells, dendritic cells); fibroblasts; natural killer cells; vascular endothelial and smooth muscle cells; neutrophils; among others. While many cell types are capable of producing IL-1, monocytes and macrophages produce the highest levels of IL-1 within the body. IL-1 is produced when IL-1 producing cells receive some sort of stimulus (i.e. tissue injury). However, IL-1 production can also be induced by various cytokines, including TNFα, transforming growth factor-β (TGF-β), macrophage colony-stimulating factor (M-CSF), as well as IL-1 itself [15, 17, 18].

Interleukin – 1 is able to exert effects on most tissues within the body, with the primary role being to improve wound repair and the remodeling of tissues [15]. While IL-1 is important in mediating host defense against injury and infection, it is also capable of contributing to disease and damage [17]. Interleukin – 1 has the ability to excite cell-mediated, humoral, and natural immune responses by directly stimulating T-cells, B-cells, and natural killer cells. However, IL-1 is also able to indirectly induce these immune responses by acting on other cells and causing them to produce the appropriate cytokines that will stimulate T-cells, B-cells, and natural killer cells [17]. For example, IL-1 is able to elicit the function of macrophages. Most importantly, IL-1 is able to stimulate macrophages to increase the release of cytokines, including IL-1, IL-6, and TNFα, all of which play a role in inflammation [15].

Interleukin – 1 affects target cells that contain either type I or type II IL-1 receptors, both of which bind IL-1 with a high affinity [15]. However, only type I receptors mediate the biological activity of IL-1. Type II receptors bind IL-1, but appear to elicit no response. Therefore, it is thought that type II receptors are decoy receptors, in order to prevent over stimulation by IL-1 [18]. Substances also exist to inhibit the effect of IL-1 on target cells, particularly IL-1 receptor antagonists. These compounds bind to both type I and type II IL-1 receptors with high affinity, without activating the receptor, thereby inhibiting the binding and action of IL-1 [15, 17]. Studies have shown that treatment with IL-1 receptor antagonists protects mice from IL-1 induced inflammatory responses [17].

Another cytokine that is important to the work presented in this thesis is TNFα [17]. Tumor necrosis factor α targets many of the same cells that IL-1 targets, as well as elicits many of the same biological responses as IL-1 [17]. Tumor necrosis factor α exists in two forms, in a soluble form and also in a membrane-anchored form [18]. Upon cell activation, TNFα is produced by monocytes, macrophages, and T-cells, with the main producers being macrophages [17, 18]. However, other cell types are also capable of producing TNFα, including: natural killer cells, mast cells, astrocytes, and vascular smooth muscle cells [17].

Tumor necrosis factor α is capable of binding to two different receptors, TNF-R55 and TNF-R75, with similar affinities [17, 18]. Both receptors are present on most cell types, except erythrocytes and unstimulated lymphocytes. Upon binding to the

receptor, TNFα and its receptor are internalized into the cell. The degree of the resulting response depends on the number of internalized complexes [17].

Tumor necrosis factor α is able to elicit a number of biological responses. Like IL-1, TNFα is able to induce cytokine production by macrophages, as well as by endothelial cells and fibroblasts. Some of the cytokines that TNFα is able to induce the production of include IL-1, IL-6, and granulocyte macrophage colony-stimulating factor (GM-CSF) [17]. In order to inhibit the activity of TNFα, TNF-binding proteins exist. These proteins bind to TNFα, thereby inhibiting TNFα from binding to its receptor and eliciting a response [17].

Interleukin – 6 is another cytokine involved in inflammation. Interleukin – 6 is produced by a number of cells, including: monocytes; macrophages; fibroblasts; endothelial cells; chondrocytes; smooth muscles cells; astrocytes. However, the majority of IL-6 within the body is produced by monocytes, macrophages, fibroblasts, as well as endothelial cells. These cells produce IL-6 upon stimulation, which is usually achieved by IL-1 or TNFα [17, 18].

Interleukin – 6 elicits its effects on cells that express the IL-6 surface receptors [17]. Like IL-1 and TNFα, IL-6 is able to stimulate cells to produce various cytokines [17]. Besides stimulating the production of cytokines, IL-6 is also involved in stimulating the proliferation and differentiation of numerous hematopoietic stem cells [19]. In order to inhibit the responses elicited by IL-6, anti-IL-6 antibodies exist. These antibodies bind to and neutralize IL-6, thereby inhibiting IL-6 from binding to its receptor and eliciting a response.

The importance of IL-1, IL-6, and TNFα in the inflammatory process has been demonstrated through genetic knockout studies in the examination of autoimmune diseases. In particular, the roles of these cytokines in inflammation have been examined in regards to collagen-induced arthritis (CIA). CIA serves as an animal model for rheumatoid arthritis (RA), which is an inflammatory joint disease in humans [19].

TNF receptor knockout mice have been utilized, in which a reduced incidence and severity of CIA were observed. However, once CIA was established, the severity of disease progression was comparable to that observed in wild type mice. TNF knockout mice were also utilized, which resulted in a delay in onset of disease, reduced incidence of disease, as well as reduced severity of disease. However, these observations were not found to be statistically significant compared to the results of wild type mice. Unlike TNFα, studies performed with IL-1α/β double knockouts or IL-6 knockouts left mice partially or completely resistant to the development of CIA [19].

The findings of studies examining the effects of genetically knocking out IL-1, IL-6, or TNFα suggest that each of these cytokines play a significant role in rheumatoid arthritis, and therefore in inflammation. However, it is important to recognize that a "no effect" outcome in genetic knockouts does not mean that the particular cytokine being knocked out does not contribute to the disease being examined. It is possible that compensatory mechanisms may develop in order to take over the function of the cytokine that was initially knocked out [19]. For example, a "no effect" outcome may be observed in rheumatoid arthritis with the utilization of TNFα knockouts. However, by knocking

out TNFα, other cytokines with similar biological functions (i.e. IL-1) may be up-regulated in response, thereby making up for the loss of function of TNFα.

2.3 Inflammation and Bone Erosion

Bone is a tissue that is constantly being renewed and undergoing change, where old worn out bone is broken down and new bone is laid down in its place. Bone remodeling is the process by which bone formation, carried out by osteoblasts, and bone resorption, carried out by osteoclasts, occur at equivalent rates [14].

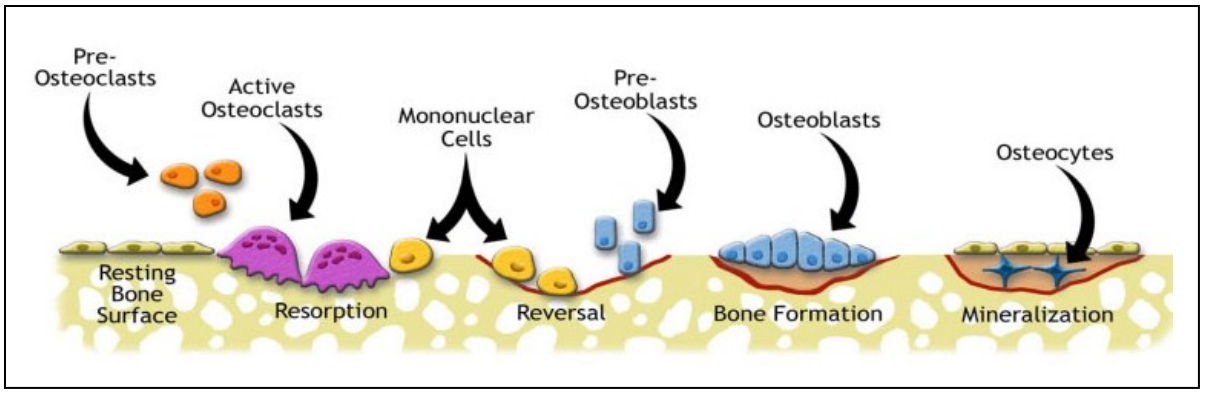

Figure 2.1 – Bone remodeling process.

http://www.ns.umich.edu/Releases/2005/Feb05/bone.html

The balanced activity between osteoblasts and osteoclasts can become altered, resulting in a number of bone diseases. One important bone disease includes osteoporosis. Osteoporosis is defined as atrophy and impaired structural integrity of bone,

which results when the rate of bone resorption is greater than bone formation. Osteoporosis leaves bones in a weakened state and at greater risk of fracture [20]. Various factors are capable of altering the bone remodeling process, including pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα. Under inflammatory conditions unrelated to irradiation, these cytokines have the ability to stimulate osteoclast formation and activity, as well as increase osteoclast lifespan [21, 22].

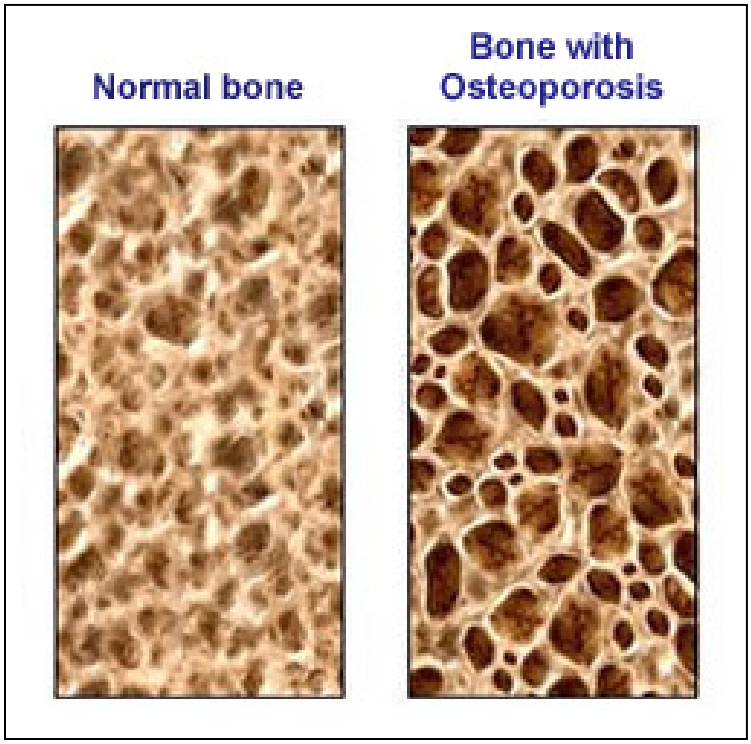

Figure 2.2 – Normal vs. osteoporotic bone

http://www.ourhealthnetwork.com/conditions/Osteoporosis.asp

In order to better understand the roles of these cytokines in bone loss, traditional osteoporosis research has focused on utilizing estrogen-deficiency mediated

postmenopausal osteoporosis animal models. In these models, animals are ovariectomized, resulting in a lack of estrogen. Estrogen is a steroid molecule that is involved in reducing the production of pro-inflammatory cytokines, including IL-1, IL-6, and TNFα. However, in the absence of estrogen, the production of pro-inflammatory cytokines is up-regulated, resulting in an accelerated loss of bone [22].

A number of studies have been performed, which have utilized this animal model, in an attempt to better understand the roles of IL-1, IL-6, and TNFα in estrogen-deficiency mediated bone loss. It has been demonstrated that ovariectomized mice had greater numbers of TNFα producing T cells. These ovariectomized mice experienced an elevated loss of bone, as compared to ovariectomized mice deficient in T cells [23]. It was also demonstrated that T cells from ovariectomized mice produced greater amounts of TNFα than T cells from non-ovariectomized mice [24]. By treating ovariectomized mice with TNF binding protein, bone loss was able to be prevented, as compared to non-ovariectomized mice treated with TNF binding protein [25]. The findings of these studies suggest that TNFα greatly contributes to estrogen-deficiency mediated bone loss.

The role of IL-6 in estrogen deficiency mediated bone loss is not as clear. Bone loss was shown not to be prevented in ovariectomized mice treated with anti-IL-6 antibody, as compared to non-ovariectomized mice treated with anti-IL-6 antibody [25]. These findings suggest that the role of IL-6 in estrogen-deficiency mediated bone loss is not as great as that of TNFα.

To further identify the roles of these cytokines in inflammatory mediated bone loss, as well as to better model an in vivo environment, combination therapies blocking

the effects of these proteins have been employed. A study was performed in which increased levels of IL-1 and TNFα, but not IL-6, were observed in ovariectomized mice, as compared to non-ovariectomized mice [26]. Bone resorption was also decreased in ovariectomized mice treated with IL-1 receptor antagonist or TNF binding protein, but not anti-IL-6 antibody, as compared to non-ovariectomized mice treated with IL-1 receptor antagonist, TNF binding protein, or anti-IL-6 antibody [26]. These findings suggest that IL-1 and TNFα play significant roles in estrogen-deficiency mediated bone loss, while IL-6 does not play as significant a role, if any. To further confirm these findings, another study was performed in which ovariectomized mice treated with IL-1 receptor antagonist or TNF binding protein experienced a decreased amount of bone loss, as compared to ovariectomized mice treated with placebo [27]. It was also reported that ovariectomized mice treated with IL-1 receptor antagonist and TNF binding protein combined experienced no bone loss at all, as compared to ovariectomized mice treated with either IL-1 receptor antagonist or TNF binding protein individually [27].

The findings of the previous work in this field indicate that IL-1 and TNFα play significant roles in estrogen-deficiency mediated bone loss, while IL-6 seems to play a less significant role. These studies also identify the significance that estrogen-deficiency mediated bone loss can be prevented with treatments involving IL-1 receptor antagonists and TNF binding proteins.

2.4 Radiation and Bone

Exposure to ionizing radiation, particularly in the context of cancer patients receiving radiation therapy, results in damage to normal (non-tumor) tissue. This normal tissue can include bone. It has been observed that patients who receive radiotherapy experience bone loss that occurs at a much greater and more severe rate than that of normal bone loss, leaving these individuals at an elevated risk of bone fracture [3, 28-31]. Baxter *et al.* [3] demonstrated that the risk of hip fracture increased 66%, 65%, and 216% for cervical, rectal, and anal cancer, respectively, for patients who underwent radiation therapy.

Many patients who experience osteoporosis related fractures also require hospitalization, followed by some form of rehabilitation in a long-term care facility [32]. After a hip fracture, it is estimated that only 15% of individuals are able to walk unaided. It is also estimated that 24% of patients, of age 50 years or older, who suffer from a hip fracture will die within the first year following their fracture. Normal osteoporosis related fractures greatly decrease an individual's quality of life, as well as increase an individual's risk for further complications, including death [1].

Fractures that occur after radiation exposure are attributed to a reduction in bone mineral content and volume after exposure [3, 33]. Fractures of the femoral neck, knee, sacrum, sacroiliac joint, clavicle, pubis, humerus, ribs, ilium, acetabulum, and mandible after radiation exposure have been well documented [29, 30, 34-39]. Previous work has developed a murine model that describes gross changes in bone after radiation exposure.

This study utilized therapeutic and space-flight relevant doses of radiation, which revealed significant losses of bone in the tibiae, as compared to mice that were not exposed to radiation [40]. The findings of this study confirmed that exposure to radiation results in a loss of bone. Further studies were performed, in which mice were exposed to different whole-body doses (0.5, 1, or 2 Gy) of proton radiation [41]. Significant bone loss was observed in the tibiae of mice exposed to a 2 Gy dose of proton radiation, while a non-significant trend toward a loss of bone was observed in mice exposed to a 1 Gy dose and no bone loss was observed in mice exposed to a 0.5 Gy dose of radiation. These findings suggest that radiation-induced bone loss occurs in a dose dependent manner.

