MOLECULAR AND CELLULAR MEDIATORS IN RADIATION-INDUCED LUNG INJURY

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology and Laboratory Medicine.

Chapel Hill

2009

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Abstract

Xuebin Yang: Molecular and Cellular Mediators In Radiation-Induced Lung Injury

(Under the direction of Suzanne Kirby, M.D. Ph.D)

Radiation-induced lung injury is a common adverse effect in patients receiving thoracic irradiation and for which, there is currently no effective therapy. Using a murine model of thoracic irradiation, we found that mice deficient in either the chemokine CCL3 or one of its receptors, CCR1, are significantly protected from radiation lung injury. This protected phenotype includes improved survival, virtually no pneumonitis or fibrosis, and preserved lung function when compared to wild-type mice. We further showed that a specific CCR1 inhibitor, BX471 provided similar protection. Therefore, CCR1 is a promising target for reducing radiation lung injury.

To investigate the mechanisms by which CCL3/CCR1 signalling mediates radiation lung injury, we evaluated their influence on lung inflammation after irradiation. When compared with irradiated WT mice, irradiated CCL3- and CCR1-deficient mice had less lung infiltration of $CD4^+$ and $CD8^+$ T cells; however, CD4-deficient mice showed only partial protection, while CD8-deficient mice had slightly worse fibrosis. We further analyzed inflammatory cytokines and different subsets of $CD4^+$ lymphocytes, T_H1, T_H2, T_H17 and Treg cells, in our model. We found no differences in lung $Foxp3^+$ Treg cells between WT and CCR1-deficient mice. Notably however, irradiated CCR1-deficient mice had less mRNA

expression of the T_H2 cytokines, IL-4 and IL-13, suggesting that T_H2 cells may mediate radiation lung injury. In addition, the T_H2-enhanced IL-10/12 double knockout mice (IL-$10/12^{-/-}$) have increased expression of IL-13 and IL-4 with an associated earlier onset and enhanced degree of lung fibrosis. Even more striking was our finding that irradiated IL-$10/12^{-/-}$ mice had an earlier onset of increased IL-17 mRNA expression, as well as increased lung infiltration of T_H17 cells, suggesting that IL-17 cells may also be important mediators in radiation lung injury.

In summary, our studies show that CCL3 and its receptor, CCR1, are key mediators of radiation lung injury that act at least in part by recruiting T_H17 and T_H2 cells into the irradiated lungs. Furthermore, our findings suggest that the specific inhibitor of CCR1, BX471, looks promising as a potential therapeutic agent for ameliorating radiation lung injury.

ACKOWLEGEMENTS

First, I feel grateful beyond measure to my advisor, Dr. Suzanne Kirby. Dr. Kirby has served a mentor to me since 2004. I am deeply indebted to her for believing in me and fostering my scientific career. I will never forget her tireless efforts to motivate and encourage me. I am always grateful to her for patience and understanding to foster me as a doctor of philosophy in molecular and cellular pathology. It has been a privilege and a pleasure to work with an advisor who has also been a good friend.

My sincere gratitude also goes out to Dr. J. Charles Jennette, chair of the Pathology Department and Dr. William Coleman, director of Graduate Students, who have both been so supportive for the Pathology graduate students. I thank them for their guidance and support.

I also want to specifically thank William Walton who has been always here to help and support me along the way. He provided me with most of my technical scientific training and enormous help in experiments. He also assisted me on a daily basis with everything from mouse room chores to tedious benchwork all throughout my doctoral training.

I would like to thank the other members of my committee too! Thanks to Dr. Donald N. Cook, Dr. Virginia L. Godfrey, Dr. Nobuyo N. Maeda, Dr. James M. Samet, and Dr. Stephen Tilley for all of their great ideas, encouragment, and for supporting me through my doctoral training.

I also thank past and present members of the Kirby/Serody Laboratoies, including but not limited to Christin Buehler, Joe Burgents, Dr. Meredith Burgents, Dr. Michael Carlson, Dr. Jay Coghill, Dr. Jennifer Gilner, Laura Greene, Karen Hogan, Robert Mango, Dr. Karen

McKinnon, Dr. Timothy P. Moran, Dr. Jonathon Serody, Jenelle Vargas, Shelly West, Cole Wilson, and Dr. Chris Wysocki, who have taught me many new techniques and offered lots of advice and discussion.

I am also very appreciative of the friends I have made within the Pathology Department, who provided me with support and inspiration: Hind Mullaen, Diane Bender, Jeremiah Hinson, Mehmet Karaca, Matt Medlin and Elizabeth Merricks. I would also like to recognize the supporting staff Dorothy Poteat who has provided me with encouragement, dependable administrative and technical support over the years.

I also owe a great deal of thanks to my parents and sisters for their unwaiving support of me. I am proud to make them proud. I also thank my parents-in law who have provided me with a much needed helping hand in caring for my daughter, Lydia. I could not have completed my dissertation without their daily help.

Lastly, I will forever be grateful to my wife Jane (Huaizhi) who has supported me and kept me motivated all along the way. Without her love, friendship and encouragement, none of this would have been possible.

Finally, There are many others listed below who have helped, supported and taught me along the way. I never could have done this without all of you! Thanks!

C. Robert Bagnell, Jr. PhD
William Blackstock, MD
W. June Brickey, PhD
Yoshizumi Deguchi, MD.
Hendrik Deventer, MD
Christopher Haskell, PhD (Bayer)
Xiaoyang Hua, MD
Qingping Hu, MD
Jenny Ting, PhD
Tong Zhou, MD PhD

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ANA	antinuclear autoantibody
BAL	bronchoalveolar lavage
CCR1	C chemokine receptor 1
CCR5	C-C chemokine receptor 5
ECM	extracellular matrix
Gusb	glucuronidase, beta
IFN-γ	interferon-gamma
IPF	idiopathic pulmonary fibrosis
MCP-1	CCL2; monocyte chemoattractant protein 1
MIP-1α	CCL3; Macrophage inflammatory protein-1 alpha
RANTES	CCL5; regulated on activation, normal T cell expressed and secreted
STAT	signal transducers and activator of transcription
TGF-β	transforming growth factor-beta
T_H1	type 1 T helper cell
T_H2	type 2 T helper cell
T_H17	type 17 T helper cell
TNF-α	tumor necrosis factor-alpha
Treg	regulatory T cell
UIP	Usual interstitial pneumonia

I. Introduction

Radiation-induced lung injury is an adverse effect in 20-40% of patients receiving thoracic irradiation [1-6] and is characterized as radiation pneumonitis that in some cases progresses to lethal fibrosis [7, 8]. Concurrent chemotherapeutic drug administration increases the incidence of radiation pneumopathy to 40-60% [4]. The current treatment, steroid therapy, has not only limited efficacy in preventing fibrosis but also has extensive side effects [9, 10]. Therefore, understanding the mechanisms of radiation pneumopathy may provide insight towards developing novel preventative and therapeutic interventions.

I.A. General Principles of Radiation Injury

Radiation causes mitotic cell death [11]. The radiosensitivity of a tissue is directly related to its mitotic activity and inversely proportional to the degree of cell differentiation. Liver, kidney, bone, muscle, lung and connective tissues are relatively radioresistant, while more proliferative tissues (bone marrow, the germinal cells of the testis, and epithelial cells of the skin and gut) are relatively radiosensitive.

Mammalian tissue reactions to radiation consist of cell death and damage followed by tissue repair and remodeling. The extent of damage depends on the radiation dose, quality, fractionation, and tissue radiosensitivity of the tissues, and the intrinsic repair and repopulation capacity; the latter being the most important factor [12]. Both dividing progenitor cells and nondividing mature cells are involved in the repair process. The former begin to die at their first or second post-irradiation division, while the latter remain relatively unaffected by radiation, continue to function and then die at their normal rate. However, the

damage to the stem cell compartment may prevent efficient repopulation injury is not apparent until the number of the functional cells falls below a critical level. Thus, the onset of recognizable injury depends on tissue population kinetics, while radiation dose determines the severity and duration of the cellular depletion. Therefore, cell death and depletion in rapidly dividing tissues, e.g. skin and gut, occurs earlier than in the slowly dividing tissues, e.g. kidney and lung.

Although biochemical, subcellular, and cellular damage takes place immediately after ionizing radiation exposure, the clinical and morphological features are often delayed for weeks, months, or even years after treatment. For example, only mild changes are seen 6 weeks after a high dose of irradiation to the lung, but by 6 months there may be widespread fibrosis. Acute effects occur within a few weeks after treatment. Later effects appear months to years after radiation exposure and may be due to persistent acute damage or a combination of changes in the connective tissues, the parenchyma, and the vascular elements. As such, radiation injury is commonly classified as acute (early) and late (consequential) effects, according to the time of symptom appearance [12].

I.B. Radiation-induced Lung Injury

1. Pathophysiology

The lung contains over 40 types of cells, most of which are considered relatively radioresistant. However, since the lung has little regenerative capacity, it can not tolerate large doses of radiation without risking radiation-induced injury that leads to impaired ventilation and diffusion capacity. This radiosensitivity is the major dose limiting factor in chest radiotherapy.

Radiation-induced lung injury has classically been divided into two distinct, yet still tightly connected, phases [6, 7]. The early acute inflammatory phase (radiation pneumonitis) typically occurs 1 to 3 months after irradiation and is characterized by alveolar cell depletion, with edema and inflammatory cell accumulation in both the interstitial and air spaces. Early radiation-induced damage from vascular injury throughout the lungs results in vascular congestion and increased capillary permeability. Clinically, patients present with symptoms of shortness of breath, congestion, cough, fever, and chest pain. This pneumonitis phase usually responds well to steroids. The late radiation fibrosis phase typically occurs about 6 months after irradiation and is characterized by fibroblast proliferation, collagen accumulation, thickening of alveolar septa and destruction of the normal lung architecture [13]. Patients may have progressive chronic dyspnea, cough, and chest discomfort with radiologic evidence of lung fibrosis, including lung contraction, pleural thickening, tenting of the diaphragm, and deviation of trachea or mediastinum toward the irradiation region. Each phase of radiation pneumopathy is characterized by distinct histologic lesions [14]. Although there is an overlap in the regression of one phase and the progression of the next phase, an intermediate exudative phase may occur at few weeks to several months after irradiation, which is characterized with marked intra-alveolar edema. Although the histopathologic features of radiation-induced lung damage have been well documented, the pathophysiology of radiation pneumonitis and fibrosis remains unclear and somewhat controversial.

Table 1.1 Histologic changes in radiation-induced lung injury

Phase	Events and Histological Lesions	Span (week)
Acute	macrophage infiltration of air spaces Mononulcear inflammatory cells in alveolar walls Edema in the alveolar wall and/or air space Fibrin in air space	0-22 Onset 4 week
Intermediate	Increased septal cellularity (plasma cells, septal cells, fibroblasts) Large cells (macrophages) with foamy cytoplasm in air space	28-36
Late (fibrotic)	Pathological organization (a replacement of normal lung tissue with fibrous connective tissue in the air space) Collagen deposition	Beyond 36 Onset 22 week

Modified from: Travis EL. The sequence of histological changes in mouse lungs after single doses of x-rays. Int J Radiat Oncol Biol Phys. 1980;6(3):345-7. and Van den Brenk, H.A.S 1971. Radiation effects on the pulmonary system. In Pathology of Padiation, edited by C.C. Berjis. Williams and Wilkins, Baltimore, pp. 569-591.

The initial oxidative injuries after radiation induce resident fibroblasts, epithelial and endothelial cells to alter their expression of inflammatory and chemotactic cytokines. The resultant cytokines subsequently serve as mediators to recruit and alter the numbers (or populations) of inflammatory cells in the inflamed tissue. Once recruited into the disturbed microenvironment, the inflammatory cells are stimulated, producing other mediators and initiating a complex ‘‘cytokine cascade.’’ This persistent perturbation of normal cellular communication can expand the inflammatory cell population, leading to an overt, functionally significant, pneumonitis. In addition, the activated cells, both inflammatory and parenchymal, produce mediators that directly affect the overall pattern of proliferation and/or extracellular matrix gene expression of lung fibroblasts, leading to progressively increased collagen synthesis and eventually the development of fibrosis. Thus, there are numerous cellular and molecular mediators involved in the development of radiation-induced lung fibrosis, which makes identifying targets for therapeutic intervention difficult [13, 15, 16].