Historically, it has been accepted that bone loss is due to reduced bone formation. Multiple studies have indicated that, after irradiation, there is a reduction in the overall number of osteoblasts, a decrease in osteoblast proliferation, as well as a loss of osteoblast function [33, 34, 37, 42]. These findings have lead to the general accepted paradigm, in which a decline in the number and activity of bone forming osteoblasts relative to bone resorbing osteoclasts following radiation exposure results in an overall loss of bone [42]. Recently, studies have been performed in which mice were exposed to 1 – 2 Gy whole-body X-ray or γ-ray doses of radiation [43, 44]. An increase in osteoclast number was observed by one week after radiation exposure, with corresponding loss of trabecular bone [43, 44]. However, blocking osteoclast activity with the use of an anti-resorptive agent (Bisphosphonate) prevented these changes without suppressing osteoblast activity [45]. These findings suggest that the activation of osteoclasts occurs early after radiation exposure, ultimately leading to a loss in bone.

2.5 Potential Cause

The cause of radiation-induced bone loss and fractures is unknown, though a causal role could likely be associated with inflammation that occurs after exposure to radiation. Pro-inflammatory cytokines, including IL-1, IL-6, and TNFα, contribute to bone loss. It has also been shown that radiation exposure is associated with both an increase in pro-inflammatory cytokine production, as well as an increase in bone loss within the irradiated volume. However, the two have never been linked within this (radiation) context. Therefore, it is believed that radiation exposure results in an inflammatory response, leading to the production of pro-inflammatory cytokines, including IL-1, IL-6, and TNFα. These cytokines then stimulate osteoclast formation and activity, resulting in an overall loss of bone.

CHAPTER 3 – IL-1, IL-6, AND TNFα GENETIC DEFICIENCIES IN THE EXAMINATION OF THE ROLES OF THESE CYTOKINES IN RADIATION-INDUCED BONE LOSS

Bone fractures can have a profound impact on an individual's quality of life. An estimated 24% of patients of age 50 years or older who suffer from a hip fracture will die within the first year following their fracture [1]. Exposure to ionizing radiation, particularly in the context of cancer patients receiving radiotherapy, results in damage to normal (non-tumor) tissue, including bone: bone loss can occur at locations that absorb dose, which increases the risk of downstream fracture [2, 3]. It has been demonstrated that the risk of hip fracture increased 66%, 65%, and 216% for cervical, rectal, and anal cancer, respectively, for patients who underwent radiation therapy [3].

Inflammation is a common response to radiation-induced tissue damage, in order to remove cell debris and prepare the injured area for repair [14]. Inflammation is characterized by leukocyte infiltration and increased presence of pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα [4, 5]. Little is known about the roles of IL-1, IL-6, and TNFα in radiation-induced bone loss, all of which are active stimulators of bone resorption. In order to better develop therapies to decrease the bone loss experienced by patients following radiotherapy, the mechanistic roles of these cytokines in the activation of osteoclasts needs to be determined.

The purpose of this study was to determine if pro-inflammatory cytokines are able to influence bone loss after radiation exposure. The approach taken in this study involved

the utilization of knockout mice, deficient in IL-1β receptor, IL-6, TNFα, or TNFα and IL-1β receptor combined. By knocking out these cytokines, either individually or combined, it will be possible to determine if each cytokine plays a critical role in radiation-induced bone loss. With this information, future studies can focus on treatments targeting individual or multiple cytokines.

3.1 Methods

3.1.1 Animal Models and Care

A total of 98 female mice were ordered from the Jackson Laboratory (Bar Harbor, ME); twelve B6.129S7-Il1r1^{tm1Imx}/J (IL-1β receptor knock out), twelve B6.129S2-Il6^{tm1Kopf}/J (IL-6 knock out), eleven B6.129S-Tnf^{tm1Gkl}/J (TNF knock out), fourteen B6.129S-Tnfrsf1a^{tm1Imx}Il1r1^{tm1Imx}/J (TNF and IL-1β receptor knock out), and forty-nine B57BL/6J (wild-type). After arrival, all mice were housed three per cage and were isolated for at least a one week period. After the isolation period, mice were grouped according to mass. Due to difficulties receiving various strains of mice of a particular age at a particular point in time, four separate experiments were performed (Table 3.1). Each experiment consisted of a Group (wild type vs. knockout), as well as a treatment (non-irradiated vs. irradiated).

Table 3.1 – Experimental groupings

Strain	Non-Irradiated	Irradiated
Experiment 1		
IL-1β receptor knock out	n = 6	n = 6
Wild type	n = 6	n = 6
Experiment 2		
IL-6 knock out	n = 6	n = 6
Wild type	n = 6	n = 6
Experiment 3		
TNFα knock out	n = 11 (left)	n = 11 (right)
Wild type	n = 11 (left)	n = 11 (right)
Experiment 4		
TNFα/IL-1β receptor knock out	n = 14 (left)	n = 14 (right)
Wild type	n = 14 (left)	n = 14 (right)

All mice were provided with food and water ad libitum and housed within a temperature (18 - 26°C) and light (12 hour light/dark cycle) controlled environment. All subsequent procedures were approved by the Institutional Animal Care and Use Committee of Clemson University.

3.1.2 Whole-Body Radiation Exposure: Experiment 1 and Experiment 2

Mice that were part of experiment 1 (10 weeks of age) and experiment 2 (8 weeks of age), were anesthetized with isoflurane prior to radiation exposure. Once anesthetized, mice in irradiated groups were placed 4.4 cm below the shield of X-ray source and were exposed to a 2 Gy whole-body dose at a rate of 1.36 Gy/min. Mice in non-irradiated groups remained under anesthesia for an equivalent period of time as the irradiated

groups. After radiation exposure, anesthesia was removed and all mice were allowed to regain consciousness.

3.1.3 Single-Limb Radiation Exposure: Experiment 3 and Experiment 4

At 10 weeks of age, all mice were anesthetized with isoflurane prior to radiation exposure. Once anesthetized, mice in irradiated groups were placed under lead shielding, leaving only the right tibia and distal femur unshielded. The unshielded limb was placed 4.4 cm below the shield of X-ray source and exposed to a 2 Gy dose at a rate of 1.36 Gy/min. Mice in non-irradiated groups remained under anesthesia for an equivalent period of time as the irradiated groups. After radiation exposure, anesthesia was removed and all mice were allowed to regain consciousness.

3.1.4 Euthanasia of Animals and Tissue Collection

Twelve days after radiation exposure, all mice were anesthetized with isoflurane. Blood was collected by cardiac puncture and exsanguination. After blood collection, cervical dislocation was performed to ensure death of all animals. After euthanasia, the right and left hind limbs (tibia and femur) of each mouse were collected. All non-osseous tissue was removed from the tibiae and femora at the time of collection. The tibiae and femora were placed in a 10% formalin solution for a 48 hour period. After 48 hours, the

tibiae and femora were removed from the 10% formalin solution and placed and stored in a 70% ethanol solution.

3.1.5 Micro-computed tomography

The tibiae of all mice were analyzed using micro-computed tomography (MicroCT) (μCT 20; Scanco Medical AG, Bassersdorf, Switzerland) at a threshold of 225. A section of each tibia, immediately distal to the epiphyseal plate and measuring 1 mm in length, was scanned with a 10 μm voxel size. Three dimensional images of each tibia were then reconstructed from all of the scans and were used to evaluate trabecular bone parameters. Trabecular bone volume fraction (BV/TV), connectivity density (ConnD.), structural model index (SMI), trabecular number (Tb. N), trabecular thickness (Tb. Th), trabecular separation (Tb. Sp), and volumetric bone mineral density (vBMD) were then analyzed for each sample using Scanco analysis software.

3.1.6 Statistical Analyses

All data are presented as mean ± standard error. Significance was determined using SigmaStat version 3.5 (Systat Software Inc., Richmond, CA) with a p-value ≤ 0.05. A two-way analysis of variance was performed with a Holm-Sidak test on all collected data. The threshold for significance for all tests was set at a 5% probability of committing a type I error ($p = 0.05$).

3.2 Results

3.2.1 Wild Type Comparison to IL-1β Receptor Knockout

Trabecular bone parameters of wild type and IL-1β receptor knockout non-irradiated and irradiated groups were collected using MicroCT (Table 3.2).

Table 3.2 – Trabecular bone parameters of non-irradiated (NR) and irradiated (IRR) wild type (WT) vs. IL-1β receptor knockout (IL-1βr KO) mice tibia

	BV/TV (%)	ConnD. ($1/mm^3$)	SMI	Tb. N (1/mm)	Tb. Th (µm)	Tb. Sp (µm)	vBMD (mg HA/cm^3)
WT							
NR	7.29 ± 0.57	115 ± 48	2.62 ± 0.22	3.97 ± 0.36	35.0 ± 0.4	254 ± 11	72.6 ± 14.2
IRR	5.55 ± 0.39*	50.6 ± 19.7*	2.90 ± 0.13*	3.48 ± 0.51*	36.5 ± 0.3	293 ± 17*	57.2 ± 12.9*
IL-1βr KO							
NR	8.08 ± 0.54	97.1 ± 22.8	2.60 ± 0.14	4.07 ± 0.46	37.5 ± 0.8#	247 ± 12	77.9 ± 13.7
IRR	5.38 ± 0.41*	51.3 ± 20.6*	2.96 ± 0.13*	3.14 ± 0.44*	38.1 ± 0.9	327 ± 22*	43.2 ± 17.2*

Note: All values are mean ± standard error. BV/TV, trabecular bone volume; ConnD., connectivity density of trabeculae; SMI, structural model index; Tb. N, trabecular number; Tb. Th, trabecular thickness; Tb. Sp, trabecular separation; vBMD, volumetric bone mineral density. #, significant difference ($p \leq 0.05$) between groups (wild type vs. knockout). *, significant difference ($p \leq 0.05$) between treatment (non-irradiated vs. irradiated).

The animal groups utilized were comparable in terms of micro-architecture. Most bone parameters for each group, regardless of treatment (e.g. for non-irradiated wild type vs. non-irradiated knockout), were similar ($p \geq 0.05$), with the exception of Tb. Th: non-

irradiated knockout was greater than non-irradiated wild type ($p \leq 0.01$). However, radiation treatment resulted in a decline of bone volume and deterioration of micro-architecture (Figures 3.1 and 3.2).

BV/TV was lower in irradiated groups, as compared to non-irradiated groups. Within irradiated groups, declines of 24% and 33% were observed for wild type and knockout individuals, respectively, as compared to non-irradiated groups. Like BV/TV, ConnD. was also greater in non-irradiated groups, as compared to irradiated groups. Declines of 56% and 47% were observed in wild type and knockout irradiated groups, respectively, as compared to non-irradiated groups.

No significant interactions (Group X Radiation treatment) occurred for any variables ($p \geq 0.05$).

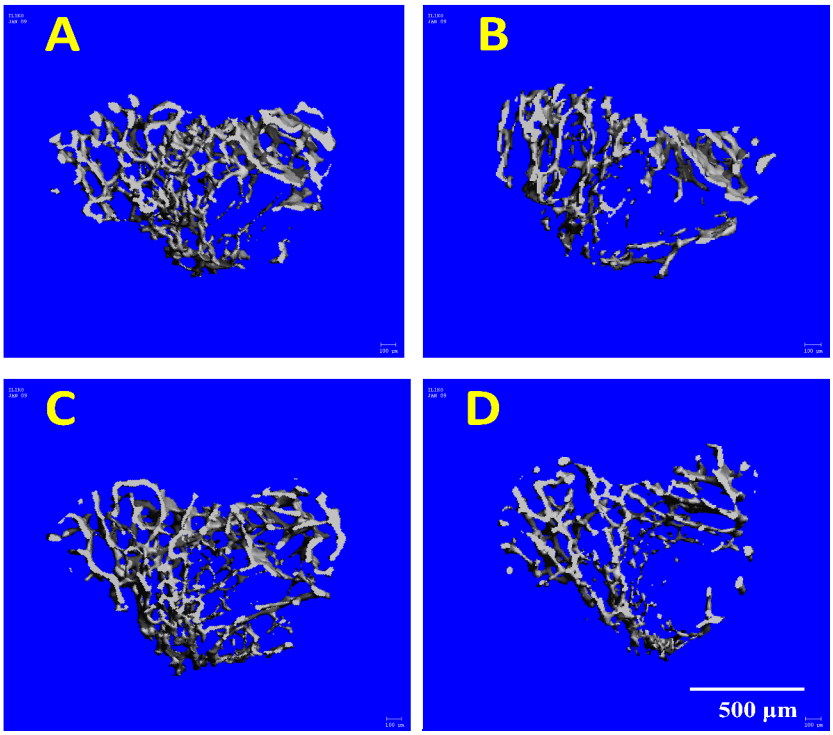

Figure 3.1 - MicroCT images of (A) wild type non-irradiated, (B) wild type irradiated, (C) IL-1β receptor knockout non-irradiated, and (D) IL-1β receptor knockout irradiated mice tibia.

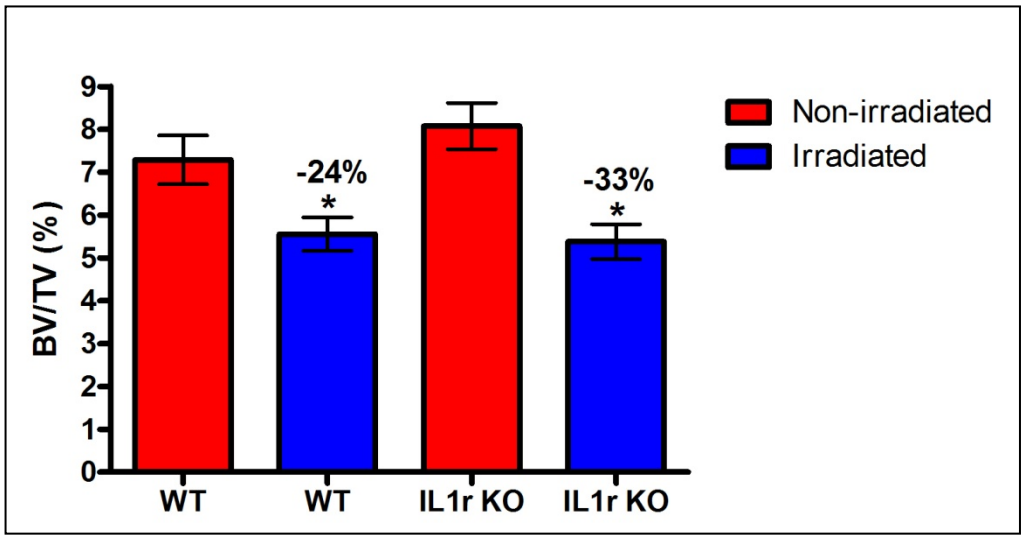

Figure 3.2 – Trabecular bone volume (BV/TV) of non-irradiated and irradiated wild type (WT) vs. IL-1β receptor knockout (IL1r KO) mice.

N = 6 for all groups. All values are mean ± standard error. Significant differences between the treatments, non-irradiated vs. irradiated, were observed for the wild type and IL-1β receptor knockout groups (*, $p<0.001$). No significant differences were observed between Groups, wild type vs. IL-1β receptor knockout, or between the treatments combined with the Groups.

3.2.2 Wild Type Comparison to IL-6 Knockout

Table 3.3 – Trabecular bone parameters of non-irradiated (NR) and irradiated (IRR) wild type (WT) vs. IL-6 knockout (IL6 KO) mice tibia

	BV/TV (%)	ConnD. (1/mm^3)	SMI	Tb. N (1/mm)	Tb. Th (µm)	Tb. Sp (µm)	vBMD (mg HA/cm^3)
WT							
NR	6.56 ± 0.37	92.0 ± 19.5	2.71 ± 0.17	4.47 ± 0.27	30.9 ± 1.0	225 ± 6	90.4 ± 10.1
IRR	4.75 ± 0.42*	52.4 ± 19.7*	2.99 ± 0.11*	3.43 ± 0.42*	32.4 ± 1.1	298 ± 14*	61.5 ± 14.1*
IL-6 KO							
NR	6.84 ± 0.36	111 ± 29	2.54 ± 0.08$^{\#}$	4.26 ± 0.48	31.8 ± 0.8	238 ± 12	88.1 ± 11.2
IRR	4.70 ± 0.15*	44.4 ± 13.5*	2.89 ± 0.05*	3.55 ± 0.27*	31.3 ± 0.4	287 ± 9*	55.9 ± 5.3*

Note: All values are mean ± standard error. BV/TV, trabecular bone volume; ConnD., connectivity density of trabeculae; SMI, structural model index; Tb. N, trabecular number; Tb. Th, trabecular thickness; Tb. Sp, trabecular separation; vBMD, volumetric bone mineral density. $^{\#}$, significant difference ($p \leq 0.05$) between groups (wild type vs. knockout). *, significant difference ($p \leq 0.05$) between treatment (non-irradiated vs. irradiated).

The animal groups utilized were comparable in terms of micro-architecture. All bone parameters for each group, regardless of treatment (e.g. for non-irradiated wild type vs. non-irradiated knockout), were similar ($p \geq 0.05$), with the exception of SMI: non-irradiated knockout was less than non-irradiated wild type ($p \leq 0.01$; Table 3.3). However, radiation treatment resulted in a decline of bone volume and deterioration of micro-architecture (Figures 3.3 and 3.4).

BV/TV was lower in irradiated groups, as compared to non-irradiated groups (Table 3.3). Within irradiated groups, declines of 28% and 31% were observed for wild type and knockout groups, respectively, as compared to non-irradiated groups. Like BV/TV, ConnD. was lower in irradiated groups, as compared to non-irradiated groups. Declines of 43% and 60% were observed for wild type and knockout irradiated groups, respectively, as compared to non-irradiated groups.

No significant interactions (Groups X Radiation treatment) occurred for any variables ($p \geq 0.05$).

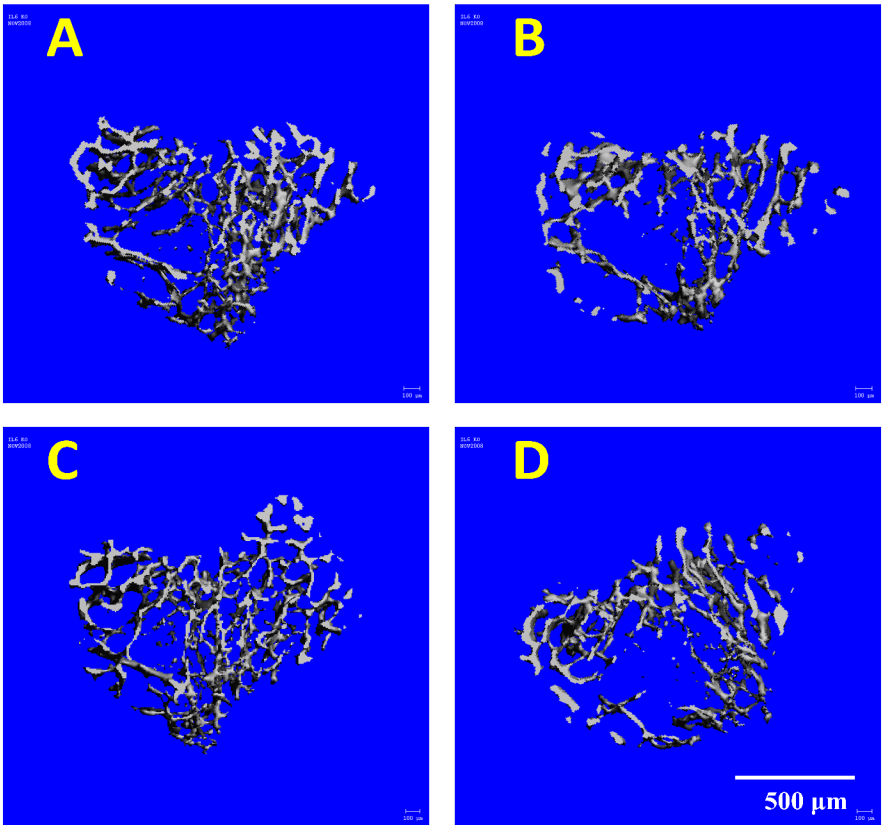

Figure 3.3 – MicroCT images of (A) wild type non-irradiated, (B) wild type irradiated, (C) IL-6 knockout non-irradiated, and (D) IL-6 knockout irradiated mice tibia.

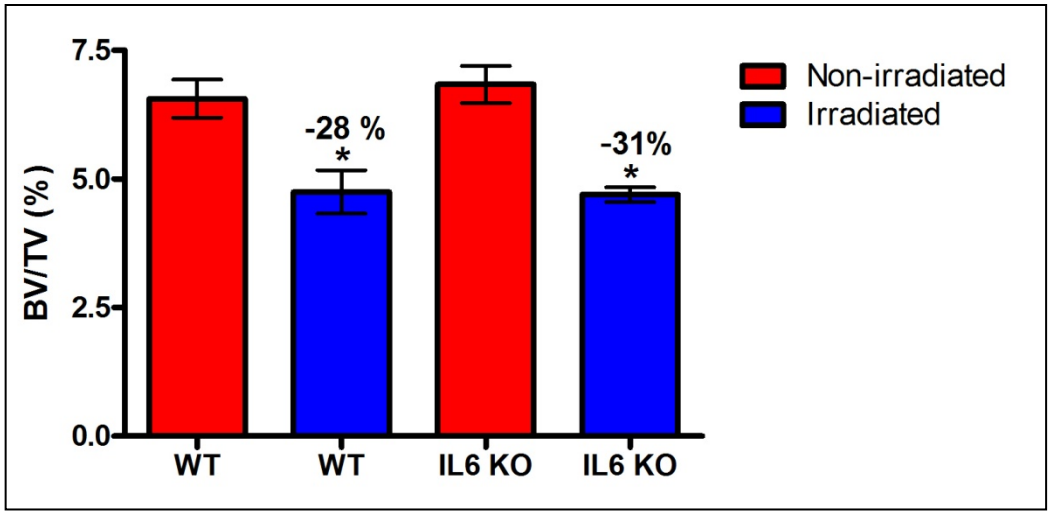

Figure 3.4 – Trabecular bone volume (BV/TV) of non-irradiated and irradiated wild type (WT) vs. IL-6 knockout (IL6 KO) mice.

N=6 for all groups. All values are mean ± standard error. Significant differences between treatments, non-irradiated vs. irradiated, were observed for the wild type and IL-6 knockout groups (*, $p<0.001$). No significant differences were observed between Groups, wild type vs. IL-6 knockout, or between the treatments combined with the Groups.

3.2.3 Wild Type Comparison to TNFα Knockout

Table 3.4 – Trabecular bone parameters of non-irradiated (NR) and irradiated (IRR) wild type (WT) vs. TNFα knockout (TNFα KO) mice tibia

	BV/TV (%)	ConnD. (1/mm^3)	SMI	Tb. N (1/mm)	Tb. Th (µm)	Tb. Sp (µm)	vBMD (mg HA/cm^3)
WT							
NR	5.49 ± 0.32	43.2 ± 5.78	2.82 ± 0.08	3.51 ± 0.06	38 ± 0.0	0.29 ± 0.01	70.6 ± 3.99
IRR	4.42 ± 0.26*	25.9 ± 2.93*	3.07 ± 0.07*	3.07 ± 0.08*	39 ± 0.0	0.33 ± 0.01*	54.8 ± 3.17*
TNFα KO							
NR	3.44 ± 0.18	15.7 ± 1.38	3.04 ± 0.07	2.58 ± 0.09	39 ± 0.0	0.39 ± 0.02	41.3 ± 3.46
IRR	2.59 ± 0.1*$^{\#}$	7.3 ± 1.13*$^{\#}$	3.23 ± 0.06*$^{\#}$	2.35 ± 0.06*$^{\#}$	40 ± 0.0	0.43 ± 0.01*$^{\#}$	26.1 ± 2.27*$^{\#}$

Note: All values are mean ± standard error. BV/TV, trabecular bone volume; ConnD., connectivity density of trabeculae; SMI, structural model index; Tb. N, trabecular number; Tb. Th, trabecular thickness; Tb. Sp, trabecular separation; vBMD, volumetric bone mineral density. $^{\#}$, significant difference ($p \leq 0.05$) between groups (wild type vs. knockout). *, significant difference ($p \leq 0.05$) between treatment (non-irradiated and irradiated).

A significant group effect was observed for most bone parameters ($p \leq 0.01$; Table 3.4), with the exception of Tb. Th ($p \geq 0.05$), where knockout mice, regardless of radiation treatment, experienced significantly lower bone parameters as compared to wild type. However, knockout groups appeared to have started with a lower overall bone mass, as compared to wild type groups. Therefore, the significant decrease in bone parameters between knockout non-irradiated and irradiated individuals is comparable to the decrease in bone parameters between wild type non-irradiated and irradiated individuals.

Radiation treatment also resulted in a decline of bone volume and deterioration of micro-architecture (Figures 3.5 and 3.6). BV/TV was lower in irradiated groups, as compared to non-irradiated groups. Within irradiated groups, declines of 19% and 25% were observed for wild type and knockout groups, respectively, as compared to non-irradiated groups. Like BV/TV, ConnD. was also lower in irradiated groups, as compared to non-irradiated groups (Table 3.4). Declines of 40% and 54% were observed for wild type and knockout irradiated groups, respectively, as compared to non-irradiated groups.

No significant interactions (Groups X Radiation treatment) occurred for any variables ($p \geq 0.05$).

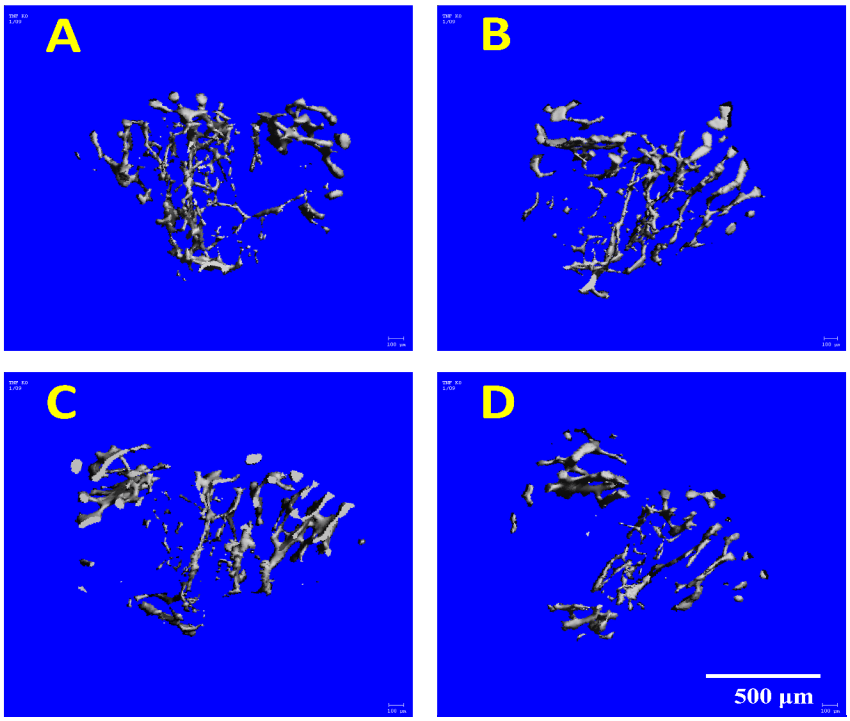

Figure 3.5 – MicroCT images of (A) wild type non-irradiated, (B) wild type irradiated, (C) TNFα knockout non-irradiated, and (D) TNFα knockout irradiated mice tibia.

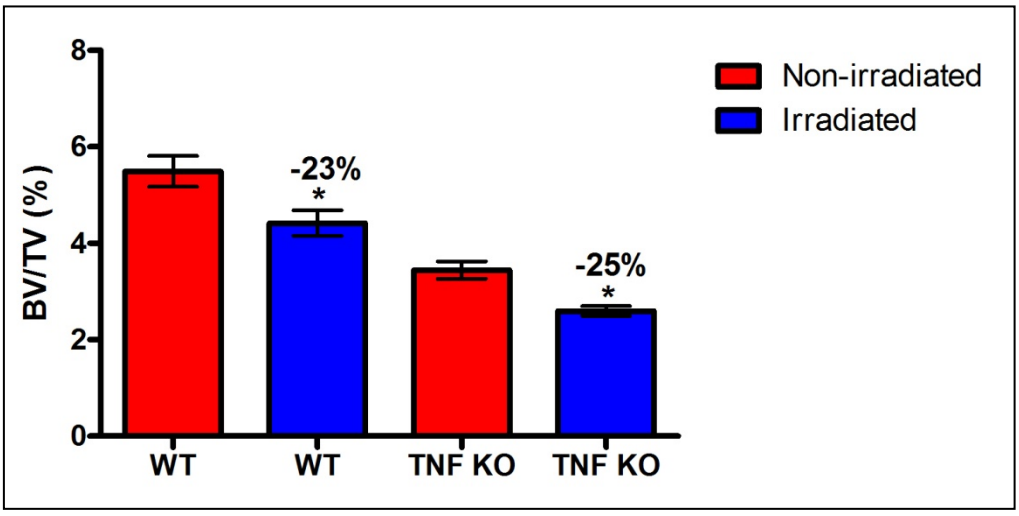

Figure 3.6 – Trabecular bone volume (BV/TV) of non-irradiated and irradiated wild type (WT) vs. TNFα knockout (TNF KO) mice.

N=11 for all groups. All values are mean ± standard error. Significant differences between the treatments, non-irradiated vs. irradiated, were observed for the wild type and TNF knockout groups (*, $p<0.001$).