2. Chronic Inflammation and Fibrosis

A common host defense to lung injury is to trigger an inflammatory response. Appropriate repair after tissue injury and inflammation requires resolution of the inflammatory response and removal of extracellular matrix breakdown products. Failure to resolve inflammation and return to its normal histology and physiologic function can lead to a chronic, persistent inflammatory response that often leads to tissue fibrosis. However, the relationship between pneumonitis and fibrosis is controversial, not only in radiation models, but also in idiopathic lung fibrosis (IPF) and chemical-induced fibrosis [17, 18]. Clinically, fibrosis invariably occurs after pneumonitis; however, fibrosis can occur independently and even in the absence of an overt pneumonitic phase [7]. For example, there is little

inflammation in advanced usual interstitial pneumonia (UIP) and idiopathic pulmonary fibrosis (IPF) [19]. Also, bleomycin-induced inflammation can occur without fibrosis in mice deficient in either the epithelial cell integrin αvß6 (ß6-/-), which activates latent TGF-ß, or CD44, a transmembrane adhesion receptor that is involved in T-cell recruitment [20]. Furthermore, the endothelial cell adhesion molecule $ICAM1^{-/-}$ mice do not develop pneumonitis after irradiation and develop fibrosis only at high doses, which suggests that the inflammatory response is not the sole factor underlying the development of radiation-induced fibrosis [21]. Lastly, although steroid therapy suppresses radiation pneumonitis, it does not alter the ultimate fibrosis in rat, and often in humans [10].

Evidence supports cellular interactions between lung parenchymal cells and inflammatory cells in mediating fibrosis through a variety of cytokines, i.e., proinflammatory cytokines, chemokines, adhesion molecules, profibrotic cytokines and growth factors [17, 22].

3. Effects on Lung Function

Many patients (50–90%) receiving irradiation to the lung experience a reduction in whole-lung function, as assessed by radiographic and lung function tests [23]. Severe respiratory distress occurs when over 75% of the lung is involved with radiation pneumonitis wherein alveolar edema obstructs the acini. Radiotherapy-induced lung symptoms can be measured by pulmonary function test (PFT) because they are an assessment of whole lung function. Clinically, gross physiologic changes do not occur in the lung until 4 to 12 weeks after irradiation, usually coincident with the period of clinical pneumonitis. This period is associated with a decrease in lung volume. Most patients have a fall in compliance coincident with the clinical pneumonitis [1]. Gas exchange abnormalities, e.g. a decrease in diffusion capacity and arterial hypoxemia, occur at about the same time, but show some tendency

toward recovery after 6 to 12 months. Lung histologic changes correlate with lung function abnormalities in several models of lung injury and fibrosis. Lung compliance is decreased and tissue elastance is increased in mice 21 days after bleomycin intratracheal instillation [24]. Franko used CO uptake to measure respiratory function in mice after irradiation and found that a decrease of CO uptake began in all mice between 9 and 12 weeks after a dose of 9.8 Gy irradiation [18]. Pauluhn et al. used an non-invasive Buxco system to analyze radiation-induced pneumonitis and the long-term sequelae [25]. They found reduced quasistatic compliance and total lung capacity in mice at 4 months after hemithorax radiation. Single-breath carbon monoxide diffusion capacity was also significantly decreased in that study.

4. Management of Radiation Lung Injury

WR-2721 (amifostine) is a phosphorylated aminothiol and free radical scavenger. It has been shown to confer cytoprotection of normal tissues when administrated with radiation therapy. This cytoprotection is believed to result from elimination of free radicals produced by radiation. Amifostine was shown to significantly reduce acute pneumonitis in patients receiving radiation therapy (31% vs 7.4%, p=0.03); however, overall survival was not impacted [26]. Amifostine appears not to affect tumor control in cancer patient with radiation therapy.

Angiotensin-converting enzyme inhibitors have also been shown to ameliorate radiation-induced pneumonitis and lung fibrosis in a rat model [27]. Although superoxide dismutase (SOD) analogues have been shown to protect against lung fibrosis in several animal models (including radiation), clinical studies on the effects of these drug in patients has not been reported yet [28, 29]. Thus, currently corticosteroids are the commonly used agents for

treating acute radiation pneumonitis because of their capacity to induce lymphocyte apoptosis and reduce the production of pro-inflammatory cytokines. Unfortunately, corticosteroid treatment has failed to substantially improve the prognosis for many patients at risk for radiation-induced fibrosis. Steroids are also associated with toxicity and an increased susceptibility to infections [9, 10]. In summary, there is no proven radioprotective therapy for radiation lung injury, especially for the later fibrosis.

5. Molecular mediators of radiation pneumopathy

Radiation-induced pneumonitis and fibrosis is a complex process involving proinflammatory and profibrotic cytokines produced by damaged and activated cells that are resident or recruited into the lung. Ionizing radiation activates a stress response in mesenchymal cells, leading to upregulation of specific transcription factors such as NF-kB, c-abl, c-jun, Egr-1 and c-fos [30]. This cellular activation initiates a repair process involving many growth factors, cytokines, and chemokines. A key role for proinflammatory cytokines is also suggested by detecting, blocking, or augmenting cytokine expression in various experimental models of lung fibrosis [31, 32]. Increased inflammatory cytokine mRNA and proteins have been observed in the progression of radiation-induced lung injury [16]. There are also distinct temporal and spatial changes in proinflammatory cytokine gene expression in bronchoalveolar lavage (BAL) and whole lungs of the irradiated mice [33].

Transforming growth factor (TGF-β) is the pivotal mediator in the fibrotic process. TGF-β induces the phenotypic modulation of human lung fibroblasts to myofibroblasts, the chemotactic recruitment of fibroblasts, the synthesis of collagen and various other extracellular matrix components (fibronectin, glycosaminoglycans, and proteoglycans), and reduces the degeneration of extracellular matrix by inhibiting the generation of serine

proteases, metalloproteinases, and collagenases [34, 35]. In addition, TGF-ß is a potent stimulator of angiogenesis and a chemoattractant for monocytes and macrophages, inducing its own production in an autocrine fashion. Several cellular sources of TGF-ß appear to be activated during lung fibrosis. The importance of these sources may vary at different stages of the reparative process and in different forms of lung disease. After irradiation in mice, TGF-ß expression increases from macrophages during the early phase of lung injury, whereas later on, type II pneumocytes and fibroblasts may serve as important sources of TGF-ß [36]. Patients with higher plasma TGF-ß, IL-1, or IL-6, before or during irradiation, have a higher risk of developing pneumonitis [37, 38]. TGF-β in BAL fluid was upregulated and peaked between 3 and 6 weeks after irradiation, coincident with the initial influx of inflammatory cells and preceding the pulmonary fibrosis, suggesting a pathogenetic role of TGF-β in the development of radiation fibrosis in humans [39]. Work by Franko et al. showed 10% of fibroblasts were active-TGF-β positive by immunohistochemical stain in the lesions in the early stage of fibrosis while only 0.7% of cells were positive in lesions without fibrosis. They concluded that the association of active TGF-β with fibroblasts was related to the initiation of fibrosis in this model [40].

TGF-β also downregulates the inflammatory response, while promoting fibrosis [20]. TGF-β released soon after tissue injury serves primarily as a proinflammatory molecule because of its potent neutrophil chemotactic effects, but then, its function switches to promote the resolution of inflammation and repair during the healing phase. TGF-β might suppress, rather than induce, tissue remodeling in some settings. TGF-β-producing regulatory T cells increased IL-10 production and then suppressed bleomycin-induced lung fibrosis

[41]. Therefore, it seems that macrophage-derived TGF-β is often pro-fibrotic, whereas Treg-cell-derived TGF-β may be suppressive.

Several members of the fibroblast growth factor (FGF) family stimulate DNA synthesis in endothelial and epithelial cells and also protect endothelial cells from radiation-induced damage by inhibiting apoptosis [42]. bFGF is produced by endothelial cells within hours after radiation and promotes fibroblast growth and differentiation. Serum concentrations of bFGF (together with TNF-α and IL-6) are consistently greater in patients undergoing lung radiotherapy [38, 43].

TNF-α is produced by activated macrophages during the fibrotic process and has proinflammatory and immunoregulatory effects. Various cytokines control the production of TNF-α: IL-2, IFN, and GM-CSF stimulate its production, whereas IL-6 and TNF-β inhibit it. TNF-α stimulates fibroblast proliferation and the secretion of extracellular matrix proteins, collagenase production, and other proinflammatory cytokines, e.g., IL-1 and IL-6. Treatment with a recombinant TNF-α receptor that blocks TNF-α activity, ameliorates the fibrotic lesions in lungs of bleomycin-treated mice [44]. TNF-α is implicated in the early phase of radiation pneumonitis as irradiated C3HeB/Fe mice show increased macrophage-produced TNF-α in BAL; however, this reaction settles within 4 months [45]. Nonetheless, there are only minor differences in TNF-α expression in the fibrosis-sensitive strain, C57LB6/J, and nonsensitive strain, C3HeB/Fe, suggesting that it may not be biologically meaningful in radiation pneumopathy [40].

A number of cytokines involved in T cell subset differentiation have also been implicated in radiation pneumopathy. Naive murine $CD4^+$ T helper cells (T_H0) can be induced to differentiate towards type 1 T helper (T_H1), T_H2, and T_H17 phenotypes according

Table 1.2 Cytokines implicated in lung fibrosis

Cytokine	Species	Model	Change	Reference
TGF-β	Human	XRT	Increased TGF-ß expression	[37]
TGF-β	mouse	XRT	Increased TGF-ß expression	[27]
TGF-β	Rat	XRT	Increased TGF-ß in IHC	[46]
bFGF	Human	XRT	Increased bFGF expression	[38, 43]
TNF-α	human	XRT	Increased TNF-α in BAL	[45]
IL-1β	human	XRT	Increased TNF-α in BAL	[38]
IL-6	human	XRT	Increased IL-6 in BAL	[43]
IL-4	Rat	XRT	Increased IL4 in tissue and BAL	
IL-4	human	IPF	Increased IL4 in tissue and BAL	[47]
IL-10	mouse	XRT	Increased IL-10 expression	[48]

bFGF- basic fibroblast growth factor; TNF-α-tumor necrosis factor-alpha

to the local cytokine milieu (**Figure 1.1**). There is also another $CD4^+$ T subset, regulatory T cells (Treg). The presence of interleukin IL-12 [signaling through STAT-4] skews differentiation towards T_H1, IL-4 (signaling through STAT-6) towards T_H2, TGF-β towards Treg, and IL-6 and TGF-β towards T_H17 [49, 50]. T_H1 cells produce mainly the pro-inflammatory cytokines IL-1, IL-2, IL-12, IL-15, IL-18, IFN-γ and lymphotoxin (TNF-β), while T_H2 cells produce IL-4, IL-5, IL-6, and IL-13. Both T_H1 and T_H2 produce IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), both of which stimulate leukocyte production from bone marrow. T_H1 cells orchestrate responses to pathogens that have overcome epithelial barriers and attack internal tissues. Conversely, T_H2 cells arm epithelial and mucosal sites to make life difficult for pathogens attempting to prosper there. The T_H1 cytokines IL-2, IFN-γ, and TNF-β activate cytotoxic T cells (Tc) and macrophages to stimulate the cellular immunity and inflammation associated with autoimmune disorders and allograft rejection, whereas the T_H2 cytokines IL-4, IL-5, IL-6, and IL-10 stimulate antibody production by B cells and mediate allergic inflammation and chronic fibroproliferative disorders, such as asthma, atopic dermatitis, IPF and systemic sclerosis. T_H17 cells have been shown to express IL-17, IL-17F, IL-21 and IL-22 (and IL-26 in humans) and to regulate inflammatory responses [51].