3.2.4 Wild Type Comparison to TNFα/IL-1β Receptor Knockout

Table 3.5 – Trabecular bone parameters of non-irradiated (NR) and irradiated (IRR) wild type (WT) vs. TNFα and IL-1β receptor knockout (TNFα/IL-1βr KO) mice tibia

	BV/TV (%)	ConnD. (1/mm^3)	SMI	Tb. N (1/mm)	Tb. Th (µm)	Tb. Sp (µm)	vBMD (mg HA/cm^3)
WT							
NR	6.34 ± 0.64	34.9 ± 5.93	3.0 ± 0.09	3.42 ± 0.14	43 ± 0.0	0.3 ± 0.02	78.7 ± 8.45
IRR	5.33 ± 0.5*	29.2 ± 5.27	3.16 ± 0.09*	3.19 ± 0.14*	43 ± 0.0	0.33 ± 0.02	68.2 ± 7.27*
TNFα/IL1βr KO							
NR	6.68 ± 0.4	52.8 ± 7.36	2.93 ± 0.09	3.82 ± 0.08	40 ± 0.0	0.26 ± 0.01	90.7 ± 5.05
IRR	5.17 ± 0.31*	34.8 ± 6.1	3.23 ± 0.11*	3.55 ± 0.07*$^{\#}$	40 ± 0.0$^{\#}$	0.28 ± 0.01$^{\#}$	73.6 ± 3.34*

N=6 for all groups. All values are mean ± standard error. BV/TV, trabecular bone volume; ConnD., connectivity density of trabeculae; SMI, structural model index; Tb. N, trabecular number; Tb. Th, trabecular thickness; Tb. Sp, trabecular separation; vBMD, volumetric bone mineral density. $^{\#}$, significant difference ($p \leq 0.05$) between Groups (wild type vs. knockout). *, significant difference ($p \leq 0.05$) between treatment (non-irradiated vs. irradiated).

The animal groups utilized were comparable in terms of micro-architecture (Table 3.5). Most bone parameters for each group, regardless of treatment (e.g. for non-irradiated wild type vs. non-irradiated knockout), were similar ($p \geq 0.05$), with the exception of Tb. N, Tb. Th, and Tb. Sp: Tb. Sp and Tb. Th non-irradiated wild type was greater than non-irradiated knockout, while Tb. N non-irradiated wild type was less than non-irradiated knockout ($p \leq 0.01$). However, radiation treatment resulted in a decline of bone volume and deterioration of micro-architecture (Figures 3.7 and 3.8). Most parameters within each group showed a significant response to irradiation, with the

exception of ConnD., Tb. Sp, and Tb. Th, which generally indicated loss of bone after exposure.

BV/TV was lower in knockout irradiated individuals, as compared to knockout non-irradiated individuals. Irradiation resulted in a decline of 23% for knockout irradiated individuals, as compared to knockout non-irradiated individuals. Unlike BV/TV, ConnD. was not lower in knockout irradiated individuals, as compared to knockout non-irradiated individuals.

No significant interactions (Group X Radiation treatment) occurred for any variables ($p \geq 0.05$).

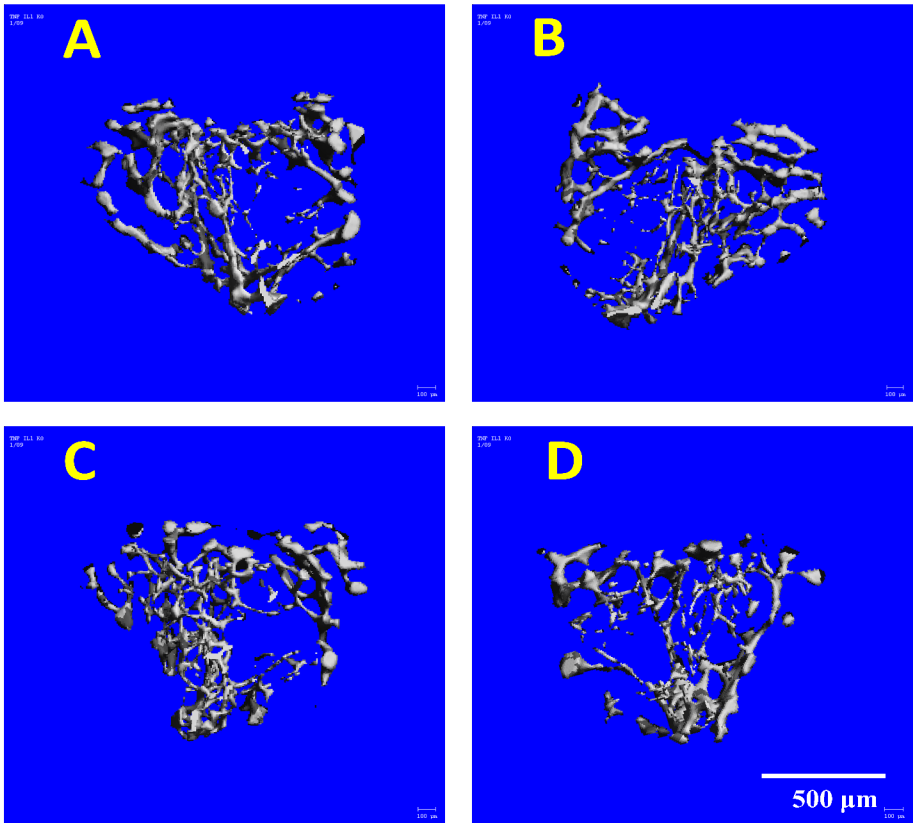

Figure 3.7 – MicroCT images of (A) wild type non-irradiated, (B) wild type irradiated, (C) TNFα/IL-1β receptor knockout non-irradiated, and (D) TNFα/IL-1β receptor knockout irradiated mice tibia.

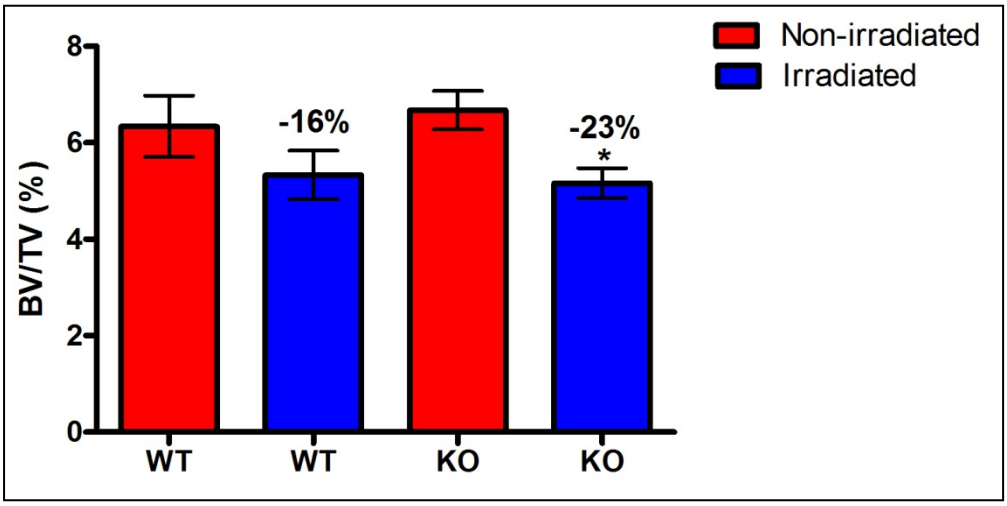

Figure 3.8 – Trabecular bone volume (BV/TV) of non-irradiated and non-irradiated wild type (WT) vs. TNFα and IL-1β receptor knockout (KO) mice. N=14 for all groups. All values are mean ± standard error.

3.3 Discussion

The presence of pro-inflammatory cytokines is elevated within tissue after exposure to radiation. As these substances have the capacity to induce bone loss by activating osteoclasts, we investigated whether inhibiting the production of these agents using genetic knockout mice could reduce bone loss after irradiation. However, this did not appear to be true: we did not observe preservation of bone after radiation exposure in knockout mice.

By genetically knocking out IL-1β, IL-6, and TNFα, either individually or combined, it was expected that radiation-induced bone loss would be prevented, leaving irradiated samples with a bone volume comparable to non-irradiated samples. However, all of the cytokine deficiencies examined failed to inhibit bone loss in irradiated samples,

as compared to non-irradiated samples. In each of the four experiments performed, trabecular bone parameters of knockout non-irradiated and irradiated samples were comparable to the trabecular bone parameters of wild type non-irradiated and irradiated samples, respectively.

3.4 Limitations and Recommendations

This study involved genetically knocking out various pro-inflammatory cytokines, including IL-1β receptor, IL-6, and TNFα, either individually or combined. While the findings of this study suggest that, within this animal model, IL-1, IL-6, and TNFα do not play a significant role in radiation-induced bone loss, the contribution of these cytokines to radiation-induced bone loss cannot be discounted entirely. It is possible that by genetically knocking out these cytokines before birth, other compensatory mechanisms were able to develop in order to account for the loss of function of the particular cytokine that is absent. For example, knocking out TNFα may have caused a compensatory up-regulation of IL-1. This compensatory up-regulation may have allowed for IL-1 to make up for the loss of function due to the absence of TNFα, since these cytokines share similar biological properties.

In order to better understand the roles played by each of these cytokines in radiation-induced bone loss, studies involving therapeutically blocking each of these cytokines must be performed. By therapeutically blocking these cytokines, compensatory mechanisms will not be able to be developed quickly enough to account for the loss of

function of the particular cytokine being blocked. Therefore, a better understanding of the roles played by each of these cytokines in radiation-induced bone loss will be able to be determined.

CHAPTER 4 – THERAPEUTIC BLOCKAGE OF IL-1 AND TNFα IN THE EXAMINATION OF THE ROLES OF THESE CYTOKINES IN RADIATION-INDUCED BONE LOSS

Exposure to ionizing radiation, particularly in the context of cancer patients receiving radiotherapy, results in damage to normal (non-tumor) tissue, including bone: bone loss can occur at locations that absorb dose, which increases the risk of downstream fracture [2, 3]. Inflammation is a common response to radiation-induced tissue damage, characterized by increased presence of pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα, all of which are active stimulators of bone resorption [4, 5].

In order to begin investigating the roles of IL-1, IL-6, and TNFα in radiation-induced bone loss, studies were performed which utilized knockout mice deficient in IL-1β receptor, IL-6, TNFα, or TNFα and IL-1β receptor combined (Chapter 3). The findings of these studies suggested that, within the particular animal model utilized, IL-1, IL-6, and TNFα do not play a significant role in radiation-induced bone loss. However, the roles of these cytokines in radiation-induced bone loss cannot be completely discounted, due to the possibility of the development of compensatory mechanisms in the knockouts utilized. For example, it is possible that by knocking out TNFα, an up-regulation of IL-1 will occur to make up for the loss of function of TNFα.

In order to better understand the roles of IL-1, IL-6, and TNFα in radiation-induced bone loss, while reducing the possibility of the development of compensatory mechanisms, therapeutic blockage of these cytokines was performed. A TNF-binding

protein, Enbrel (Etanercept), and an IL-1 receptor antagonist, Kineret (Anakinra), were administered, either individually or in combination, to better determine the roles played by these cytokines in radiation-induced bone loss.

4.1 Methods

4.1.1 Animal Models and Care

Eighty eight 12-week old C57BL/6 female mice were ordered from Taconic (Germantown, NY). Upon arrival, all mice were housed three per cage and were isolated for at least a one week period. After the isolation period, mice were grouped according to mass (Table 4.1). Group refers to the various drug treatments individuals received. Treatment refers to exposure to radiation.

Table 4.1 – Experimental groupings

Group	Non-Irradiated	Irradiated
Placebo (PBS)	11	11
Enbrel	11	11
Kineret	11	11
Enbrel + Kineret	11	11

All mice were provided with food and water ad libitum and housed within a temperature (18 - 26°C) and light (12 hour light/dark cycle) controlled environment. All

subsequent procedures were approved by the Institutional Animal Care and Use Committee of Clemson University.

4.1.2 Drug Administration

At 16-weeks of age, all mice received daily intraperitoneal injections for a 14-day period. The dose of each treatment administered was equivalent to the once weekly or once monthly doses administered to humans; 5 mg/kg phosphate buffered saline, 1 mg/kg Enbrel, 10 mg/kg Kineret, or 1 mg/kg Enbrel + 10 mg/kg Kineret.

4.1.3 Radiation Exposure

On the third day of injection, all mice were anesthetized with isoflurane prior to radiation exposure. Once anesthetized, mice in irradiated groups were placed 4.4 cm below the shield of X-ray source and were exposed to a 2 Gy whole-body dose at a rate of 1.36 Gy/min. Mice in non-irradiated groups remained under anesthesia for an equivalent period of time as the irradiated groups. After radiation exposure, anesthesia was removed and all mice were allowed to regain consciousness.

4.1.4 Euthanasia of Animals and Tissue Collection

Twenty four hours after radiation exposure, three mice from each group were euthanized, while all remaining mice were euthanized thirteen days after radiation exposure. In order to euthanize, all mice were anesthetized with isoflurane and blood was collected by cardiac puncture and exsanguination. After blood collection, cervical dislocation was performed to ensure death of all animals. After euthanasia, the right and left hind limbs (tibia and femur) of each mouse was collected. All non-osseous tissue was removed from the tibiae and femora at the time of collection. The tibiae and femora were placed in a 10% formalin solution for a 48 hour period. After 48 hours, the tibiae and femora were removed from the 10% formalin solution and placed and stored in a 70% ethanol solution.

4.1.5 Micro-computed tomography

The tibiae of mice euthanized thirteen days after radiation exposure were analyzed using micro-computed tomography (MicroCT) (μCT 20; Scanco Medical AG, Bassersdorf, Switzerland) at a threshold of 215. A section of each tibia, immediately distal to the epiphyseal plate and measuring 1 mm in length, was scanned with a 10 μm voxel size. Three dimensional images of each tibia were then reconstructed from all of the scans and were used to evaluate trabecular bone parameters. Trabecular bone volume fraction (BV/TV), connectivity density (ConnD.), structural model index (SMI),

trabecular number (Tb. N), trabecular thickness (Tb. Th), trabecular separation (Tb. Sp), and volumetric bone mineral density (vBMD) were then analyzed for each sample using Scanco analysis software.

4.1.6 Immunohistochemistry

The tibiae of mice euthanized twenty four hours after radiation exposure were decalcified in a weak formic acid solution (Immunocal™) overnight. The tibiae were then processed and embedded in paraffin, and cut into sagittal sections with a thickness of 5μm. Slides were deparaffinized and incubated with an anti-TNF-α, anti-IL-1β, or anti-IL-6 specific polyclonal antibody (AbCam, SantaCruz, and AbCam respectively), and Vectastain Elite ABC kit and Vector VIP kit (Vector Laboratories, Inc.) were used to visualize primary antibody binding.

4.1.7 Statistical Analyses

All data are presented as mean ± standard error. Significance was determined using SigmaStat version 3.5 (Systat Software Inc., Richmond, CA) with a p-value ≤ 0.05. A two-way analysis of variance was performed with a Holm-Sidak test on all collected data. The threshold for significance for all tests was set at a 5% probability of committing a type I error ($p = 0.05$).

4.2 Results

4.2.1 Comparison of Non-irradiated and Irradiated Placebo and Drug Treated Trabecular Bone Parameters

Trabecular bone parameters of placebo and drug treated non-irradiated and irradiated groups were collected using MicroCT (Table 4.2).

Table 4.2 – Trabecular bone parameters of non-irradiated (NR) and irradiated (IRR) mice tibia, with groups receiving various treatments; phosphate buffered saline (PBS), Enbrel (E), Kineret (K), Enbrel + Kineret (E+K)

		BV/TV (%)	ConnD. ($1/mm^3$)	SMI	Tb. N (1/mm)	Tb. Th (µm)	Tb. Sp (µm)	vBMD (mg HA/cm^3)
PBS								
	NR	5.69 ± 0.75	25.9 ± 5.78	2.70 ± 0.11	3.0 ± 0.12	45.7 ± 1.41	337 ± 15.1	66.2 ± 8.27
	IRR	4.31 ± 0.28*	16.2 ± 2.52*	2.86 ± 0.11	2.82 ± 0.09*	44.6 ± 1.28	358 ± 12.1*	49.1 ± 3.27*
E								
	NR	5.61 ± 0.5	23.1 ± 2.72	2.90 ± 0.07	3.03 ± 0.11	46.9 ± 2.18	333 ± 12.4	65.2 ± 5.67
	IRR	3.48 ± 0.24*	13.1 ± 2.03*	3.07 ± 0.11	2.53 ± 0.08*	43.2 ± 1.06	402 ± 13.6*	39.4 ± 3.19*
K								
	NR	5.68 ± 0.23	27.6 ± 2.52	2.80 ± 0.08	2.92 ± 0.08	47.1 ± 1.14	347 ± 9.50	60.8 ± 3.76
	IRR	4.13 ± 0.22*	20.4 ± 1.80*	2.87 ± 0.08	2.65 ± 0.07*	42.9 ± 1.17	381 ± 9.35*	42.4 ± 3.95*
E+K								
	NR	5.56 ± 0.36	27.1 ± 3.19	2.79 ± 0.07	3.14 ± 0.11	44.8 ± 1.70	322 ± 13.2	60.1 ± 5.51
	IRR	4.32 ± 0.26*	14.2 ± 2.71*	2.88 ± 0.09	2.50 ± 0.12*	47.6 ± 1.23	416 ± 17.8*	45.7 ± 2.86*

Note: All values are mean ± standard error. BV/TV, trabecular bone volume; ConnD., connectivity density of trabeculae; SMI, structural model index; Tb. N, trabecular number; Tb. Th, trabecular thickness; Tb. Sp, trabecular separation; vBMD, volumetric bone mineral density. #, significant difference ($p \leq 0.05$) between groups (placebo vs. Enbrel, placebo vs. Kineret, placebo vs. Enbrel + Kineret). *, significant difference ($p \leq 0.05$) between treatment (non-irradiated and irradiated).

The animal groups utilized were comparable in terms of micro-architecture. All bone parameters for each group, regardless of treatment (e.g. for non-irradiated placebo vs. non-irradiated Enbrel), were similar ($p \geq 0.05$). However, radiation treatment resulted in a decline of bone volume and deterioration of micro-architecture (Figures 4.1 and 4.2). Most parameters within each group showed a significant response to irradiation ($p \leq$

0.01), with the exception of Tb. Th and SMI, which generally indicated loss of bone after exposure.

BV/TV was lower in irradiated groups, as compared to non-irradiated groups. Within irradiated groups, declines of 24%, 38%, 27%, and 22% were observed in placebo, Enbrel, Kineret, and Enbrel + Kineret groups, respectively, as compared to non-irradiated groups. Like BV/TV, ConnD. was also greater in non-irradiated groups, as compared to irradiated groups. Declines of 37%, 43%, 26%, and 48% were observed in placebo, Enbrel, Kineret, and Enbrel + Kineret irradiated groups, respectively, as compared to non-irradiated groups.

No significant interactions (Group X Radiation treatment) occurred for any variables ($p \geq 0.05$), with the exception of Tb. Sp.

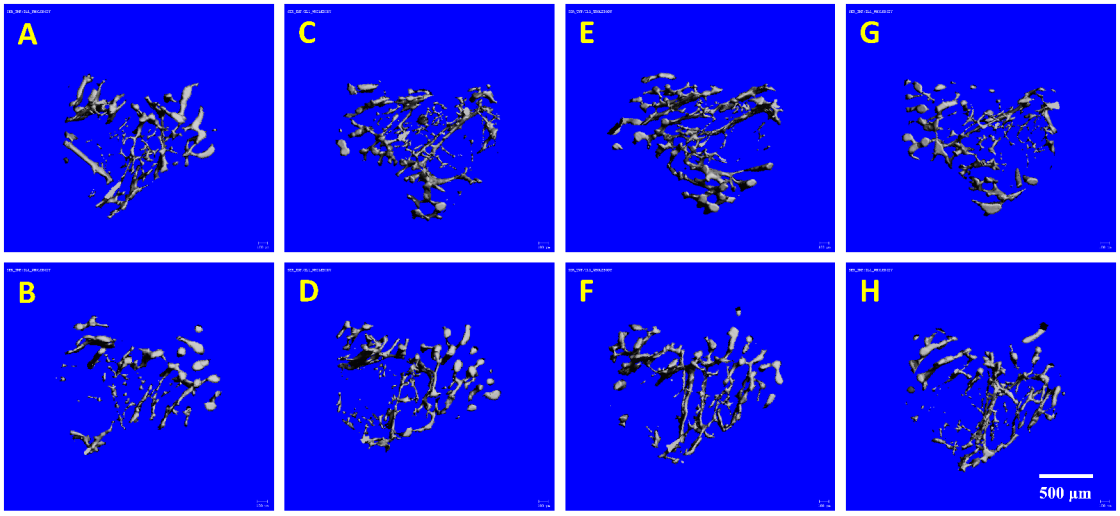

Figure 4.1 – MicroCT images of (A) PBS non-irradiated, (B) PBS irradiated, (C) Enbrel non-irradiated, (D) Enbrel irradiated, (E) Kineret non-irradiated, (F) Kineret irradiated, (G) Enbrel + Kineret non-irradiated, and (H) Enbrel + Kineret irradiated.

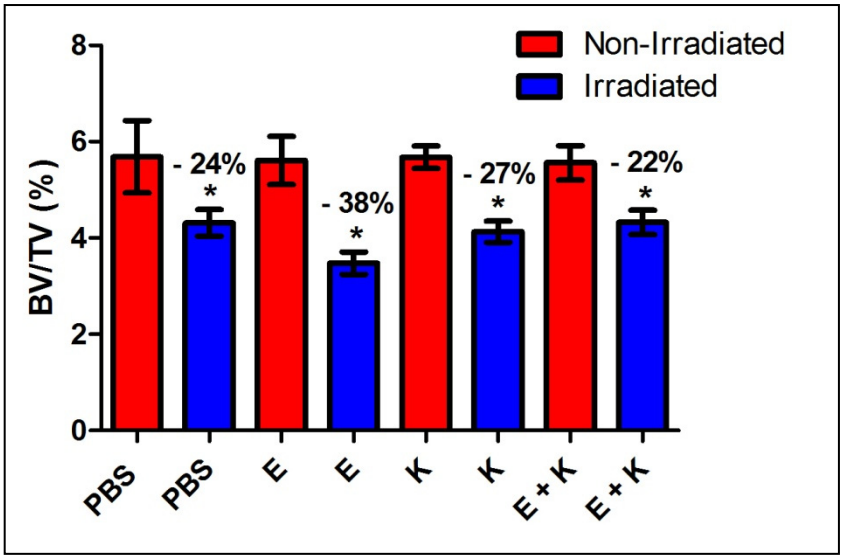

Figure 4.2 – Trabecular bone volume (BV/TV) of non-irradiated and irradiated mice tibia, with groups receiving various treatments; phosphate buffered saline (PBS), Enbrel (E), Kineret (K), or Enbrel + Kineret (E+K). N=8 for all groups. All values are mean ± standard error.

4.2.2 Comparison of Non-irradiated and Irradiated Placebo and Drug Treated TNFα Presence

Immunohistochemistry was performed to detect the presence of TNFα in tibiae sections of all groups twenty-four hours post-irradiation (Figure 4.3).

Figure 4.3 – Immunohistochemistry staining in detection of presence of TNFα. TNFα presence indicated by purple coloring.

TNFα was detected in non-irradiated and irradiated samples in all groups. However, an increased amount of TNFα was detected in irradiated samples, as compared to non-irradiated samples, for all groups. Increased TNFα levels were also observed in

Enbrel treated mice and Kineret treated mice, as compared to placebo and Enbrel + Kineret treated mice.

4.2.3 Comparison of Non-irradiated and Irradiated Placebo and Drug Treated IL-1β Presence

Immunohistochemistry was performed to detect the presence of IL-1β in tibiae sections of all groups twenty-four hours post-irradiation (Figure 4.4).

Figure 4.4 – Immunohistochemistry staining in detection of presence of IL-1β. IL-1β presence is indicated by purple coloring.

IL-1β was detected in non-irradiated and irradiated samples in all groups. However, similar to what was observed for TNFα, irradiated samples contained increased

amounts of IL-1β, as compared to non-irradiated samples, for all groups. The amount of IL-1β detected in Enbrel and Enbrel + Kineret irradiated samples were comparable to the amount of IL-1β detected in placebo treated irradiated samples. However, it was observed that Kineret treated irradiated samples contained a decreased amount of IL-1β, as compared to placebo treated irradiated samples. There were also elevated levels of IL-1β in non-irradiated Enbrel, Kineret, and Enbrel + Kineret treated groups, as compared to non-irradiated placebo treated samples.

4.2.4 Comparison of Non-irradiated and Irradiated Placebo and Drug Treated IL-6 Presence

Immunohistochemistry was performed to detect the presence of IL-6 in tibiae sections of all groups twenty-four hours post-irradiation (Figure 4.5).

Figure 4.5 – Immunohistochemistry staining in detection of presence of IL-6. IL-6 presence is indicated by purple coloring.

Increased levels of IL-6 were observed in irradiated placebo, Enbrel, and Enbrel + Kineret treated groups, as compared to the respective non-irradiated groups. The levels of IL-6 detected in irradiated Kineret treated samples was comparable to IL-6 levels detected in non-irradiated Kineret treated samples.

4.3 Discussion

The conclusions drawn from the findings of this study are consistent with those of the previous study performed (Chapter 3). The data collected from this study further support the suggestion that, within the particular animal model utilized, pro-inflammatory

cytokines, including IL-1 and TNFα, do not play a significant role in radiation-induced bone loss. By therapeutically blocking these cytokines, it was expected that any compensatory mechanisms that may have developed when genetically knocking out these cytokines would be avoided, thereby preventing radiation-induced bone loss. However, therapeutically blocking these cytokines, either individually or combined, did not prevent radiation-induced bone loss, with declines in bone volume fractions comparable to those observed in Chapter 3.

Immunohistochemistry was also performed to detect the presence of IL-1, IL-6, and TNFα in all groups. Increased levels of IL-1, IL-6, and TNFα were expected to be observed in irradiated placebo treated groups, as compared to non-irradiated placebo treated groups. It was also thought that treatment with TNF-binding protein and/or IL-1 receptor antagonist would result in an increase in the production of the cytokine being therapeutically targeted; an increase in TNFα and/or IL-1 production would result in order to compensate for the functional block of these cytokines, thereby resulting in increased production of TNFα and/or IL-1 within irradiated groups, as compared to non-irradiated groups. Qualitatively, it appeared that, in some instances, treatment with TNF-binding protein, IL-1 receptor antagonist, or a combination of the two did result in increased production of TNFα and IL-1.

Previous studies have been performed which have examined the contributions of pro-inflammatory cytokines in estrogen-deficiency mediated bone loss. It has been demonstrated that ovariectomized mice had greater numbers of TNFα producing T-cells, as well as T-cells that produced greater amounts of TNFα, each of which resulted in an

elevated loss of bone as compared to controls [23, 24]. These studies suggested that TNFα played a significant role in estrogen-deficiency mediated bone loss. In further support of these findings, another study demonstrated that treatment with TNF-binding protein prevented bone loss in ovariectomized mice, as compared to non-ovariectomized mice [25]. Studies were also performed with IL-1 receptor antagonist, which produced similar findings to those of the studies utilizing TNF-binding protein. It was demonstrated that treatment with IL-1 receptor antagonist prevented bone loss in ovariectomized mice as compared to non-ovariectomized mice [26]. These findings suggested that, like TNFα, IL-1 played a significant role in estrogen-deficiency mediated bone loss. While multiple studies have provided evidence that TNFα and IL-1 play significant roles in estrogen-deficiency mediated bone loss, evidence has also been presented suggesting that IL-6 does not play as significant a role, if any, in estrogen-deficiency mediated bone loss. Studies demonstrated that treatment with anti-IL-6 antibody was not able to prevent bone loss in ovariectomized mice, as compared to non-ovariectomized mice [25, 26].

Multiple studies provide information supporting the significance of the roles that TNFα, IL-1, and IL-6 play in estrogen-deficiency mediated bone loss. Due to the fact that exposure to radiation results in an inflammatory response, it was hypothesized that IL-1, IL-6, and TNFα would play similar roles in radiation-induced bone loss as they do in estrogen-deficiency mediated bone loss [4, 5]. However, from the findings presented in this study, the roles of these cytokines are not identical to the roles played in estrogen-deficiency mediated bone loss.

4.4 Limitations and Recommendations

This study involved therapeutically blocking various pro-inflammatory cytokines, including IL-1 and TNFα, either individually or combined. In addition to the conclusions drawn from the experiments performed in Chapter 3, the findings of this study further support the suggestion that, within the particular animal model utilized, IL-1 and TNFα do not play a significant role in radiation-induced bone loss. However, it is possible that therapeutically blocking a particular cytokine resulted in the up-regulation of the transcription of a cytokine that could account for the loss of function of the cytokine being blocked.

In order to better determine whether IL-1, IL-6, and TNFα play any significant role in radiation-induced bone loss, a study involving therapeutically blocking the transcription of these cytokines must be performed. By therapeutically blocking the transcription of these cytokines, compensatory mechanisms will not be able to develop quick enough to account for the loss of function of the cytokines being blocked and the up-regulation of transcription of another pro-inflammatory cytokine cannot occur to account for the loss of function of the cytokines being blocked. Therefore, a better understanding of whether these pro-inflammatory cytokines, including IL-1, IL-6, and TNFα, play any significant role in radiation-induced bone loss will be able to be better determined.

CHAPTER 5 – THERAPEUTIC BLOCKAGE OF P38 MAPK TRANSCRIPTION FACTOR IN THE EXAMINATION OF THE ROLES OF IL-1, IL-6, AND TNFα IN RADIATION-INDUCED BONE LOSS

Exposure to ionizing radiation results in bone loss, which increases the risk of downstream fracture [2, 3]. Inflammation is a common response to radiation-induced tissue damage, characterized by increased presence of pro-inflammatory cytokines, including IL-1, IL-6, and TNFα [4, 5]. This thesis tested the possibility that bone loss is mediated by radiation-induced inflammation. However, previous studies performed, that have attempted to reduce the effects of inflammation after radiation exposure, have not prevented bone loss.