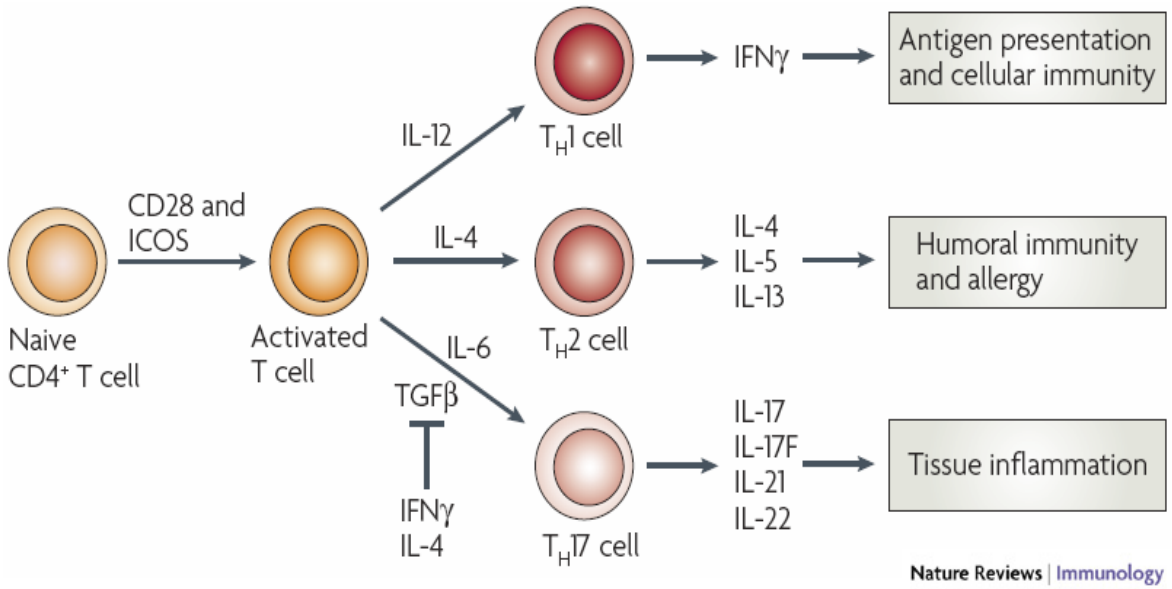


Figure 1.1 General scheme of T-helper-cell differentiation. Naive $CD4^+$ T cells, after activation by signaling through the T-cell receptor and co-stimulatory molecules such as CD28 and inducible T-cell co-stimulator (ICOS), can differentiate into one of three lineages of effector T helper (T_H) cells — T_H1, T_H2 or T_H17 cells. These cells produce different cytokines and have distinct immunoregulatory functions. Interferon-γ (IFN-γ) produced by T_H1 cells is important in the regulation of antigen presentation and cellular immunity. The T_H2-cell cytokines interleukin-4 (IL-4), IL-5 and IL-13 regulate B-cell responses and anti-parasite immunity and are crucial mediators of allergic diseases. T_H17 cells have been shown to express IL-17, IL-17F, IL-21 and IL-22 (and IL-26 in humans) and to regulate inflammatory responses. TGF-β, transforming growth factor-β. *Cheng Dong*, *Nat Rev Immunol. 2008, 8:337-48.*

The opposing effects of T_H1 and T_H2 cytokines have been shown in a number of studies [34]. The predominant T_H1 cytokine IFN-γ inhibits the proliferation of T_H2 cells, while IFN-γ and IL-2 stimulate B cells to secrete IgG2a and inhibit secretion of IgG1 and IgE. The T_H2 cytokine IL-10 inhibits T_H1 secretion of IFN-γ and IL-2; it also suppresses Class II MHC expression and the production of bacterial killing molecules and inflammatory cytokines by macrophages. IL-4 stimulates B cells to secrete IgE and IgG1. The balance between T_H1 and T_H2 activity may steer the immune response in the direction of cell-mediated or humoral immunity.

In fibrosis, IL-4, IL-5 and IL-13, the signature T_H2 cytokines, are important mediators of fibroblast activation and have powerful anti-inflammatory effects while IFN-γ has profound suppressive effects on the production of extracellular matrix proteins [52]. For example, a predominant T_H2 cytokine (IL-4 and IL-5) profile is seen in IPF by immunostaining and in situ hybridization of open lung biopsy specimens, while BAL findings in sarcoidosis and hypersensitivity pneumonitis were characterized by a T_H1-dominant profile [47, 53]. A shift from a T_H1 to a T_H2 cytokine profile is likely to be a key event in the progression of inflammation to fibrosis in IPF. T_H1 cytokines, particularly IFN-γ, appear to have antifibrotic effects by inhibiting myofibroblasts [54, 55].

IL-1 is mainly produced by activated macrophages, but also by other cells, e.g., endothelial cells. There are two nearly equivalent functional forms of IL-1, designated IL-1α and IL-1β. Both forms of IL-1 bind to the same receptors (type 1 and type 2 receptors) and show similar biological activities. They act on target cells both directly and via the induction of further cytokines. The main biological activity of IL-1 is the stimulation of T_H1 cells, which are induced to secrete IL-2 and to express IL-2 receptors. IL-1 also acts directly on B

cells, promoting their proliferation and the synthesis of immunoglobulins. IL-1 also upregulates adhesion molecule expression on endothelial cells and is a strong chemoattractant for leukocytes. Increased production of both IL-1α and IL-1β was observed in alveolar macrophages in vitro after radiation [56]. IL-1β has been shown to stimulate IL-6 production by human lung fibroblasts [43]. IL-1β increased in the lungs of irradiated fibrosis-resistant (C3H/HeJ) mice, but not in fibrosis-sensitive (C57BL/6), suggesting it may have a protective function in radiation lung injury [57].

IL-6 is an acute phase proinflammatory cytokine produced by activated alveolar macrophages, T helper lymphocytes, lung fibroblasts, and Type II pneumocytes. High pre-treatment or post-treatment levels of IL-6 and IL-1α correlated with the development of radiation pneumonitis in humans, suggesting that pre-treatment IL-6 levels may serve as a predictor for radiation pneumonitis [38, 43].

IL-10 is a known suppressive cytokine for T-cell proliferation and cytokine production. Although IL-10 is produced mainly by T_H2 cells, Treg, T_H0, T_H1, B cells, monocytes and keratinocytes are additional source of IL-10 [58]. IL-10 mRNA expression increases in a radiation-dose-dependent manner and may also explain some of the immunosuppressive effects of ionizing radiation. IL-10 has shown some efficacy in the treatment of fibrosis. Mice treated with IL-10 had less carbon tetrachloride (CCl4)-induced liver fibrosis [59] and bleomycin-induced lung fibrosis [59, 60]. IL-10 inhibited radiation-induced transendothelial cell migration by leukocytes in mice through the inhibition of ICAM-1 expression, but the IL-10 deficiency had little effect on the development of fibrosis [48].

IL-4 has potent pro-fibrotic activity. IL-4 is increased in idiopathic pulmonary fibrosis [53], cryptogenic fibrosing alveolitis [47], periportal fibrosis [61], and in the lungs of rats undergoing hemi-thoracic radiation [62]. In addition, neutralizing antibody for IL-4 consistently reduced hepatic collagen deposition in *schistosomiasis* infected mice [63].

IL-5 exacerbates bleomycin-induced lung fibrosis, but IL-$5^{-/-}$ mice had no improvement in fibrosis, suggesting that IL-5 may act as an amplifier, rather than as a direct mediator, of lung fibrosis [64].

IL-12 acts a central mediator of the cell-mediated immune response by promoting T_H1 development. Cells known to produce IL-12 include macrophages, dendritic cells, monocytes, Langerhans cells, neutrophils, and keratinocytes. Biologically active mouse IL-12 is a disulfide-linked, 70 kDa (p70) heterodimeric glycoprotein composed of a 40 kDa (p40) subunit and a 35 kDa (p35) subunit. While the p40 and p35 subunits by themselves do not have IL-12 activity, the p40 homodimer has been shown to bind the IL-12 receptor and is an IL-12 antagonist. The p40 subunit was previously shown to be secreted in excess of the heterodimeric IL-12 in cells expressing both the p35 and p40 mRNAs; however, free p35 subunits were not detected in supernatants of cultured cells expressing either only p35 or both p35 and p40 mRNAs.

IL-13 and IL-4 have many similar functions as both use the same IL-4 receptor α-chain (IL-4Rα) and STAT-6 signaling pathway. IL-13 has been shown to play a dominant role in the pathogenesis of pulmonary fibrosis in some experimental models [65-67]. Subepithelial airway fibrosis was induced without any additional inflammatory stimulus, by overexpressing IL-13 in the mouse lung whereas IL-13-specific antibodies markedly reduced collagen deposition in the lungs of animals that were challenged with *Aspergillus fumigatus*

conidia or bleomycin. In addition to direct effects on fibroblasts that support fibroproliferation, IL-13 stimulates the production of CC-chemokines by epithelial cells.

In summary, although many studies support T_H2 cells as the main mediators in IPF and other models of fibrosis [34, 68], it is still unknown whether radiation-induced lung fibrosis may be T_H1 or T_H2-mediated.

6. Cellular Mediators of Radiation Pneumopathy

The lung parenchyma consists of respiratory bronchioles, alveolar ducts, and alveoli. The alveolar wall is covered by endothelium that is connected to the epithelium by a basement membrane. Alveolar epithelial and endothelial cells, as well as fibroblasts, synthesize and maintain the basement membrane. The interalveolar interstitial space is composed of fibroblasts, alveolar macrophages, and extracellular matrix (ECM). ECM contains proteoglycans, fibronectin, laminin, entactin, and Type IV and VII collagen. The quantity and quality of the ECM changes during radiation pneumopathy, via gene activation in fibroblasts. Increases in the collagens I/III/IV and fibronectin occurs early and persists until 8 weeks after radiation [69].

There are two types of alveolar epithelial cells: type I and type II **pneumocytes**. The squamous type I pneumocytes cover ≥90% of the surface of the alveolar epithelium. The cuboidal type II pneumocytes (granular pneumocytes) synthesize and secrete the lung surfactant that covers the alveolar surface and regulates surface tension. When injury occurs, type I pneumocytes are the first to be affected. Type I pneumocyte apoptosis leads to the proliferation of type II pneumocytes, thus inducing a regenerative response to repopulate the alveolar epithelium. Changes in type II pneumocytes are consistent findings in both clinical and experimental radiation pneumonitis. Because of their role in synthesizing surfactant, a

loss or change in the normal physiology of type II pneumocytes can profoundly alter lung function. Type II cell hyperplasia is a nonspecific marker of alveolar injury and repair and has been reported following other lung insults [70]. These cells respond by increasing alveolar surfactant production during the first 2–6 weeks after irradiation [23]. Thus, the early response to radiation is thought to be primarily driven by lung epithelial and endothelial cells [15].

There are two types of **fibroblasts** in lung interstitium: the **common fibroblast**, which parallels the epithelium and is intimately connected to the fiber elements of the ECM, and the **myofibroblast**, which is stellate and oriented perpendicularly to the alveolar wall. In response to injury, fibroblasts transdifferentiate into myofibroblasts, migrate, and generate the matrix components of scar tissue [7]. Myofibroblasts express matrix metalloproteinase (MMPs) and tissue inhibitors of metalloproteinase (TIMPs), through which they regulate the degradation of matrix components [71]. Myofibroblasts are either recruited from bone marrow, i.e. fibrocyte ($CD45^+$, $Cd34^+$, collagen I^+), or are derived by epithelial-mesenchymal transdifferentiation (EMT) [72-74]. Fibrocytes are associated with skin lesions, lung fibrosis, and tumors and contribute to the remodeling response by secreting matrix metalloproteinases [75, 76]. Recent data indicate that the CXC and CC chemokine families are involved in promoting the mobilization and trafficking of fibrocytes [75-77]. These functions of CXC chemokines are important in the pathogenesis of lung fibrosis and other fibroproliferative disorders.

While lung fibroblasts are probably the most central cell population in the process of radiation-induced lung fibrosis, it is clear that regulation of the fibroblast responses to injury is influenced by specific interactions with multiple inflammatory cell types. Numerous lung

inflammatory cells, both resident and recruited, are involved in the development of radiation pneumonitis and fibrosis in experimental models. A selective increase of $CD4^+$ T cells was observed, peaking 4 weeks after irradiation in the rat lung [62], 70]. A number of macrophages and lymphocytes were found in both BAL and lung in mice after irradiation [15]. T cell depletion prevents radiation-induced pneumonitis in specific pathogen free (SPF) mice, which suggests an autoimmune component to this injury [78].

Activated **macrophages** produce numerous cytokines with mitogenic or chemotactic properties for neutrophils and lymphocytes and also act directly on fibroblasts and endothelial cells [7]. Macrophage activation was first described as a T_H1 cell–IFN-γ-mediated process; however, it is now clear that macrophages differentiate into at least two functionally distinct populations depending on whether they are exposed to T_H1 or T_H2 cytokines. T_H1 cytokines activate nitric-oxide synthase 2 (NOS2) expression in classically activated macrophages, whereas the T_H2 cytokines IL-4 and IL-13 preferentially stimulate arginase-1 (ARG1) activity in "alternatively activated" macrophages [79]. Macrophages are found at diverse sites of inflammation and have been linked to the process of inflammation and repair. Activated macrophages regulate inflammatory cells, tissue debridement, cell killing, recruiting and activating myofibroblasts, and regulating spontaneous recovery of fibrosis in physiological or aberrant wound healing [80-83]. Macrophages also affect the transcription of a variety of other cytokines, chemokines, and growth factors, including PDGF, TGFα, TGFβ, FGF2, IL-10, TNF, IL-1a, macrophage-derived chemokine (MDC, CCL22), monocyte chemotactic protein 1 (MCP-1; CCL2), CCL3, MIP-1β and regulated upon activation, normal T expressed and secreted (RANTES; CCL5). Therefore, it is no surprise that alveolar macrophages are another cell population important in the

pathophysiology of lung fibrosis. In a hapten-induced lung fibrosis murine model, depletion of alveolar macrophages reduced the number of TNF-α-containing cells, as well as the number of inflammatory cells recruited into the alveolar space, with subsequent decreased collagen deposition [84]. Macrophages were recruited into the lungs of mice and rats after thoracic irradiation [15, 46, 85, 86]. The conditioned media of alveolar macrophages isolated from irradiated rabbit lungs evoked a substantial proliferation of fibroblasts [86]. Cultured alveolar macrophages dramatically increase their TGF-β secretion after radiation. Mice with radiation-induced fibrosis exhibit increased TGF-β expression by macrophages during the early phase of lung injury that was associated with lung inflammatory foci [36]. The regions of inflammatory infiltration and focal fibrotic lesions after irradiation contains numerous macrophages [18]. Recruited macrophages have severe hypoxia at 6 months after XRT, suggesting that the oxygen consumption by activated macrophages contributes to the systemic hypoxia [6]. Although macrophage-derived TGF-ß may promote fibrosis by directly activating resident fibroblasts to become collagen-producing myofibroblasts, prior macrophage depletion by liposome-clodronate did not influence later cell recruitment [15]. Thus, the specific role for alveolar macrophages in radiation-induced lung fibrosis is still likely complex and not fully understood.

Lastly, the role of neutrophils has not been clearly defined; however, at least one study suggests that neutrophils are not important in radiation lung injury [38].

In summary, radiation-induced pneunomitis and fibrosis is a complex multicellular process in which the fibroblasts respond to the macrophages, the lymphocytes, the type II epithelial cells, and the endothelial cells.

I.C. Chemokines and Chemokine receptors

1. Chemokine Structure

Chemokines (chemotactic cytokines) are single polypeptides ranging from approximately 70 to 120 KD in length and share 20% to 30% of amino acid sequence identity. Chemokines are rich in basic amino acids and contain several four cysteine motifs forming disulfide bonds between the first and third cysteines and between the second and fourth cysteines [87, 88]. The general structure of chemokines is a β-barrel consisting of 3 anti-parallel β strands flanked by a basic C-terminal α-helix, a disordered N-terminal region, and the connecting loops (**Figure 1.2**).

2. Nomenclature

Chemokines are divided into four subgroups (CXC, CC, C, and CX3C chemokines) based on the organization of the first pair of conserved cysteine motifs (Table 2). Chemokine receptors are defined by their ability to signal or bind one or more members of the chemokine family: CXCR (receptors for CXC chemokines), CCR (receptors for CC chemokines), XCR (a receptor for C chemokines), and CX3CR (a receptor for CX3C chemokines) [88, 89].

3. *Chemokine Receptors*

Chemokines are often induced rather than constitutively produced, act locally rather than systemically and mediate their effects through 7-transmembrane-spanning G-protein-coupled receptors. Most chemokine receptors recognize more than one chemokine and several chemokines bind to more than one receptor. Chemokine receptors signal through the G protein subunit to directly activate phospholipase C and phosphoinosidide 3-kinase (PI3K) and subsequently activate cytoskeletal regulatory kinases, such as FAK, that mediate

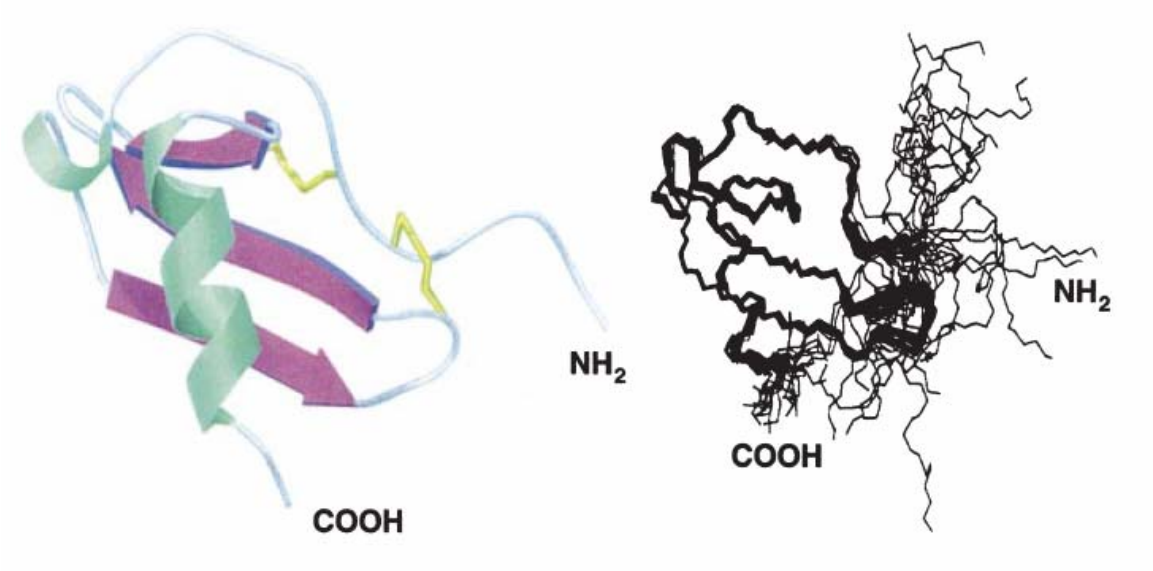


Figure 1.2 Three-dimensional structure of the CXC chemokine SDF-1 as obtained by nuclear magnetic resonance (NMR) analysis in solution. A model ribbon structure is shown on the left. On the right, 30 single structures are projected onto each other. This presentation shows that the structure of the core is rigid as judged by the fact that it is virtually identical in all single views. The amino- and carboxyl-terminal regions, by contrast, can move freely as shown by their disordered structure. Crump et al. [90].

chemotaxis. A systematic nomenclature for chemokine receptors has been established (**Table 1.3**). Chemokine receptor interacts with two main sites on a chemokine, one in the N-terminal region and the other within an exposed loop of the backbone that extends between the second and the third cysteine. The N-terminal binding site is essential for triggering signal transduction from the receptor. However, the receptor recognizes the loop region first: it is this interaction that is necessary for the correct presentation of the triggering domain. Analogues generated by amino-acid deletion or modification of the N-terminal region still bind effectively, but do not signal, and thus act as receptor antagonists [91].

4. Chemokine Sources and Regulation

While virtually every cell in the body can produce chemokines, some chemokines are produced constitutively by specific cell types. Leukocytes, vascular endothelial cells, smooth muscle cells and fibroblasts all have receptors for chemokines (**Table 1.4**). CXC chemokine receptors are primarily expressed by neutrophils and lymphocytes, whereas CC chemokine receptors are expressed on a wider range of cells, such as lymphocytes, monocytes, macrophages, and basophils. Of interest to our model, T_H1 and T_H2 cells show distinct patterns of chemokine receptor expression: CCR5 and CXCR3 are characteristic for T_H1 cells and their expression can be suppressed by IL-10, whereas CCR3 and CCR4 are characteristic for T_H2 cells and their expression requires the synergistic effects of IL-2 and IL-4 [92]. Chemokines are also classified by their functions as being either inflammatory or homeostatic. Homeostatic chemokines, also called homing lymphoid chemokines, including CCL19, CCL21, CXCL12 and CXCL13, are constitutively produced in lymphoid tissue and maintain the activity of leukocytes in these organs by directing the traffic and homing of

Table 1.3 Chemokines and Chemokine Receptors, Christopherson,2004 [88]

Name	Gene (Human)	Protein Synonyms	Chemokine Receptor(s)
C subfamily			
XCL1	SCYC1	Lymphotactin α, SCM-1α, ATAC	XCR1
XCL2	SCYC2	Lymphotactin β, SCM-1β, ATAC	XCR2
CC subfamily			
CCL1	SCYA1	I-309,TCA3 (mouse)	CCR8
CCL2	SCYA2	MCP-1, MCAF, JE (mouse)	CCR2
CCL3	SCYA3	MIP-1α, LD78α	CCR1, CCR5
CCL4	SCYA4	MIP-1β	CCR5
CCL5	SCYA5	RANTES	CCR1, CCR3, CCR5
CCL6	SCYA6	C10 (mouse), MRP-1 (mouse)	Unknown
CCL7	SCYA7	MCP-3	CCR1, CCR2, CCR3
CCL8	SCYA8	MCP-2	CCR3, CCR5
CCL9	SCYA9	MRP-2 (mouse), MIP-1γ (mouse)	CCR1
CCL10	SCYA10	CCF18	CCR1
CCL11	SCYA11	Eotaxin	CCR3
CCL12	SCYA12	MCP-5 (mouse)	CCR2
CCL13	SCYA13	MCP-4, CKβ10	CCR2, CCR3
CCL14	SCYA14	CC-1,HCC-1,NCC-2,CCCK-1/3,MCIF	CCR1, CCR5
CCL15	SCYA15	HCC-2,Lkn-1,MIP-5,CC-2,NCC-3, MIP-1δ	CCR1, CCR3
CCL16	SCYA16	NCC-4,LEC,HCC-4,LMC,Mtn-1,LCC-1	CCR1, CCR2
CCL17	SCYA17	TARC	CCR4
CCL18	SCYA18	DC-CK1,PARC,MIP-4,AMAC-1,CKβ7	unknown
CCL19	SCYA19	Exodus-3,ELC, MIP-3β,CKβ11	CCR7
CCL20	SCYA20	Exodus-1,MIP-3α,LARC,ST38 (mouse)	CCR6
CCL21	SCYA21	Exodus-2,SLC, 6Ckine,TCA4,CKβ9	CCR7
CCL22	SCYA22	MDC,ABCD-1,DC/B-CK(mouse)	CCR4
CCL23	SCYA23	MIP-3,MPIF-1,CKβ8-1	CCR1
CCL24	SCYA24	MPIF-2, CKβ6,Eotaxin-2	CCR3
CCL25	SCYA25	TECK, CKβ15	CCR9
CCL26	SCYA26	Eotaxin-3, MIP-4α,	CCR3
CCL27	SCYA27	ALP,Skinkine,ITL,ESkine,CTAK	CCR10
CCL28	SCYA28	MEC	CCR3, CCR10
CXC Subfamily			
CXCL1	SCYB1	GROα,MGSA,N51/KC(mouse),MIP-2	CXCR2,CXCR1
CXCL2	SCYB2	GROβ, MIP-2α	CXCR2
CXCL3	SCYB3	GROγ, MIP-2β	CXCR2
CXCL4	SCYB4	Platelet factor-4	unknown
CXCL5	SCYB5	ENA-78	CXCR2
CXCL6	SCYB6	GCP-2	CXCR1, CXCR2
CXCL7	SCYB7	PBP,CTAPIII, β-TG, NAP-2	CXCR2
CXCL8	SCYB8	IL-8	CXCR1, CXCR2
CXCL9	SCYB9	Mig	CXCR3
CXCL10	SCYB10	γIP-10, crg-2 (mouse)	CXCR3
CXCL11	SCYB11	H174,β-R1, I-TAC,IP-9	CXCR3
CXCL12	SCYB12	SDF-1α,SDF-1β,PBSF	CXCR4
CXCL13	SCYB13	BLC, BCA-1	CXCR5
CXCL14	SCYB14	BRAK	unknown
CXCL15	SCYB15	Lungkine,Weche	unknown
CXCL16	SCYB16	Bonzo/STRL33 ligand	CXCR6
CX_3CL1	SCYD1	Fractalkine, Neurotactin (mouse) CX3C Subfamily	CX_3CR1

Table 1.4 Expression of CC Chemokine Receptors in Mouse Leukocyte Populations

	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7	CCR8
T lymphocytes	+	+	+	+	+	+	+	+
B lymphocytes		+			+	+	+	+
Macrophages	+	+			+			+
Eosinophils	+		+					
Basophils	+	+	+					
Neutrophils	+							
Mast cells	+		+	+	+			
Immature Dendritic cells	+			+	+	+		
Mature Dendritic cells					+		+	
NK cells	+	+		+				
T_H1 cells		+			+			
T_H2 cells			+	+				+
T_H17 cells	+*	+			+*	+		

adapted from Saeki, Curren Pharm Des, 2003, [93] and Von Andrian, N Engl J Med, 1998, [94]. Potzl, PLoS ONE, 2008, [95]

* unpublished data from Dr. Mike Carlson.

lymphocytes and dendritic cells [94, 96]. Inflammatory (inducible) chemokines are mainly secreted by activated leukocytes, endothelial cells, and epithelial cells and function as chemoattractants for neutrophils, monocytes, and other immune cells in the host defenses to infection and inflammation. The expression of inflammatory chemokines and their receptors is up-regulated by a variety of inflammatory stimuli, e.g., pro-inflammatory cytokines (IL-1β, TNF-α), or lipopolysaccharides. Inflammatory chemokines include the MCP and MIP families, RANTES and eotaxin. These chemokines bind and signal through chemokine receptors, CCR1, CCR2, CCR4 and CCR5. T cells express most chemokine receptors constitutively; but, under the influence of cytokines, e.g. IL-2, IL-4, IFN-γ, will overexpress CCR1, CCR2, CCR5 and CXCR3 and chemotax to RANTES, CCL4, IP-10 and others.