Previous studies have demonstrated that mice deficient in IL-1β receptor, IL-6, TNFα, or TNFα and IL-1β receptor combined experienced comparable bone loss after radiation exposure, as compared to wild type mice (Chapter 3). In addition, it has also been demonstrated that treatment with TNF-binding protein and/or IL-1 receptor antagonist did not prevent radiation-induced bone loss, as compared to placebo treated individuals (Chapter 4). The findings of these studies suggested that, within the particular animal model utilized, IL-1, IL-6, and TNFα do not play a significant role in radiation-induced bone loss. However, it is possible that therapeutically blocking a particular cytokine may have resulted in the up-regulation of the transcription of the same cytokine, or another cytokine, that could account for the loss of function of the cytokine being blocked.

In order to better determine whether IL-1, IL-6, and TNFα play any significant role in radiation-induced bone loss within the particular animal model utilized, a study involving therapeutically blocking the transcription of these cytokines was performed. A p38 blocking molecule, AR-447, was utilized, which blocks the action of p38 mitogen-activated protein kinase (MAPK), which is important in the expression and production of IL-1, IL-6, and TNFα [6-8].

5.1 Methods

5.1.1 Animal Models and Care

Fifty 12-week old C57BL/6 female mice were ordered from Taconic (Germantown, NY). Upon arrival, all mice were housed three per cage and were isolated for at least a one week period. After the isolation period, mice were grouped according to mass (Table 5.1). Group refers to the drug treatments the individuals received. Treatment refers to exposure to radiation.

Table 5.1 – Experimental groupings

Group	Non-Irradiated	Irradiated
Placebo	12	13
AR-447	12	13

All mice were provided with food and water ad libitum and housed within a temperature (18 - 26°C) and light (12 hour light/dark cycle) controlled environment. All subsequent procedures were approved by the Institutional Animal Care and Use Committee of Clemson University.

5.1.2 Drug Administration

Prior to administration, AR-447 was prepared in 1% CMC/0.5% Tween at 3 mg/mL. The mixture was then sonicated until a homogenous suspension was achieved.

At 19-weeks of age, all mice received daily oral gavage for a 14-day period. All mice were anesthetized with isoflurane prior to oral gavage. The dose of treatment administered was 30 mg/kg twice per day, administered at 12 hour increments. After treatment administration, all mice were allowed to regain consciousness.

5.1.3 Radiation Exposure

On the third day of drug administration, all mice were anesthetized with isoflurane prior to radiation exposure. Once anesthetized, mice in irradiated groups were placed 4.4 cm below the shield of X-ray source and were exposed to a 2 Gy whole-body dose at a rate of 1.36 Gy/min. Mice in non-irradiated groups remained under anesthesia for an equivalent period of time as the irradiated groups. After radiation exposure, anesthesia was removed and all mice were allowed to regain consciousness.

5.1.4 Euthanasia of Animals and Tissue Collection

Twenty-four hours after radiation exposure, four mice from non-irradiated groups and five mice from irradiated groups were euthanized, while all remaining mice were euthanized thirteen days after radiation exposure. All mice were euthanized within a CO_2 chamber. Cervical dislocation was performed after euthanasia to ensure death of all animals. Of the eighteen mice euthanized twenty four hours post-irradiation, bone marrow was harvested from two mice from each group, while the right and left hind limbs (tibia and femur) were collected from the remaining ten mice. Of the thirty two mice euthanized thirteen days after radiation exposure, the right and left hind limbs (tibia and femur) were collected. All non-osseous tissue was removed from the tibiae and femora at the time of collection. All collected tibiae and femora were placed in a 10% formalin solution for a 48 hour period. After 48 hours, the tibiae and femora were removed from the 10% formalin solution and placed and stored in a 70% ethanol solution.

5.1.5 Micro-computed tomography

The tibiae of mice euthanized thirteen days after radiation exposure were analyzed using micro-computed tomography (MicroCT) (μCT 20; Scanco Medical AG, Bassersdorf, Switzerland) at a threshold of 240. A section of each tibia, immediately distal to the epiphyseal plate and measuring 1 mm in length, was scanned with a 10 μm

voxel size. Three dimensional images of each tibia were then reconstructed from all of the scans and were used to evaluate trabecular bone parameters. Trabecular bone volume fraction (BV/TV), connectivity density (ConnD.), structural model index (SMI), trabecular number (Tb. N), trabecular thickness (Tb. Th), trabecular separation (Tb. Sp), and volumetric bone mineral density (vBMD) were then analyzed for each sample using Scanco analysis software.

5.1.6 Immunohistochemistry

The tibiae of mice euthanized twenty four hours after radiation exposure were decalcified in a weak formic acid solution (Immunocal™) overnight. The tibiae were then processed and embedded in paraffin, and cut into sagittal sections with a thickness of 5 μm. Slides were deparaffinized and incubated with an anti-TNF-α, anti-IL-1β, or anti-IL-6 specific polyclonal antibody (AbCam, SantaCruz, and AbCam respectively), and Vectastain Elite ABC kit and Vector VIP kit (Vector Laboratories, Inc.) were used to visualize primary antibody binding.

5.1.7 RNA Isolation and Real-Time Polymerase Chain Reaction

Mouse whole bone marrow was frozen in RNA*later*™ after isolation. The cells were processed for mRNA isolation using the Qiagen RNeasy® mini-column protocol

with on-column DNase digestion. Purity and RNA concentration was determined using a spectrophotometer (Eppendorf BioPhotometer).

Real-time polymerase chain reaction (RT-PCR) was performed using the QuantiTech SYBR Green kit (Qiagen). Individual RT-PCR reactions were established with 10 ng target gene mRNA, 0.5 μM (IL-1β or GAPDH) or 0.35 μM (IL-6 and TNFα) primers for each target, 2.5 mM $MgCl_2$, and all other components for the reactions using the kit's manufacturer's recommendations. Amplification and detection of target genes was performed using an Eppendorf Mastercycler® *realplex*^{4}. The following cycling profile was used for all reactions (T. Scott, MD Owens/Molecular Immunology 45 (2008) 1001-1008): one cycle of 50°C for 30 minutes (reverse transcriptase), 95°C for 15 minutes (DNA polymerase), then 40 cycles of: 94°C for 15 seconds (denaturation), 57°C for 20 seconds (annealing), and 72°C for 20 seconds (extension). The following melting curve was added to the cycling profile: 95°C for 15 seconds, 57°C for 15 seconds, and 95°C for 15 seconds. The cycle threshold (C_t) values were determined from fluorescence data and relative fold expression. These values were based upon the C_t values of our housekeeping gene, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and were calculated according to Scott & Owens (2008).

mRNA	Primer Sequence (5'-3')	Accession number
GAPDH	F:TCAACAGCAACTCCCACTCTTCCA	NM_008084
	R:ACCACCCTGTTGCTGTACCGTATT	
IL-1β	F:AAGGGCTGCTTCCAAACCTTTGAC	NM_008361
	R:ATACTGCCTGCCTGAAGCTCTTGT	
IL-6	F:ATCCAGTTGCCTTCTTGGGACTGA	NM_031168
	R:TAAGCCTCCGACTTGTGAAGTGGT	
TNF-α	F:AGCCGTGACTGTAATTGCCCTACA	NM_Y00467
	R:TTTAGGCCTCCGCAAAGAGATGGA	

Figure 5.1 – Real-time polymerase chain reaction primer sequences

5.1.8 Statistical Analyses

All data are presented as mean ± standard error. Significance was determined using SigmaStat version 3.5 (Systat Software Inc., Richmond, CA) with a p-value ≤ 0.05. A normality test, equal variance test, and two-way analysis of variance was performed on all collected data. The threshold for significance for all tests was set at a 5% probability of committing a type I error ($p = 0.05$).

5.2 Results

5.2.1 Comparison of Non-irradiated and Irradiated Placebo and Drug Treated Trabecular Bone Parameters

Trabecular bone parameters of placebo and drug treated non-irradiated and irradiated groups were collected using MicroCT (Table 5.2).

Table 5.2 – Trabecular bone parameters of non-irradiated (NR) and irradiated (IRR) mice tibia, with groups receiving either placebo or AR-447 treatment

	BV/TV (%)	ConnD. (1/mm^3)	SMI	Tb. N (1/mm)	Tb. Th (µm)	Tb. Sp (µm)	vBMD (mg HA/cm^3)
PBS							
NR	4.15 ± 0.36	22.3 ± 4.43	2.72 ± 0.11	2.9 ± 0.13	38.4 ± 1.09	352 ± 19.5	63.2 ± 5.07
IRR	3.15 ± 0.2*	15 ± 1.51*	2.95 ± 0.12*	2.64 ± 0.14*	37.4 ± 0.98	396 ± 21.7*	50.1 ± 4.46*
AR-447							
NR	4.25 ± 0.19	21 ± 2.61	2.73 ± 0.08	2.94 ± 0.10	38.1 ± 1.26	346 ± 13.2	66.3 ± 2.62
IRR	2.93 ± 0.15*	14.9 ± 2.92*	2.92 ± 0.05*	2.72 ± 0.03*	35.1 ± 1.02	370 ± 4.45*	51.6 ± 2.44*

Note: All values are mean ± standard error. BV/TV, trabecular bone volume; ConnD., connectivity density of trabeculae; SMI, structural model index; Tb. N, trabecular number; Tb. Th, trabecular thickness; Tb. Sp, trabecular separation; vBMD, volumetric bone mineral density. $^{\#}$, significant difference ($p \leq 0.05$) between groups (placebo vs. AR-447). *, significant difference ($p \leq 0.05$) between treatment (non-irradiated vs. irradiated).

The animal groups utilized were comparable in terms of micro-architecture. All bone parameters for each group, regardless of treatment (e.g. for non-irradiated placebo

vs. non-irradiated AR-447), were similar ($p \geq 0.05$). However, radiation treatment resulted in a decline of bone volume and deterioration of micro-architecture (Figures 5.2 and 5.3). Most parameters within each group showed a significant response to irradiation ($p \leq 0.05$), with the exception of Tb. Th, which generally indicated loss of bone after exposure.

BV/TV was lower in irradiated groups, as compared to non-irradiated groups. Within irradiated groups, declines of 24% and 31% were observed in placebo and AR-447 groups, respectively, as compared to non-irradiated groups. Like BV/TV, ConnD. was also greater in non-irradiated groups, as compared to irradiated groups. Declines of 33% and 29% were observed in placebo and AR-447 irradiated groups, respectively, as compared to non-irradiated groups.

No significant interactions (Group X Radiation treatment) occurred for any variables ($p \geq 0.05$).

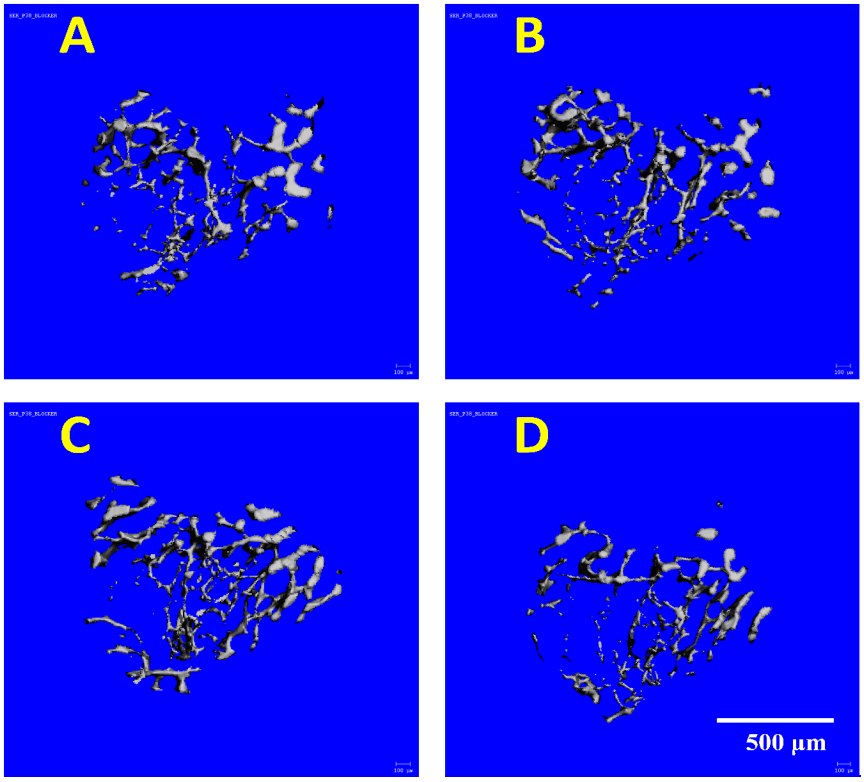

Figure 5.2 – MicroCT images of (A) placebo non-irradiated, (B) placebo irradiated, (C) AR-447 non-irradiated, and (D) AR-447 irradiated mice tibia.

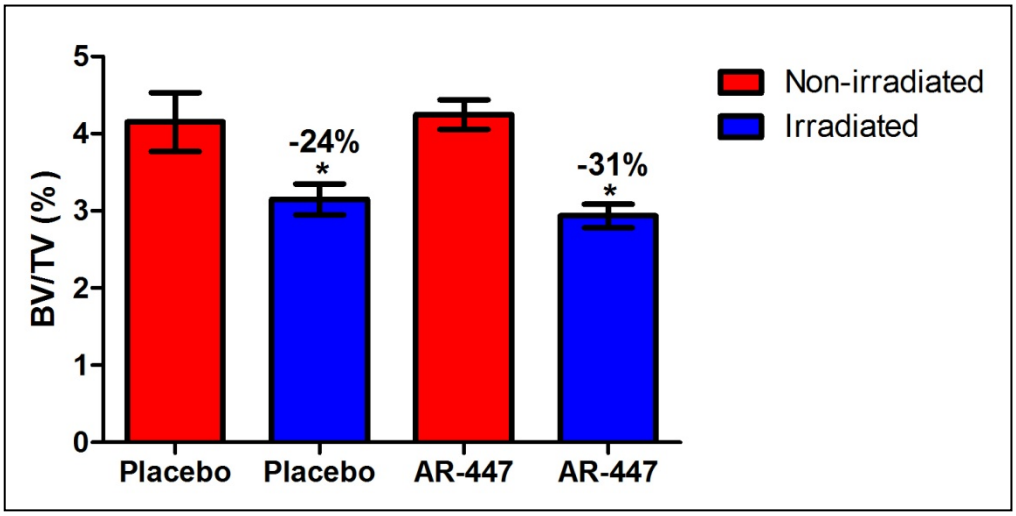

Figure 5.3 – Trabecular bone volume (BV/TV) of non-irradiated and irradiated mice tibia, with groups receiving placebo or AR-447 treatment. N=8 for all groups. All values are ± standard error.

5.2.2 Comparison of Non-irradiated and Irradiated Placebo and Drug Treated TNFα Presence and Expression

Real-time polymerase chain reaction was performed to detect quantitative changes in TNFα gene expression in placebo and AR-447 treated non-irradiated and irradiated individuals (Figure 5.4).

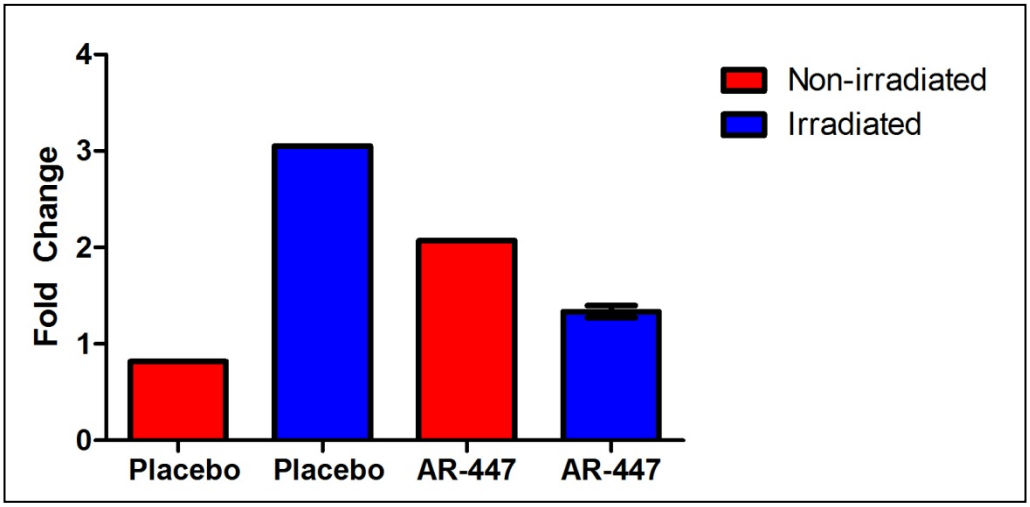

Figure 5.4 – TNFα mRNA expression

TNFα expression at the transcriptional level appeared to increase in placebo irradiated individuals, as compared to placebo non-irradiated individuals. However, the expression of TNFα appeared to decrease in AR-447 irradiated individuals as compared to AR-447 non-irradiated individuals. Due to small sample sizes (N=2), it was not possible to determine whether these changes in TNFα expression were considered significant or not.

To further demonstrate the presence of TNFα, immunohistochemistry was performed (Figure 5.5).

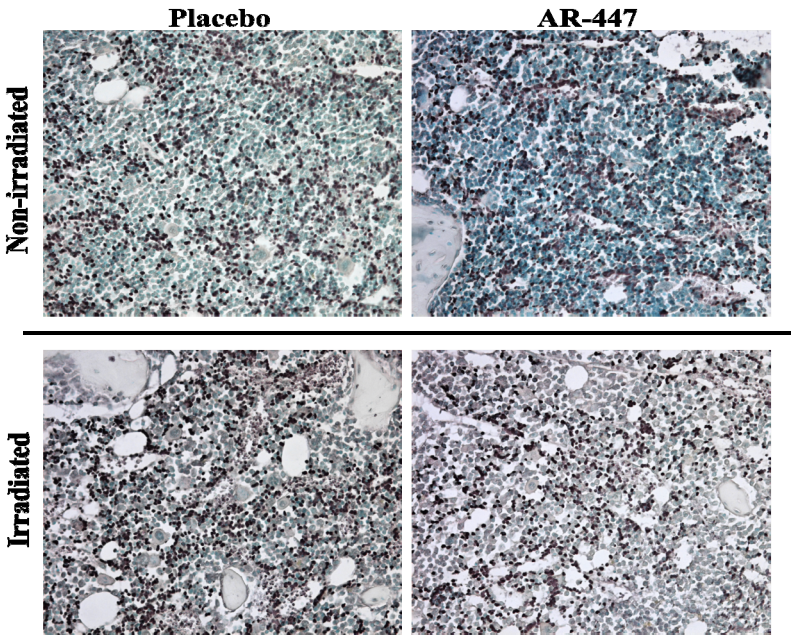

Figure 5.5 – Immunohistochemistry staining in detection of presence of TNFα. TNFα presence indicated by purple coloring.

As was seen with the change in TNFα mRNA expression, an increase in TNFα presence was observed in placebo irradiated individuals, as compared to placebo non-irradiated individuals. It was also observed that AR-447 irradiated individuals experienced a decreased presence of TNFα, as compared to AR-447 non-irradiated individuals.

5.2.3 Comparison of Non-irradiated and Irradiated Placebo and Drug Treated IL-1β Presence and Expression

Real-time polymerase chain reaction was performed to detect quantitative changes in IL-1β gene expression in placebo and AR-447 treated non-irradiated and irradiated individuals (Figure 5.6).

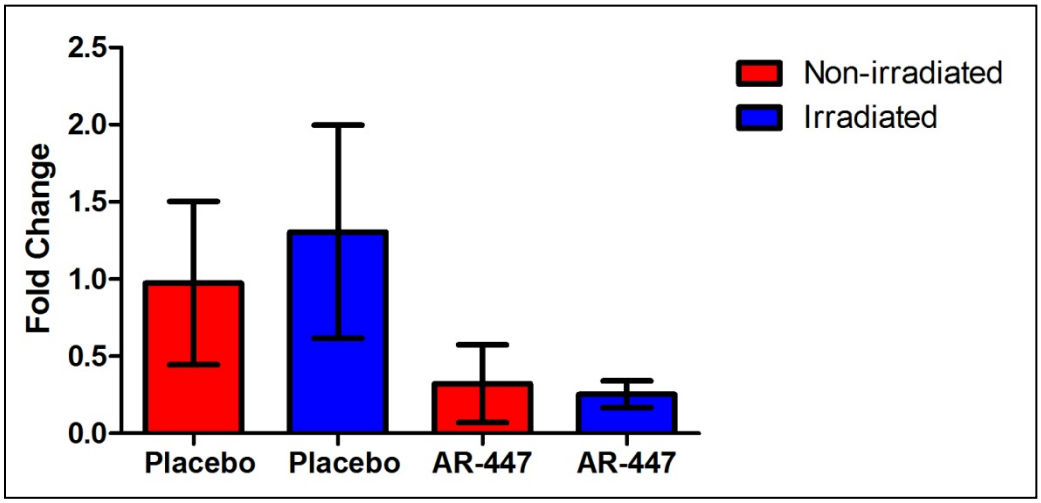

Figure 5.6 – IL-1β mRNA expression

A non-significant increase in IL-1β expression occurred in placebo irradiated individuals, as compared to placebo non-irradiated individuals. However, there appeared to be no changes in IL-1β expression in AR-447 irradiated individuals, as compared to AR-447 non-irradiated individuals.

To further demonstrate the presence of IL-1β, immunohistochemistry was performed (Figure 5.7).

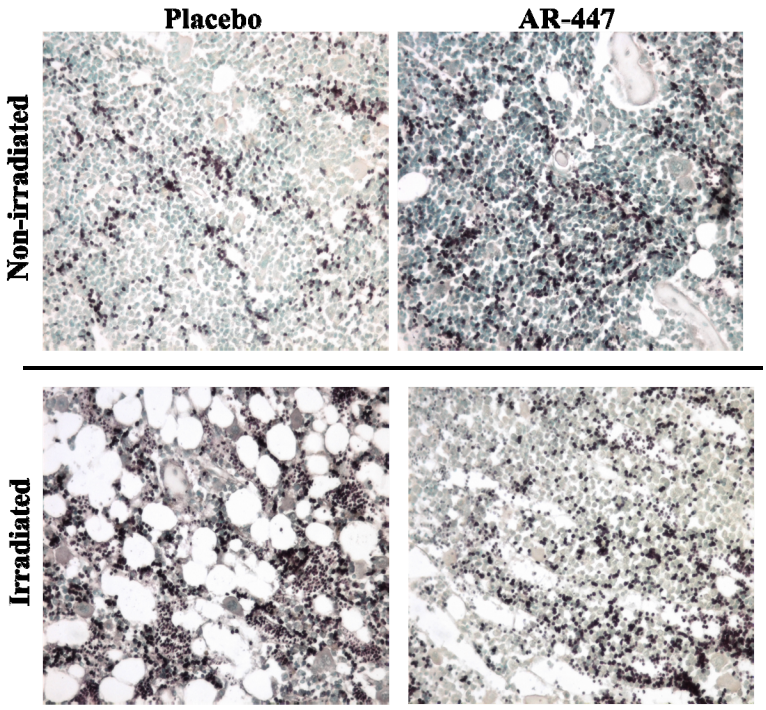

Figure 5.7 – Immunohistochemistry staining in detection of presence of IL-1β. IL-1β presence indicated by purple coloring.

An increase in IL-1β presence was observed in placebo irradiated individuals, as compared to placebo non-irradiated individuals. In addition, there was also an increase in IL-1β presence observed in AR-447 non-irradiated individuals, as compared to placebo non-irradiated individuals. However, IL-1β presence appeared to decrease in AR-447 irradiated individuals, as compared to AR-447 non-irradiated or placebo irradiated individuals.

5.2.4 Comparison of Non-irradiated and Irradiated Placebo and Drug Treated IL-6 Presence and Expression

Real-time polymerase chain reaction was performed to detect quantitative changes in IL-6 gene expression in placebo and AR-447 treated non-irradiated and irradiated individuals (Figure 5.8).

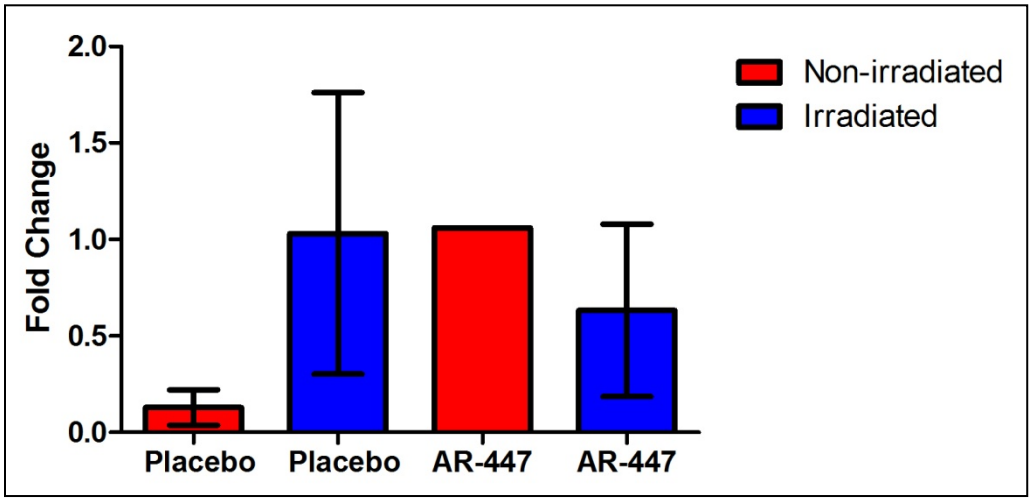

Figure 5.8 – IL-6 mRNA expression

A significant increase in IL-6 expression occurred in placebo irradiated individuals, as compared to placebo non-irradiated individuals. However, there was a non-significant decrease in IL-6 expression in AR-447 irradiated individuals, as compared to AR-447 non-irradiated individuals.

To further demonstrate the presence of IL-6, immunohistochemistry was performed (Figure 5.9).

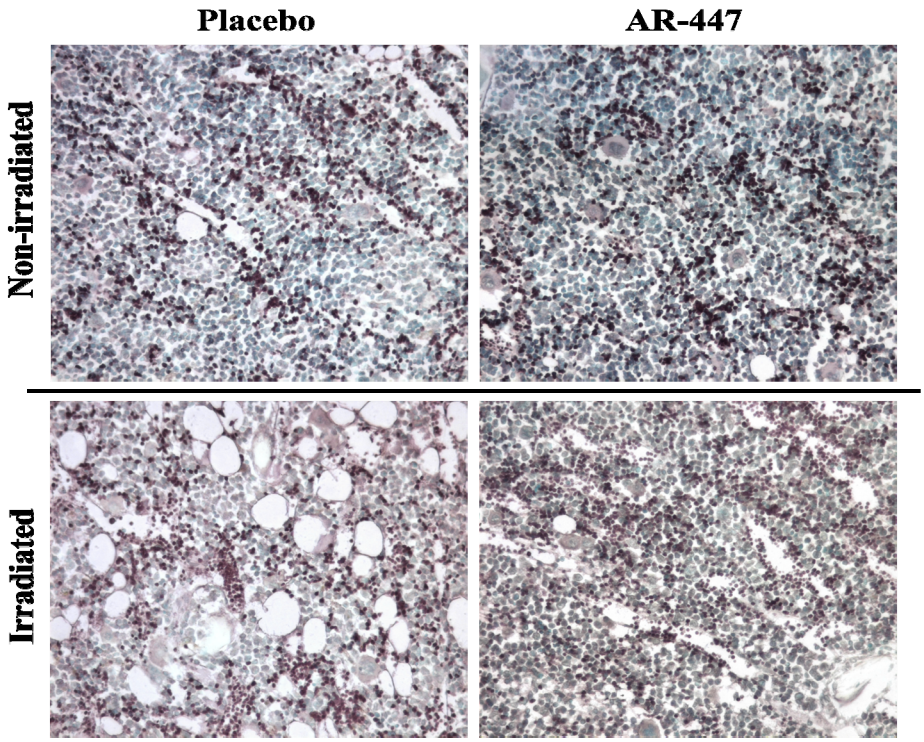

Figure 5.9 – Immunohistochemistry staining in detection of presence of IL-6. IL-6 presence indicated by purple coloring.

An increase in IL-6 presence was observed in placebo irradiated individuals, as compared to placebo non-irradiated individuals. In addition, the presence of IL-6 observed in AR-447 non-irradiated individuals was comparable to that observed in placebo non-irradiated individuals. The presence of IL-6 appeared to remain unchanged in AR-447 irradiated individuals, as compared to AR-447 non-irradiated individuals.

5.3 Discussion

The conclusions drawn from the findings of this study are consistent with those of the previous studies performed (Chapters 3 and 4). The data collected from this study further supports the suggestion that, within the particular animal model utilized, pro-inflammatory cytokines, including IL-1, IL-6, and TNFα, do not play a significant role in radiation-induced bone loss. By therapeutically blocking the transcription of these cytokines, it was expected that the up-regulation of any cytokines in response to the functional block of another cytokine would be inhibited, thereby preventing radiation-induced bone loss. However, therapeutically blocking the transcription of these cytokines did not prevent radiation-induced bone loss, with declines in bone volume fractions comparable to those observed in Chapters 3 and 4.

Real-time polymerase chain reaction was performed to detect the levels of mRNA expression of IL-1, IL-6, and TNFα in all groups. Increased expression levels of IL-1, IL-6, and TNFα were expected to be observed in placebo irradiated samples, as compared to placebo non-irradiated samples. It was also expected that AR-447 treatment would decrease the expression of IL-1, IL-6, and TNFα in non-irradiated and irradiated groups, as compared to the respective placebo treated groups. Treatment with AR-447 did reduce the expression of IL-1, IL-6, and TNFα in irradiated groups, as compared to non-irradiated groups.

In addition to real-time polymerase chain reaction, immunohistochemistry was also performed in order to detect the presence of IL-1, IL-6, and TNFα in all groups. It

was expected that the presence of all cytokines would correlate to the levels of mRNA expression of the respective cytokines. The presence of IL-1, IL-6, and TNFα appeared to increase in placebo irradiated individuals, as compared to placebo non-irradiated individuals, while AR-447 irradiated individuals experienced a decreased presence of all cytokines, as compared to AR-447 non-irradiated individuals.