5. Chemokine Functions

A vast literature documents the roles of chemokines in a variety of diseases (**Table 1.5**). Their major function is to stimulate leukocyte migration to sites of tissue injury or inflammation, and to activate many cell types, including T lymphocytes and monocytes [87, 89, 92, 97-99]. Chemokine-induced leukocyte migration is a multistep process as described in **Figure 1.3**. Chemokines also regulate the order and timing of integrin adhesion. For example, it has been recently reported that CXCL1 (GROα) and CX3CL1 (fracktalkine) mediate the initial firm adhesion whereas CCL2 (MCP-1) is required for subsequent leukocyte spreading and diapedesis. Chemokines can stimulate leukocyte degranulation and release of inflammatory mediators. CCL2 is a potent stimulator of histamine release from basophils [94].

Table 1.5 Chemokine-related diseases

Category	Human diseases	Animal models
Autoimmune disease	Rheumatoid arthritis; systemic lupus erythematosis; multiple sclerosis	Autoimmune arthritis (for example, collagen-induced arthritis); MRL-Fas_{lpr}; experimental allergic encephalitis
Graft rejection	Heart allograft rejection	kidney heterotopic heart allografts; sponge allograft rejection allografts
Infection	Acute and chronic bacterial and viral infections (especially HIV and mycobacteria)	Rodent models using the same or analogous pathogens; cecal ligation and puncture-induced sepsis
Inflammation or allergy	Asthma; arthritis; colitis; psoriasis	Antigen sensitization and anatomically specific delivery (for example, inhaled antigen challenge in asthma models)
Neoplasia	Leukocyte recruitment in cancer angiogenesis	Therapeutic vaccination; *in vivo* angiogenesis models
Vascular	Atherosclerosis; hypertension; ischemia-reperfusion	Hypercholesterolemic rodents; genetic models of hypertension arterial injury models

adapted from Baggiolini, Nature, 1998, [91, 96]

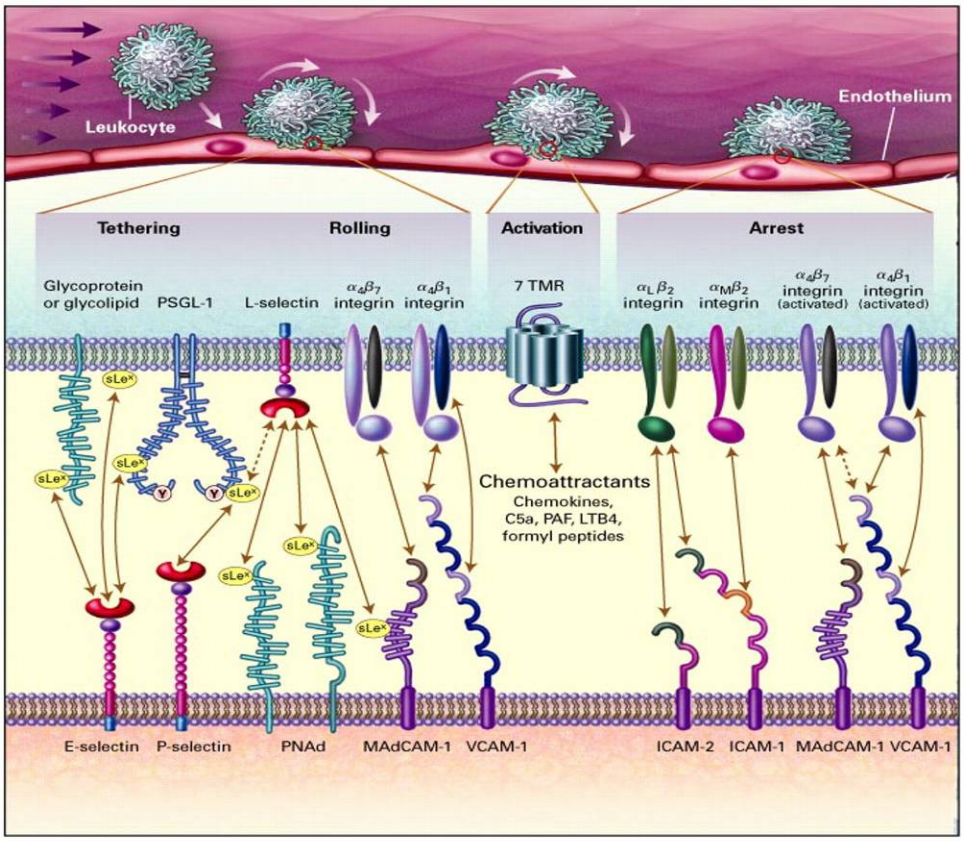


Figure 1.3 Chemokines in leukocyte trafficking. The migration of leukocytes from the blood into the tissue is a multisetp process involving a series of interaction between leukocytes and endothelial cells. Initially, leukocytes in the blood become tethered to endothelial cells and roll slowly downstream. **Tethering** is greatly facilitated by the recognition of L-selectin, E-selectin, P-selectin glycoprotein ligand 1 [PSGL-1], and α_4 integrins by their receptors. E-selectin and the α_4 integrins can tether some leukocytes, but their predominant function is to reduce the velocity of rolling. Selectin-mediated bonds arrest leukocytes at the vessel wall, resulting in subsequently leukocytes **rolling** (margination) along the vessel wall under the pressure of the blood flow. The rolling leukocytes response to a chemoattractant gradient formed by the accumulation of chemokines and other molecules on endothelial cells. These rolling leukocytes are then activated through their cell surface chemokine receptors with seven transmembrane domains which transmit intracellular signals through G proteins. The activating signal induces rapid activation of $ß_2$ integrins, $\alpha 4$ integrins, or both, which then bind to members of the endothelial immunoglobulin superfamily, resulting in **firm arrest** of leukocytes. Next, the leukocytes will cross the endothelial cell barrier and follow the chemoattractant gradient by a process called **extravasation**. Finally the leukocytes migrate to the site of inflammation. Adapted from Ian Mackay, N Eng J Med, 1998, [94].

Chemokines are clearly important in directing leukocyte trafficking by providing chemoattractant gradients as well as by activating integrins. Chemokines that are highly expressed in response to the pathogens or other stimuli both initiate and sustain leukocyte recruitment to the affected sites. Initially, this process acts to clear the infectious agents or irritants with only minimal damage; however, this response can become an overwhelming attack on normal tissues, causing autoimmune or other chronic inflammation. Chemokines also control the local milieu of inflammatory cytokines. Therefore, locally produced chemokines crucially influence disease progression and contribute to the chronicity of many types of inflammatory diseases (see **Table 1.5**).

The specific leukocytes recruited by chemokines into disease sites reflect the nature and chronicity of the diseases. Massive infiltration of neutrophils is the main characteristic of many acute inflammatory diseases and correlates with a striking increase in CXC chemokines, e.g., IL-8 (CXCL8) and MIP-2 (CXCL2). In contrast, many chronic inflammatory diseases often show infiltration of lymphocytes and monocytes/macrophages, which is associated with CC chemokines. For example, CCL3 and CCL1 levels are increased in rheumatoid arthritis, a chronic inflammatory disease characterized by the infiltration of memory T lymphocytes and monocytes [100]. The levels of CCL3 and CCL2 are elevated in multiple sclerosis and the deficiency of their receptors, CCR2 or CCR1, diminishs symptoms in murine model of EAE [88]. CCL3 and CCR5 are important in graft versus host disease (GVHD) because CCL3 attracts $CD8^+$ T lymphocytes to the liver by means of CCR5 expressed on their surface [101]. In addition to controlling leukocyte trafficking and activation, chemokines may help determine the character of local immune responses and

contribute to the systemic organization of the immune system by mediating tissue-selective trafficking of memory and effector T and B cells [102, 103].

In hematopoiesis, chemokines function to guide hematopoietic stem and precursor cells to the diverse microanatomical niches in the bone marrow and thymus [89]. CCL3 and CCL4 both affect the proliferation of myeloid progenitor cells. CCL3 has complex effects on hematopoiesis, enhancing the proliferation of lineage-committed progenitor cells, but suppressing the proliferation of immature progenitor cell proliferation, and mobilizing mature and immature myeloid progenitor cells to the blood [93, 104].

Some chemokines, such as CXC with ERL motif and CCL2, have very strong angiogenic activity and promote capillary formation [97]. These angiogenic properties of chemokines may affect tumor growth, inflammation and wound healing, all processes where angiogenesis is an important requirement [105].

Finally, there is overwhelming evidence that chemokines are also involved in tumor progression. Many tumor cells and tumor-associated stromal cells generate chemokines, which may attract the neutrophils, macrophages and lymphocytes involved in anti-tumor immune responses [105, 106]. A variety of effects from these chemokines have been described: some promote angiogenesis, metastasis and tumor growth, while others can inhibit angiogenesis or improve anti-tumor immunity [105]. The CXCL12-CXCR4 axis facilitates tumor metastasis to distant organs and CCL5/RANTES, that binds to CCR1, CCR4, and CCR5, has been shown to promote tumor growth [106]. Therefore, small molecule inhibitors of these receptors could affect tumor growth, metastases, angiogenesis, and anti-tumor responses, depending on the duration and dose given.

6. Chemokines and Chemokine Receptors in Airway Diseases

Many airway diseases, e.g., asthma, chronic bronchitis and chronic obstructive pulmonary disease (COPD), are characterized by the influx of inflammatory cells into the airway. Given to the important role of chemokines and their receptors in leukocyte migration, it is not surprising that they are important in the development of airway diseases (**Table 1.6**). The influx of eosinophils, TH2 cells, and mast cells characterizes asthma [107, 108]. Dysregulated CXCL1 expression is associated with reduced Treg activities in allergic asthma [109]. In contrast, chronic bronchitis and COPD characteristically have a large influx of neutrophils, macrophages and CD8+ lymphocytes. The increased expression of CCL2 and CCR2 correlates with the accumulation of mast cells and macrophages in the lungs of COPD and chronic bronchitis. Both CCL2 and CCR2 are expressed on mast cells and macrophage, suggesting that CCR2 may be an interesting target for therapeutic intervention in the patients with COPD and chronic bronchitis [110].

Table 1.6 Chemokines in respiratory diseases

CC-chemokine receptors	Chemokine ligands	Cell-type/tissue-expression	Potential treatment for:
CCR1	MIP-1α, MIP-1β, MCP-2, MCP-3, RANTES	NK cells, T lymphocyte, macrophage, basophil, eosinophil, neutrophil	severe asthma
CCR2	MCP-1,-2,-3,-4,-5	monocytes, memory T lymphocytes, B lymphocytes, basophils, neutrophils	COPD, chronic bronchitis; asthma
CCR3	Eotaxin, -2,-3, RANTES, MCP-2, -3, -4, MIP-1α, MIP-1β	eosinophils, T_H2 cells, basophils, mast cells	asthma, allergic diseases
CCR4	MDC, TARC, MCP-1	T_H2 lymphocytes, NK cells	allergic diseases
CCR8	I-309, TARC	T_H2 lymphocytes, monocytes in brain, spleen, lymph node	allergic diseases
CXCR1	IL-8, GCP-2, ENA-78	neutrophil, macrophage	COPD, chronic bronchitis, severe asthma
CXCR2 IL-8	IL-8, NAP-2, ENA-78, GRO-α,β,γ	neutrophil, macrophage, eosinophil	COPD, chronic bronchitis, severe asthma

Charles Owen, Pulm Pharmaco Ther, 2001,[107]

7. Chemokines Receptor Antagonists-New Perspective for Therapy

With the extensive biological roles for chemokines and their receptors in a variety of diseases(**Table 1.5**), the chemokine system may provide novel therapeutic opportunities for cardiovascular, allergic, and autoimmune diseases, as well as in transplantation, neuroinflammation, cancer and HIV-associated disease [34, 92, 97]. Since chemokines act through G-protein coupled seven-transmembrane receptors, and there is a redundancy in which chemokines binding to each receptor, neutralization at the chemokine receptor level is a more promising target for therapeutic intervention. Thus, identifying which chemokine receptors are involved in the pathogenesis of a particular disease is crucial for therapeutic drug development.

Strategies for interfering with chemokine-chemokine receptor system include neutralizing antibodies, peptide antagonists and non-peptide antagonists (**Table 1.7**). Some inhibitors specifically bind to certain chemokine receptors, whereas other broad-spectrum chemokine inhibitors (BSCIs) inhibit a number of chemokine receptors. Many chemokine inhibitor molecules have been tested by using mutant animals lacking only the desired protein expression or by blockade with specific antagonists. Some antagonists have already been used successfully in vivo. For example, TAK-779, a CCR5 and CXCR3 antagonist has been shown to inhibit the development of experiment-induced arthritis in mice by modulating the migration of $CCR5^{+}/CXCR3^{+}$ T cells into joints [111]. Anti-CCL3 antibodies were reported to ameliorate the severity of multiple sclerosis in a murine EAE model [112]. Similarly, the CCR1 antagonist, BX-471, effectively reduced symptoms in a rat EAE model [93]. BX471 also prolonged survival of cardiac

Table 1.7 Chemical and Peptide Antagonists of CC Chemokines and receptors

Inhibitors	Affect Chemokine/Chemokine Receptor	References
tRANTES & tMCP-3 (t = truncated)	antagonists of CCR1, CCR2, CCR3, CCR5	[113]
BX 471	inhibits CCR1 and leukocyte migration	[114]
2q-1	inhibits human & murine CCR1 & CCR3	[115]
4HPs (2,2DPCPV)	inhibits CCL3 / CCR1 binding & signaling	[116, 117]
MCP-3	natural antagonist of CCR5	[118]
AOP-RANTES	inhibits CCR5 & monocyte chemotaxis	[119]
Met-RANTES	inhibits CCR5 & monocyte chemotaxis	[119]
vMIP-II	antagonist at CCR1, 2, 3, 5, and CXCR4	[120]
TAK-779	specifically inhibits CCR5	[121]
Intrakines	CCR5 specific inhibition	[122]
Hammerhead ribozymes	CCR5 specific inhibition	[123]
CCR5Δ32	natural inactivating mutant allele	[124]
Met-chemokine β7	specific antagonist of CCR3	[125]
MC148RvMCC-1	blocks CC & CXC chemokine-induced neutrophil, monocyte, and T cell migration	[126, 127]
T135kDa protein	broad spectrum chemokine scavenger	[128]
T7	broad spectrum CC chemokine & γ-IFN scavenger	[129]
US28	chemokine sequestration	[130]
Monoclonal ab	blocks CCR5	[131]
IL-10	inhibits chemokine release during endotoxemia	[132]

allografts in rats, possibly by inhibiting of monocyte adhesion to inflamed endothelium [133]. Thus, chemokine receptor blocking agents may indeed provide important and novel therapeutic interventions.

I.D. Chemokines in Radiation-induced Lung Injury

Chemokines are fundamental regulators of leukocyte homeostasis and inflammation, and their antagonism by small molecule chemokine receptor antagonists may be important in the future treatment of human respiratory diseases. A number of studies suggest that cytokines and chemokines are involved in the pathogenesis of radiation-induced fibrosis and other fibrosis models [16, 69, 75, 134-136]. CXCL/CXCR2 is important for neutrophil recruitment during the pathogenesis of hyperoxia-induced lung injury [137]. Chemokines are also involved in promoting the mobilization and trafficking of circulating mesenchymal progenitor cells (also known as fibrocytes) in lung fibrosis [77].

Although the involvement of chemokines in inflammation is well established, their functional role in disease progression, and particularly, in the development of fibrosis, is still unclear. The CCL and CCR families have important regulatory roles in fibrotic processes, but numerous chemokine signaling pathways are probably involved. Chemokines are expressed by a variety of leukocytes implicated in pneumonitis and fibrosis (**Table 1.4**). CCL3 and CCL2 have so far been identified as important pro-fibrotic mediators. CCL3 was required for cell recruitment in pneumonitis induced by a variety of pathogens, including influenza virus, *Aspergillus fumigatus*, as well as in GVHD [138, 139]. Lung macrophages and epithelial cells are thought to be the main cellular sources of CCL3 [140]. CCL3 specific antibodies were shown to significantly reduce bleomycin-induced fibrosis [141, 142]. Studies

in murine model of bronchiolitis obliterans syndrome show similar results for $CCR2^{-/-}$ mice and for CCL2 antibody-treated WT mice [143]. Thus, both CCL3- and CCL2-mediated signaling pathways appear significant in pathogenesis of fibrosis [76, 144].

I.E. The chemokine receptor CCR1 in Radiation-induced Lung Injury

The chemokine receptor, CCR1 is expressed by T and B cells, NK cells, dendritic cells, macrophages, eosinophils, neutrophils and basophils. CCR1 binds a number of CC chemokines, including CCL3, CCL5 (RANTES), CCL2 (MCP-1), CCL7 (MCP-3) and CCL15 (MIP-5). These ligands have potent chemotactic activity and can also be produced in an autocrine fashion. CCR1 is believed to play a crucial role in the migration of leukocytes to the sites of inflammation and in perpetuating inflammatory responses. CCR1 signaling may also contribute to tissue damage and inflammation through the enhancement of T cell activation, regulation of T_H1/T_H2 polarization, and the stimulation of macrophage function and protease secretion. These properties support CCR1 as an attractive therapeutic target to modulate leukocyte infiltration and decrease the associated tissue damage common to the autoimmune diseases.

CCR1 may also be important for murine neutrophil-mediated host defense, since CCR1-deficient mice are much more susceptible to *Aspergillus fumigatus* infection [145]. Anti-CCR1 antibodies significantly reduced the accumulation of inflammatory cells and collagen deposition, resulting in dramatic improvement in the survival of mice with bleomycin-induced lung fibrosis [146]. The CCR1 ligand CCL3 was shown to contribute to cellular recruitment during *Schistosoma* egg granuloma formation [147]. $CCR1^{-/-}$ mice have a marked reduction in lung granuloma size after infection with *Schistosoma mansoni* eggs, this effect is associated with increased expression of the T_H1 cytokine IFN-γ and decreased

expression of the T_H2 cytokine IL-4 [145]. Johntson reported a strong correlation between elevated CCL3 and development of radiation-induced late effects in fibrosis-sensitive mice [16]. The mRNA expression of chemokines BLC, C10, IP-10, CCL2, CCL7, CCL9 and CCL5, and the receptors CCR1, CCR2, CCR5 and CCR6 were elevated in fibrosis-sensitive (C57BL/6) mice at 26 weeks postirradiation at the dose of 12.5 Gy [148]. Therefore, chemokines may cooperate with pro-fibrotic cytokines in the development of fibrosis by recruiting inflammatory cells to the sites of tissue damage. Although chemokines and their receptors have begun to be investigated in radiation lung injury [16, 99, 136], studies in animal models of specific chemokine/receptor inhibitors have not yet been reported. Nonetheless, CCR1 is a contributor to airway remodeling in airway diseases and may prove to be a therapeutic target.

We hypothesize that specific chemokines/receptors induced by irradiation may mediate the recruitment and activation of inflammatory cells, and thereby may contribute to the development of radiation-induced lung injury. And we further hypothesize that inhibitors of these chemokines/receptors might represent excellent molecular therapeutic targets for reducing or eliminating radiation-induced pneumonitis and subsequent fibrosis.

Specific Aims

(i) **To explore the roles of the chemokine, CCL3, and its receptors, CCR1 and CCR5, in radiation-induced lung injury.** Mouse strains individually lacking CCL3, CCR1, and CCR5 are used to compare with radiation-sensitive wild type C57BL6/J control mice in radiation-induced lung injury, including mortality, pneumonitis, fibrosis, and lung function.

(ii) **To investigate potential therapeutic interventions based on studies in protected genotypes**, e.g. BX-471 blocking CCR1.

(iii) **To investigate the roles of inflammatory cell subtypes in mediating radiation lung injury.** We characterize the inflammatory cell infiltrates in the lung after radiation by histologic staining and flow cytometry analyses. Mouse strains individually lacking certain inflammatory cell subtypes, e.g. CD8 or CD4-deficient mice, are used in these studies.

II. Murine models of radiation lung injury

II.A. Introduction

Animals previously used in models of radiation-induced lung injury include rat [22, 25], mouse [149-151] rabbit [86], and pig [152]. Although mice offer many advantages as a model, there are some notable differences in the effects of radiation on the lungs in humans compared to mice. For example, the hyaline membrane formation which is an early response in human lungs has not been observed in mouse lung [153]. There are substantial strain-differences in the effects of irradiation on mouse lung. Radiation fibrosis was seen in C57L/J, C57BL/6J, and C57BL/10J but not in C3HeB/FeJ, C3/HeJ, or CBA/J [150]. C3H/HeMs mice may be able to clear matrix proteins and inflammation more rapidly after irradiation than C57BL/6J mice [85]. In contrast, radiation pneumonitis is much more severe in C3/HeJ than C57BL/6J mice. Similarly, irradiated C3HeB/FeJ mice develop only classical pneumonitis during the early phase, whereas C57L/J mice develop small, tightly packed areas of inflammation which undergo fibrosis during the latent period, and exhibit progressive fibrosis from large regions of intense inflammation during the early phase [40]. Radiation-induced lung response susceptibility loci were identified by a genome-wide scan in reciprocal backcrossed mice bred from C3H/HeJ and C57BL/6J strains [154]. There was a striking difference in the timing and level of expression of cytokines between these two strains after lung radiation. For example, TNF-α and IL-1α mRNA levels were increased in irradiated C57BL/6 mice but not C3H/HeJ mice, and conversely, IL-1β mRNA levels were increased in irradiated C3H/HeJ mice but not C57BL/6 mice [57].

Clinically, there is significant individual variation in the incidence and severity of lung fibrosis for the radiotherapy patients, suggesting inherent risk factors for developing radiation lung fibrosis [155]. Studies by Travis *et al.* show the spatial heterogeneity of the radiosensitivity of murine lungs with critical target cells concentrated in the lung bases [10].

In general, however, the C57BL/6J is the most commonly used strain for studies of radiation-induced lung injury. Moreover, numerous genetically-altered strains are available for studying specific individual mediators of radiation lung injury. Thus, we used C57BL/6J mice in our model.

II.B. Materials and Methods

1. Mice

All animal studies were performed under approval by the IACUC at the University of North Carolina at Chapel Hill. Wild-type mice were purchased from Jackson Laboratories. All mice were housed in microisolator cages maintained in a specific pathogen-free environment. All of the mice used, except for those used in leukocyte flow cytometry and TGF-β1 experiments, were male. Age-matched nonirradiated mice were used in all experiments.

2. Thoracic Irradiation

Anesthetized mice at the age of 10-12 weeks were immobilized with a lead shield excluding all but the thoracic cavity. Animals were then irradiated with a single dose of 14.5 Gy from a cesium source (J. L. Shepherd & Associates, San Fernando, CA, USA) at a dose rate of 1.65 Gy/min. The LD_{50} for C57BL/6J mice is 14.5 Gy and at this dose survival is sufficient to permit adequate numbers of animals for long-term analyses.

3. Hydroxyproline (HYP) and Pyridinoline (PYR) assays

Anesthetized mice were exsanguinated, and the lungs were perfused intracardiacally with sterile PBS and removed. Tissue samples were minced, lyophilized for 48 hours until dry, and then hydrolyzed in 1 ml of 6 N hydrochloric acid at 120°C for 20 hours. Samples were then dried to remove HCl in a speed vacuum for 2 h, resuspended in distilled water, applied to a filter Eppendorf tube and spun at 14000 rpm for 5 min. The resultant filtrate was assayed for hydroxyproline and pyridinoline by HPLC.

4. Elastin Assay

The right lungs of mice were homogenized in 2ml of PBS and then incubated in 0.5ml 1M acetic acid containing 6mg/ml pepsin for 24h at room temperature with stirring (1500 rpm). Supernatants were obtained by centrifugation for 30 minutes at 5,000xg, at 4 ^{o}C, and then subjected to Fastin elastin assay according to the manufacturer's protocol (Biocolar CO., County Antrim, UK).

5. Gene Expression Analyses

Mice were anesthetized and lungs were harvested after perfusion with PBS at 0, 1, 3, 4, 6, 12, 16, 20, 24, 28 and 32 weeks after thoracic irradiation. RNA was extracted from the left lungs using RNeasy Mini kits (QIAGEN INC., Valencia CA.). cDNA was generated using SuperScript II reverse transcriptase, dNTPs, and Random Hexamer Primer (Invitrogen Corporation, Carlsbad, CA). The resultant cDNA was then analyzed by quantitative real-time PCR using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Commercial TaqMan gene expression assays (Applied Biosystems, Foster City, CA) were used as the gene-specific probe and primer sets for analysis of all genes studied. Data were analyzed using the 2-ΔΔCT method as previously described [156]. Briefly, data were presented as the fold change in gene expression

normalized to an internal control gene (glucuronidase β, Gusb) and relative to a calibrator sample (from non-irradiated controls).

6. Histologic Analyses

After euthanasia, the lungs were inflated with 10% formalin via a tracheal cannula, removed from the thoracic cavity, fixed overnight in formalin and then paraffin embedded. Lung sections were stained with hematoxylin and eosin for routine histology and Masson's trichrome for evaluation of collagen deposition.