The p38 MAPK signal transduction pathway is important in the expression of pro-inflammatory cytokines, including IL-1, IL-6, and TNFα [6-8]. Studies have demonstrated the significant role that p38 MAPK plays in inflammation, by utilizing blockers of p38 MAPK to reduce inflammation and inhibit rheumatoid arthritis in rodents [46]. In addition, p38 MAPK has been shown to play a role in osteoclast differentiation, suggesting that this signal transduction pathway could be a potential target for therapies focused on preventing bone loss [8, 47].

While the mechanism of action of the p38 blocking molecule AR-447 is proprietary information, the compound did appear to down regulate the action of p38 MAPK, as demonstrated by the decreased presence and expression of IL-1, IL-6, and TNFα. However, even though the action of p38 MAPK, radiation-induced bone loss was not successfully prevented, further supporting the findings of the previous chapters; within the particular animal model utilized, IL-1, IL-6, and TNFα do not play a significant role in radiation-induced bone loss.

CHAPTER 6 – SUMMARY & CONCLUSION

Bone fractures greatly decrease an individual's quality of life, as well as increase an individual's risk for further complications, including death. Radiation exposure causes bone loss, increasing the risk of fracture [2, 3]. Exposure to ionizing radiation, particularly in the context of cancer patients receiving radiotherapy, results in damage to normal (non-tumor) tissue, including bone. Radiation-induced tissue damage results in an inflammatory response, which is characterized by the increased presence of pro-inflammatory cytokines, including IL-1, IL-6, and TNFα, all of which are active stimulators of bone resorption [4, 5]. In order to better develop therapies to decrease the bone loss experienced by patients following radiotherapy, the mechanistic roles of these cytokines in the activation of osteoclasts needed to be determined.

The objective of this thesis was to determine the individual and combined effects of IL-1, IL-6, and TNFα as they relate to bone loss after radiation exposure. It was hypothesized that the up regulation of pro-inflammatory cytokines (IL-1, IL-6, and TNFα), after irradiation leads to the rapid activation of osteoclasts and subsequent bone loss.

This thesis provided confirmation that acute exposure to ionizing radiation results in a decline of bone volume and deterioration of micro-architecture. However, no causal mechanism of radiation-induced bone loss was established from this thesis, specifically concerning the blocking of inflammatory mediators to inhibit bone loss. The main observations from this thesis include:

- As determined by RT-PCR and immunohistochemistry, an increase in pro-inflammatory cytokine (IL-1, IL-6, and TNFα) presence and expression occurred as early as 24 hours after irradiation, as compared to non-irradiated samples.
- Use of an IL-1β receptor deficient (B6.129S7-Il1r1^{tm1Imx}/J) rodent model did not prevent radiation-induced bone loss, as compared to wild type (B57BL/6J).
- Use of an IL-6 deficient (B6.129S2-Il6^{tm1Kopf}/J) rodent model did not prevent radiation-induced bone loss, as compared to wild type (B57BL/6J).
- Use of a TNFα deficient (B6.129S-Tnf^{tm1Gkl}/J) rodent model did not prevent radiation-induced bone loss, as compared to wild type (B57BL/6J).
- Use of a TNFα/IL-1β receptor deficient (B6.129S-Tnfrsf1a^{tm1Imx}Il1r1^{tm1Imx}/J) rodent model did not prevent radiation-induced bone loss, as compared to wild type (B57BL/6J).
- Administration of TNF-binding protein (Enbrel), IL-1 receptor antagonist (Kineret), or a combination of the two did not inhibit radiation-induced bone loss, as compared to treatment with placebo.
- Administration of Enbrel, Kineret, or a combination of the two did not prevent an increased presence of IL-1, IL-6, and TNFα, as compared to treatment with placebo.
- Administration of a p38 blocking molecule (AR-447) did not prevent radiation-induced bone loss, as compared to treatment with placebo.

- Administration of AR-447 reduced IL-1, IL-6, and TNFα mRNA expression, as well as IL-1, IL-6, and TNFα presence after radiation exposure, as compared to treatment with placebo.

The findings of the studies performed in this thesis suggest that, within the particular animal model utilized, the pro-inflammatory cytokines IL-1, IL-6, and TNFα do not play a significant role in radiation-induced bone loss. Increased presence and expression of IL-1, IL-6, and TNFα was observed after irradiation. Therefore, it was expected that, if these cytokines activate osteoclasts, inhibiting the action of these cytokines would prevent radiation-induced bone loss. However, by genetically or therapeutically blocking these cytokines, radiation-induced bone loss was not prevented, suggesting the possibility that these cytokines are not responsible for the radiation-induced activation of osteoclasts, as was originally hypothesized in this thesis.

Future Studies

Exposure to ionizing radiation results in the formation of reactive oxygen species (ROS) from water within tissues or cells [48]. ROS are also produced during the respiratory burst of macrophages, as macrophages respond to cell and tissue injury from radiation exposure. ROS, including superoxide anion radical, hydrogen peroxide, and hydroxyl radical, are able to damage DNA, resulting in cell and tissue damage [5]. ROS have also been shown to stimulate inflammatory conditions by activating nuclear factor

κB (NF-κB), increasing expression of receptor activator of NF-κB ligand (RANKL), as well as increasing expression of pro-inflammatory cytokines, including IL-1, IL-6, and TNFα [49-51]. ROS have the ability to: directly activate osteoclasts; stimulate differentiation of osteoclasts and osteoclast resorption; activate NF-κB pathway, which leads to the activation of osteoclasts [49, 52].

This information suggests that ROS may play a role in radiation-induced bone loss, through the activation of osteoclasts. In order to better understand the mechanisms involved in radiation-induced bone loss, it would be beneficial to investigate the roles of ROS, particularly through inhibiting their formation with an anti-oxidant compound.

BIBLIOGRAPHY

1. NOF.org. *National Osteoporosis Foundation*. Fast Facts on Osteoporosis 2010 [cited 2010; Available from: http://www.nof.org/osteoporosis/diseasefacts.htm.

2. Abe, H., et al., *Radiation-induced insufficiency fractures of the pelvis: evaluation with 99mTc-methylene diphosphonate scintigraphy.* AJR Am J Roentgenol, 1992. **158**(3): p. 599-602.

3. Baxter, N.N., et al., *Risk of pelvic fractures in older women following pelvic irradiation.* JAMA, 2005. **294**(20): p. 2587-93.

4. Polistena, A., et al., *Local radiotherapy of exposed murine small bowel: apoptosis and inflammation.* BMC Surg, 2008. **8**: p. 1.

5. Vujaskovic, Z., et al., *Radiation-induced hypoxia may perpetuate late normal tissue injury.* Int J Radiat Oncol Biol Phys, 2001. **50**(4): p. 851-5.

6. Dinarello, C.A., *Hyperosmolar sodium chloride, p38 mitogen activated protein and cytokine-mediated inflammation.* Semin Dial, 2009. **22**(3): p. 256-9.

7. O'Sullivan, A., et al., *Inhibition of Nuclear Factor-kappaB and p38 Mitogen-Activated Protein Kinase Does Not Always Have Adverse Effects on Wound Healing.* Surg Infect (Larchmt). **11**(1): p. 7-11.

8. Zwerina, J., et al., *Activation of p38 MAPK is a key step in tumor necrosis factor-mediated inflammatory bone destruction.* Arthritis Rheum, 2006. **54**(2): p. 463-72.

9. Hall, *Radiobiology for the Radiologist* 2000, Philadelphia Lippincott Williams & Wilkins

10. Holmes-Siedle, A.G. and L. Adams, *Handbook of radiation effects*. 2nd ed. 2001, Oxford ; New York: Oxford University Press. xxv, 614 p.

11. Prise, K.M., et al., *New insights on cell death from radiation exposure.* Lancet Oncol, 2005. **6**(7): p. 520-8.

12. Stone, H.B., et al., *Effects of radiation on normal tissue: consequences and mechanisms.* Lancet Oncol, 2003. **4**(9): p. 529-36.

13. Lorimore, S.A., et al., *Inflammatory-type responses after exposure to ionizing radiation in vivo: a mechanism for radiation-induced bystander effects?* Oncogene, 2001. **20**(48): p. 7085-95.

14. Marieb, E.N. and Pearson Benjamin Cummings (Firm), *Human anatomy & physiology*. 6th ed. 2004, San Francisco, CA: Pearson Benjamin Cummings. xxi, 1242 p.

15. Beutler, B., *Tumor necrosis factors : the molecules and their emerging role in medicine*. 1992, New York: Raven Press. xvii, 590 p.

16. Dinarello, C.A., *Advances in Immunology*. Vol. 44. 1989: Academic Press, Inc. .

17. Sim, E., *Humoral factors*. The Natural immune system. 1993, Oxford ; New York: Oxford University Press. xxiv, 373 p.

18. Meager, A., *The molecular biology of cytokines*. Molecular medical science series. 1998, Chichester ; New York: Wiley. 416 p.

19. Fantuzzi, G., *Cytokine knockouts*. 2nd ed. Contemporary immunology. 2003, Totowa, N.J.: Humana Press. xix, 471 p.

20. Teitelbaum, S.L., *Bone resorption by osteoclasts.* Science, 2000. **289**(5484): p. 1504-8.

21. Fuller, K., et al., *TNFalpha potently activates osteoclasts, through a direct action independent of and strongly synergistic with RANKL.* Endocrinology, 2002. **143**(3): p. 1108-18.

22. Pacifici, R., *Cytokines, estrogen, and postmenopausal osteoporosis--the second decade.* Endocrinology, 1998. **139**(6): p. 2659-61.

23. Cenci, S., et al., *Estrogen deficiency induces bone loss by enhancing T-cell production of TNF-alpha.* J Clin Invest, 2000. **106**(10): p. 1229-37.

24. Roggia, C., et al., *Up-regulation of TNF-producing T cells in the bone marrow: a key mechanism by which estrogen deficiency induces bone loss in vivo.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13960-5.

25. Kimble, R.B., et al., *Simultaneous block of interleukin-1 and tumor necrosis factor is required to completely prevent bone loss in the early postovariectomy period.* Endocrinology, 1995. **136**(7): p. 3054-61.

26. Kitazawa, R., et al., *Interleukin-1 receptor antagonist and tumor necrosis factor binding protein decrease osteoclast formation and bone resorption in ovariectomized mice.* J Clin Invest, 1994. **94**(6): p. 2397-406.

27. Kimble, R.B., S. Bain, and R. Pacifici, *The functional block of TNF but not of IL-6 prevents bone loss in ovariectomized mice.* J Bone Miner Res, 1997. **12**(6): p. 935-41.

28. Hirbe, A., et al., *Skeletal complications of breast cancer therapies.* Clin Cancer Res, 2006. **12**(20 Pt 2): p. 6309s-6314s.

29. Ogino, I., et al., *Pelvic insufficiency fractures in postmenopausal woman with advanced cervical cancer treated by radiotherapy.* Radiother Oncol, 2003. **68**(1): p. 61-7.

30. Overgaard, M., *Spontaneous radiation-induced rib fractures in breast cancer patients treated with postmastectomy irradiation. A clinical radiobiological analysis of the influence of fraction size and dose-response relationships on late bone damage.* Acta Oncol, 1988. **27**(2): p. 117-22.

31. Pierce, S.M., et al., *Long-term radiation complications following conservative surgery (CS) and radiation therapy (RT) in patients with early stage breast cancer.* Int J Radiat Oncol Biol Phys, 1992. **23**(5): p. 915-23.

32. Guise, T.A., *Bone loss and fracture risk associated with cancer therapy.* Oncologist, 2006. **11**(10): p. 1121-31.

33. Hopewell, J.W., *Radiation-therapy effects on bone density.* Med Pediatr Oncol, 2003. **41**(3): p. 208-11.

34. Bliss, P., C.A. Parsons, and P.R. Blake, *Incidence and possible aetiological factors in the development of pelvic insufficiency fractures following radical radiotherapy.* Br J Radiol, 1996. **69**(822): p. 548-54.

35. Blomlie, V., et al., *Incidence of radiation-induced insufficiency fractures of the female pelvis: evaluation with MR imaging.* AJR Am J Roentgenol, 1996. **167**(5): p. 1205-10.

36. Grigsby, P.W., H.L. Roberts, and C.A. Perez, *Femoral neck fracture following groin irradiation.* Int J Radiat Oncol Biol Phys, 1995. **32**(1): p. 63-7.

37. Howland, W.J., et al., *Postirradiation atrophic changes of bone and related complications.* Radiology, 1975. **117**(3 Pt 1): p. 677-85.

38. Huh, S.J., et al., *Pelvic insufficiency fracture after pelvic irradiation in uterine cervix cancer.* Gynecol Oncol, 2002. **86**(3): p. 264-8.

39. Moreno, A., et al., *Pelvic insufficiency fractures in patients with pelvic irradiation.* Int J Radiat Oncol Biol Phys, 1999. **44**(1): p. 61-6.

40. Hamilton, S.A., et al., *A murine model for bone loss from therapeutic and space-relevant sources of radiation.* J Appl Physiol, 2006. **101**(3): p. 789-93.

41. Bandstra, E.R., et al., *Long-term dose response of trabecular bone in mice to proton radiation.* Radiat Res, 2008. **169**(6): p. 607-14.

42. Ergun, H. and W.J. Howland, *Postradiation atrophy of mature bone.* CRC Crit Rev Diagn Imaging, 1980. **12**(3): p. 225-43.

43. Kondo, H., et al., *Total-body irradiation of postpubertal mice with (137)Cs acutely compromises the microarchitecture of cancellous bone and increases osteoclasts.* Radiat Res, 2009. **171**(3): p. 283-9.

44. Willey, J.S., et al., *Early increase in osteoclast number in mice after whole-body irradiation with 2 Gy X rays.* Radiat Res, 2008. **170**(3): p. 388-92.

45. Willey, J.S., et al., *Risedronate prevents early radiation-induced osteoporosis in mice at multiple skeletal locations.* Bone. **46**(1): p. 101-11.

46. Revesz, L., et al., *Novel p38 inhibitors with potent oral efficacy in several models of rheumatoid arthritis.* Bioorg Med Chem Lett, 2004. **14**(13): p. 3595-9.

47. Li, X., et al., *p38 MAPK-mediated signals are required for inducing osteoclast differentiation but not for osteoclast function.* Endocrinology, 2002. **143**(8): p. 3105-13.

48. Slupphaug, G., B. Kavli, and H.E. Krokan, *The interacting pathways for prevention and repair of oxidative DNA damage.* Mutat Res, 2003. **531**(1-2): p. 231-51.

49. Bai, X.C., et al., *Reactive oxygen species stimulates receptor activator of NF-kappaB ligand expression in osteoblast.* J Biol Chem, 2005. **280**(17): p. 17497-506.

50. Lean, J., et al., *Thioredoxin-1 mediates osteoclast stimulation by reactive oxygen species.* Biochemical and Biophysical Research Communications, 2004. **321**(4): p. 845-850.

51. Lean, J.M., et al., *Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation.* Endocrinology, 2005. **146**(2): p. 728-735.

52. Ha, H., et al., *Reactive oxygen species mediate RANK signaling in osteoclasts.* Exp Cell Res, 2004. **301**(2): p. 119-27.