7. Measurements of Lung Mechanics

Mice were anesthetized after thoracic radiation with 70-90 mg/kg pentobarbital sodium, tracheostomized, and mechanically ventilated at a rate of 350 breaths/min, with a tidal volume of 6ml/kg, and positive end-expiratory pressure of 3-4 cmH2O using a computer-controlled small-animal ventilator (Scireq, Montreal, Canada). Once ventilated, mice were paralyzed with 0.8 mg/kg pancuronium bromide. Using custom designed software (FlexiVent, Scireq), airway pressure, volume, and airflow measurement were recorded by using a precisely controlled piston during maneuvers to evaluate lung mechanics as previously described [157].

8. ELISA Analyses of TGF-β1

Quantitative ELISA measurements of total TGF-β protein levels from lung extracts of irradiated and control female mice were determined using the Promega TGF-β1 Emax® Immunoassay systems according to the company protocols.

9. Flow Cytometry

Lungs were harvested from irradiated female mice at 0, 1, 4, 8, 12, 16, 20, 24 and 28 weeks post-XRT. Excised whole lungs were minced and digested in RPMI with 5% FCS, 1

mg/ml collagenase A and 25 units/ml of DNAse for 1 hour. Red blood cells were lysed with ACK buffer, and nucleated cells were washed three times with PBS, layered on a 20% Percoll gradient and spun at 1500 x g for 30 minutes before resuspension in PBS with 2% FBS. Then resultant total mononuclear cells from both lungs were stained with PE- or FITC-labeled monoclonal antibodies to murine CD45, CD3, CD4, CD8, F4/80, and Gr-1 (Pharmingen, San Jose, CA) and then analyzed on a FACScan with Cytomation software.

II.C. Results

1. Survival analysis of* C57BL/6J *Mice after Thoracic Irradiation

C57BL/6J male mice received thoracic irradiation as a single dose of 14.5 Gy with a lead shield to exclude other body areas and then were monitored for up to 32 weeks. Premature death is defined as death before 32 weeks. Approximately half (48%) of irradiated WT mice died by 32 weeks after irradiation (**Figure 2.1**). End stage lung damage after radiation has both pneumonitis and fibrosis, also known as organizing alveolitis. Death appeared to be mostly due to chronic hypoxemia from poor diffusion of gas across fibrotic lung.

2. *Lung Hydroxyproline and Cross-linked Collagen is Increased after Irradiation*

Right lungs were harvested from the irradiated mice at 32 weeks after thoracic radiation. Hydroxyproline is a major component of the collagen and is found in few proteins other than collagen. Hydroxyproline content has been used as an indicator to determine collagen [158]**.** Therefore, we conducted hydroxyproline (HYP) assays of whole lungs to quantitate fibrosis. Pyridinoline (PYR) assays were performed to quantitate the cross-linked collagen. As shown in **Table 2.1**, the dry weight and hydroxyproline content of WT mouse right lungs increased (32%, $p=0.018$ and 35%, $p<0.001$) at 32 weeks post-irradiation, concurrent with the lung fibrosis seen in histology. Cross-linked collagen also increased at 32 weeks post-irradiation,

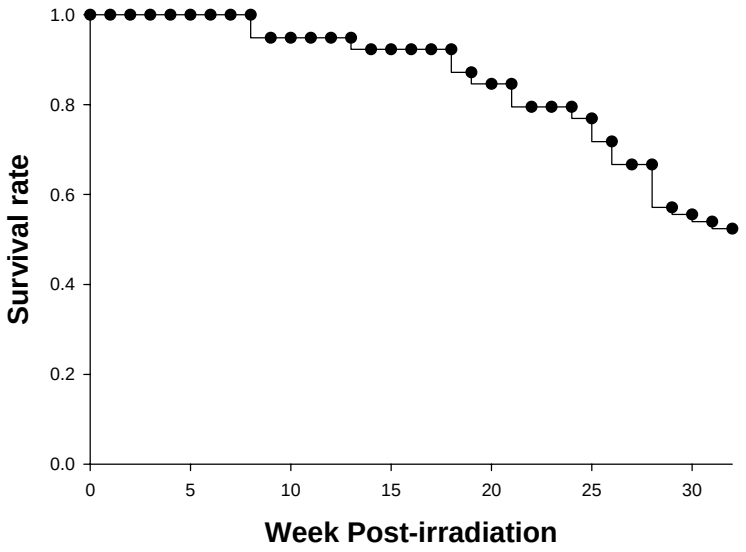


Figure 2.1 Survival analyses of irradiated WT mice by Sigmaplot 8.0. WT (n=39) animals received a single dose of 14.5 Gy of thoracic irradiation. Premature death was defined as death before 32 weeks post-radiation.

Table 2.1 Lung dry weight, hydroxyproline (HYP) and pyridinoline (PYR) content after irradiation in WT mice

Group	Dry weight(mg)	HYP(μg/right lung)	PYR(μg/right lung)
Control	23.56±1.29	232.15±29.07	1.047±0.13
IR	31.19±2.02	312.15±34.93	1.267±0.22
P value	0.018	<0.01	0.077

Values shown are mean±SEM from 5~9 mice per group

although the increase did not reach statistical significance (p=0.077).

3. *Increased Gene Expression of Lung Collagen 1α*

Collagen 1 and collagen 3 are the major components of connective tissue in the lung and are mostly synthesized by fibroblasts [158]. Previous studies have shown that type I collagen gene expression increases in bleomycin-treated mouse lungs and is associated with increased TGF-β, a profibrotic cytokine [159, 160]. However, Type III collagen is the predominant type in granulation tissue. To investigate which type of collagen contributes to radiation lung fibrosis, lung collagen 1α1 and 3α1 mRNA in lung were measured using Commercial TaqMan gene expression assays. As shown in **Figure 2.2**, collagen 3α1 gene expression significantly increased at 3 weeks following irradiation and remained elevated through 32 weeks post-irradiation. In contrast, there was no increase in collagen 1α1 gene expression. Therefore, type III collagen is predominant in radiation-induced lung fibrosis, while type I collagen predominates in bleomycin-induced lung fibrosis [160].

4. *No Increase in Lung Elastin*

Elastin is a major component of lung connective tissue. Increased elastin has been reported in the bleomycin and butylated hydroxytoluene models of lung fibrosis [161, 162]. However, we found no difference between irradiated mice and non-irradiated mice in the total amounts of elastin in the right lungs (**Figure 2.3**).

5. Histologic Analyses

As shown in **Figure 2.4**, some minor histologic changes occur in lungs at 8-12 weeks post-irradiation, including edema and mild inflammatory cell infiltration. Morphologic changes became more distinct at 24 weeks post-irradiation, including alveolar inflammatory cell accumulation, septal thickening and small, but distinguishable fibrotic foci. By 32 weeks

post-irradiation, the lungs of irradiated WT mice had septal thickening, diffuse inflammation, alveolar inflammatory cells (especially foamy macrophages), and patchy interstitial collagen deposition. In summary, the histologic characteristics of the lung responses to irradiation we observed in our model were consistent with those seen in other studies.

6. Diminished Lung Function after Thoracic Irradiation

To investigate lung function after thoracic radiation-induced lung injury, anesthetized mice were subjected to lung function tests while ventilated as described previously. Decreased compliance and increased tissue elastance are expected in radiation-induced fibrotic lung. Consistent with this expectation, the static compliance of the irradiated WT mice at 20 weeks and 32 weeks post-irradiation have a decrease of 7.7% ($p=0.007$) and 24% ($p<0.0001$), respectively. The tissue elastance of the irradiated WT mice at 20 weeks and 32 weeks post-irradiation have an increase of 14% ($p=0.007$) and 82% ($p<0.0001$), respectively. (**Figure 2.5**).

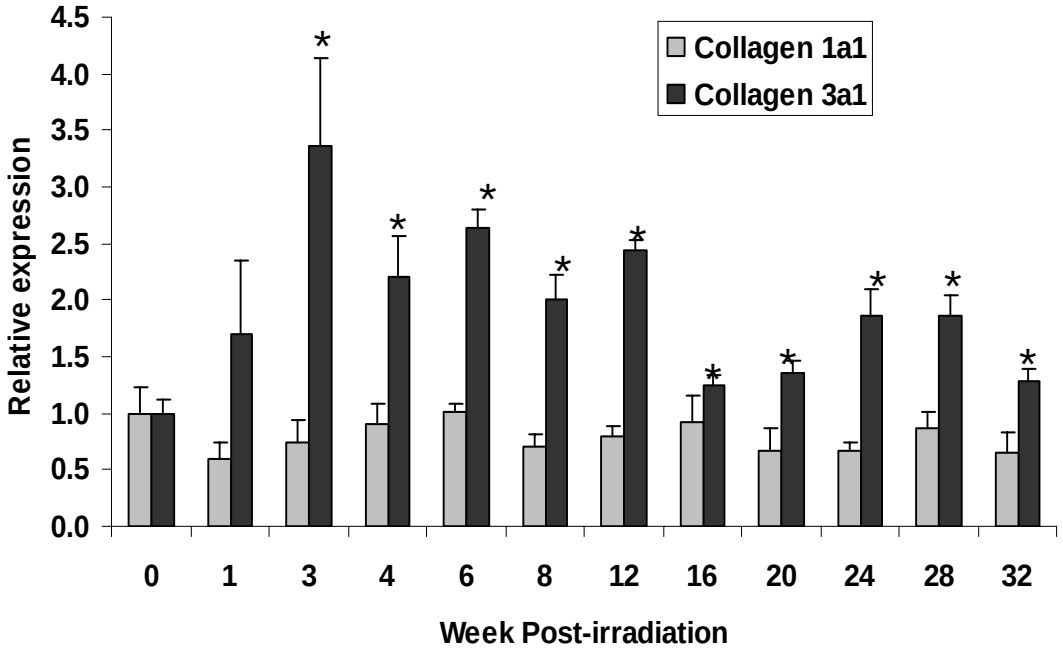


Figure 2.2 Increased lung collagen 3α1 gene expression in irradiated WT mice. Lung total RNA from non-irradiated control and irradiated WT mice was analyzed for collagen 1α1 and 3α1 mRNA expression by real-time RT-PCR. Collagen 1α1 and 3α1 gene expression is relative to Gus expression as indicated in Materials and Methods, and normalized to the levels in control mice. Values shown represent mean±SD from 5~9 mice per group. ***** $p <$ 0.05, compared to WT non-irradiated control.

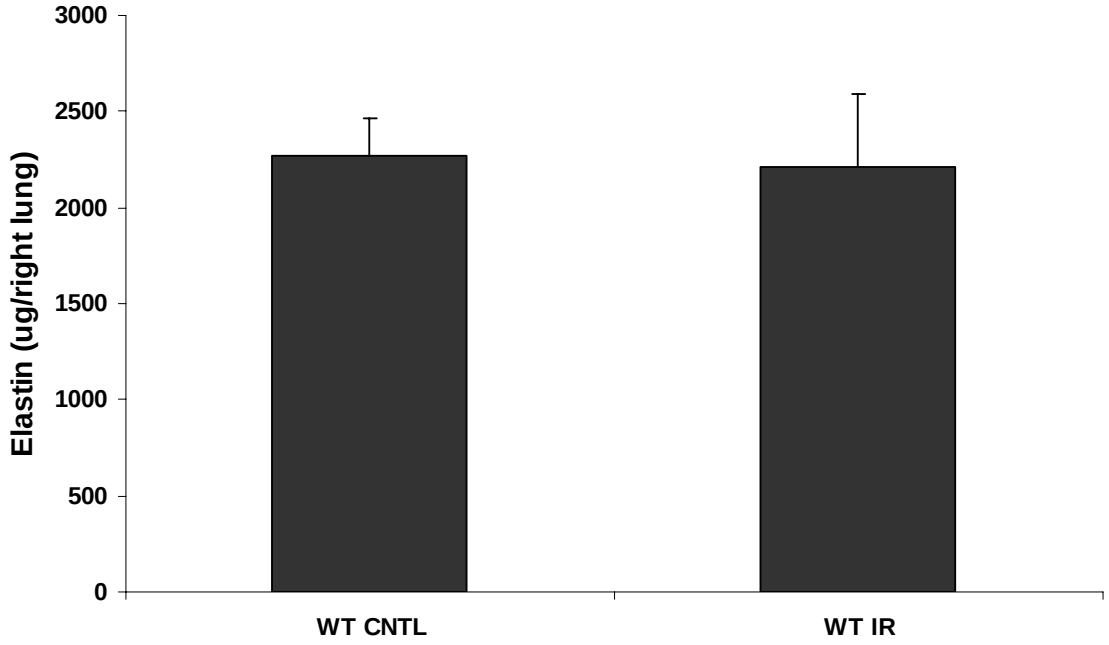


Figure 2.3 Lung elastin is not increased in irradiated WT mice. Mouse right lungs were homogenized in 2ml 1xPBS and incubated in 0.5ml 1M acetic acid containing 6mg/ml pepsin for 24h at room temperature with stirring (1500rpm). Supernatants were obtained by centrifugation for 30 minutes at 5,000xg, 4 ^{o}C, and then analyzed using the Fastin elastin assay according to the manufacturer's protocol (Biocolar CO., County Antrim, UK).

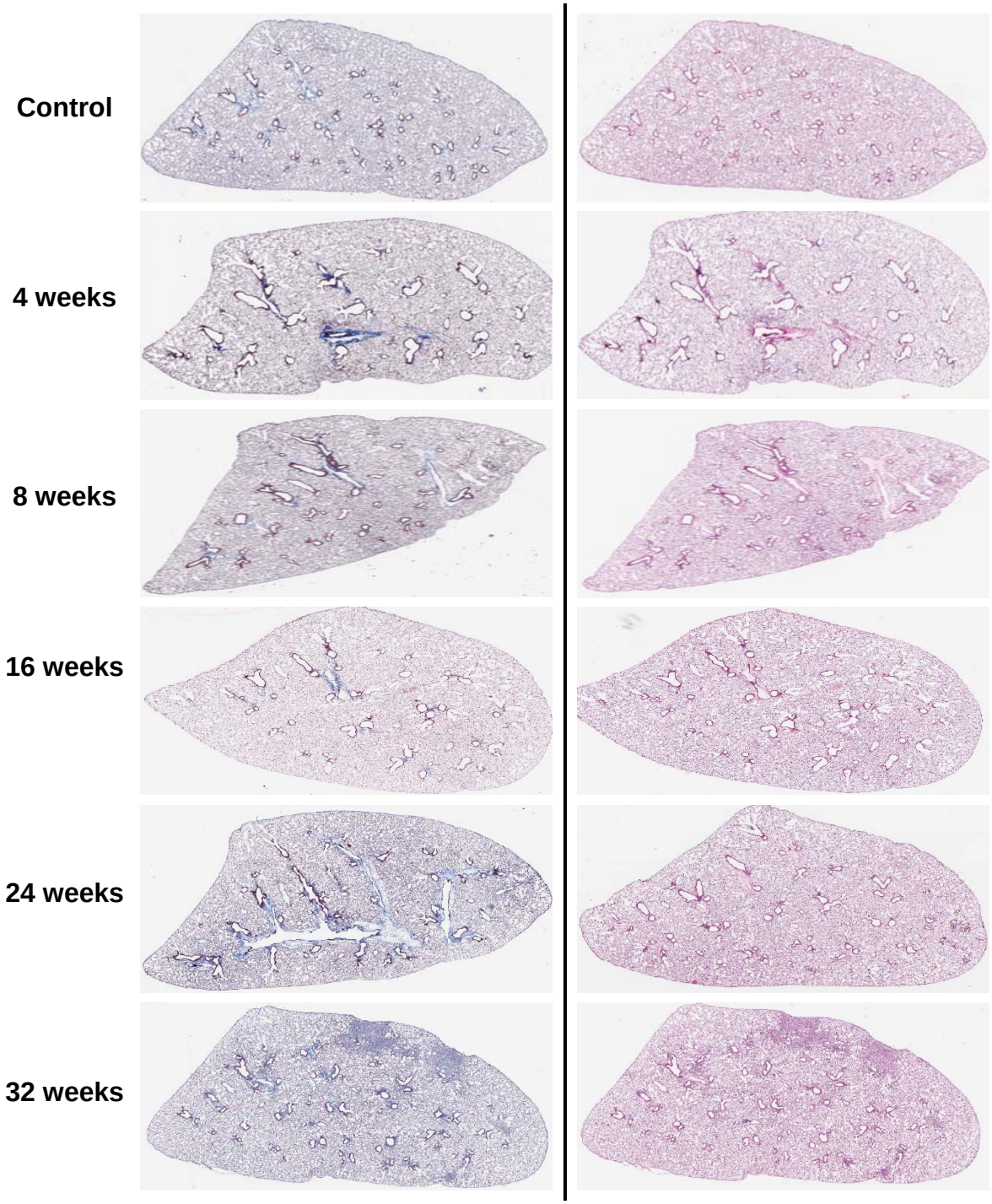
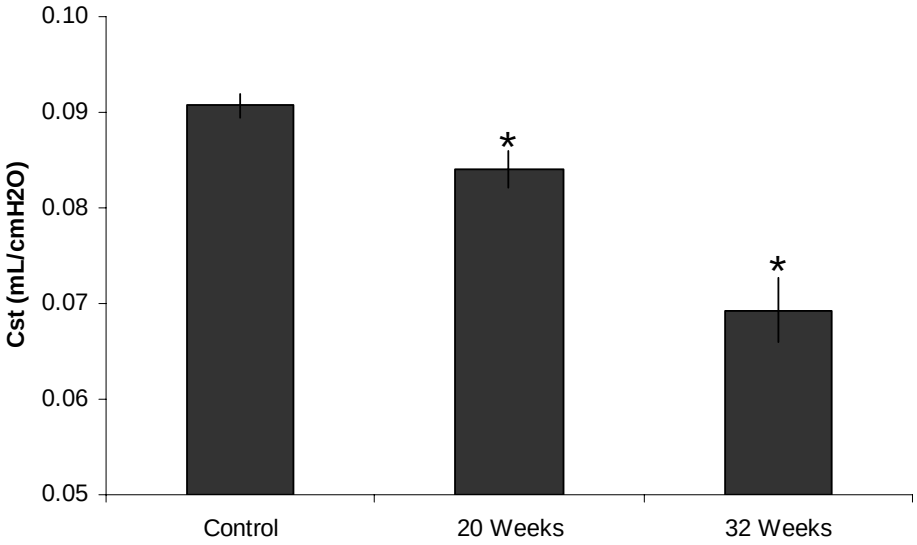


Figure 2.4 Changes in lung histology after irradiation in WT mice. Lungs were harvested at 0, 4, 8, 16, and 24 and 32 weeks post-irradiation and inflated with 10% formalin via a tracheal cannula, then removed from the thoracic cavity, and fixed overnight in formalin. Lung sections were stained with hematoxylin-eosin for routine histology (right panel) and Masson's trichrome for evaluation of collagen deposition (left panel).

A.



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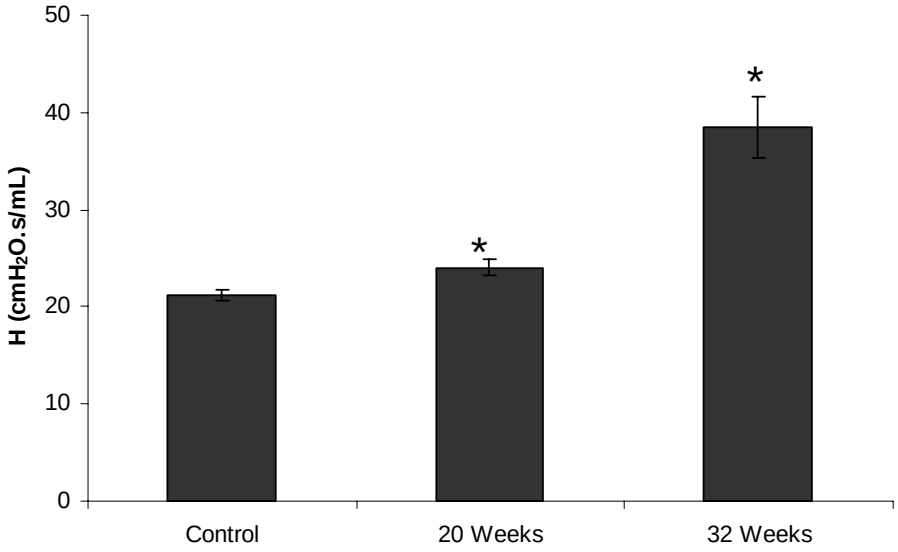


Figure 2.5 Analysis of lung mechanics demonstrates that irradiated WT mice had diminished lung function after thoracic radiation. Lung mechanics were measured by FlexiVent in anesthetized, paralyzed, and mechanically ventilated mice at 20 and 32 weeks post thoracic irradiation. **A.** Static compliance (Cst) was determined by fitting the Salazar-Knowles equation to pressure-volume curves. **B.** Tissue Elastance (H) was determined by applying prime wave impedance values to the constant phase model. Values shown are mean±SEM of 6-9 mice per group. *$p < 0.05$, compared with non-irradiated controls.

7. Increased TGF-beta1 in lung after Thoracic Irradiation

TGF-β1 protein increased in the lungs of the irradiated female WT mice at 16, 24 and 28 weeks post-irradiation (**Figure 2.6**). This upregulation of TGF-β1 preceded the fibrosis, suggesting a pathogenic role in radiation fibrosis, similar to that seen in other fibrosis models [40, 163].

8. Flow cytometric analyses of lung inflammatory infiltration

To better understand the cellular mediators involved in radiation lung injury, we analyzed the pulmonary inflammatory cell infiltrates in irradiated female mice over time. The data shown represents the average cell numbers in the right lung. As **Figure 2.7** shows, the lung $CD45^+$ leukocytes decreased within the first week after irradiation, and gradually returned to the basal level by 4 weeks, likely reflecting the initial apoptosis induced in resident cells by radiation. By 12 weeks, inflammatory cell infiltrates increased, peaking at 24-28 weeks. $CD4^+$ and $CD8^+$ T cells returned to the basal level at 8 weeks and remained elevated above the basal levels, thereafter consistent with expansion on ongoing recruitment. Analyses of $F4/80^+$ macrophages showed increases at 4 to 16 weeks, with a drop at 20 weeks, and then dramatically increasing again at 24 weeks. Interestingly, both the early and late peaks in pulmonary macrophages preceded the peaks in TGF-β1 levels (**Figure 2.6**&**2.7D**), suggesting that the macrophage may be important source of this profibrotic cytokine, as has been reported by others [37, 40, 163]. Interestingly, neutrophilic inflammation, clearly a hallmark of many acute lung injury models, does not appear be a significant factor in this model (**Figure 2.7E**). These data show that inflammatory cell infiltration begins at 4 weeks after XRT and has second higher and more prolonged peak beginning at 24-28 weeks. As we know from the histologic analyses, radiation-induced fibrosis becomes overtly apparent after

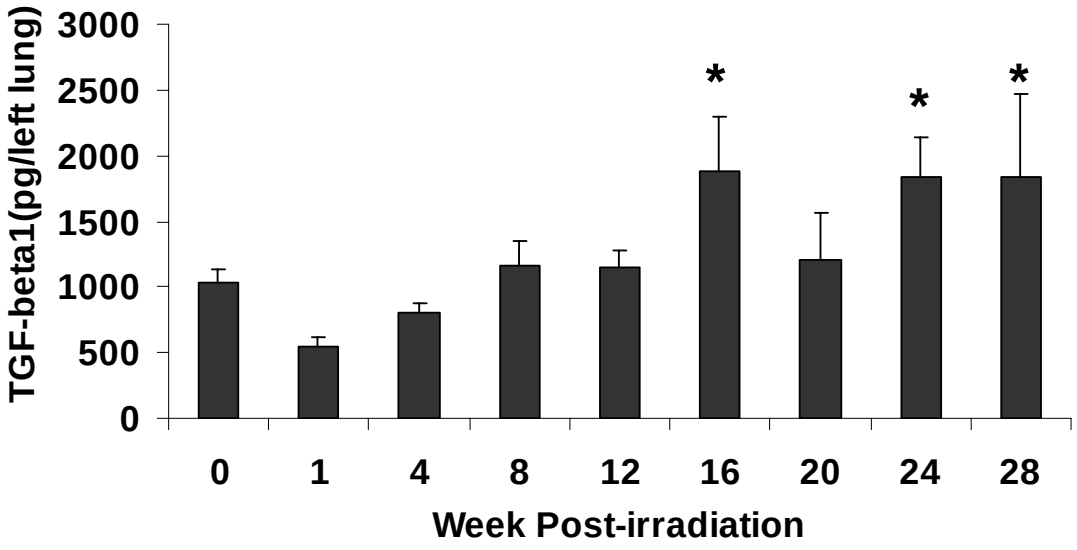


Figure 2.6 Increased levels of total TGF-β protein in the lungs of irradiated female WT mice. TGF-β in left lungs was measured by ELISA (Promega) after 14.5 Gy of thoracic irradiation. Each bar represents 6-13 animals at each time point. * $p < 0.05$ compared to the non-irradiated control mice.

Figure 2.7A

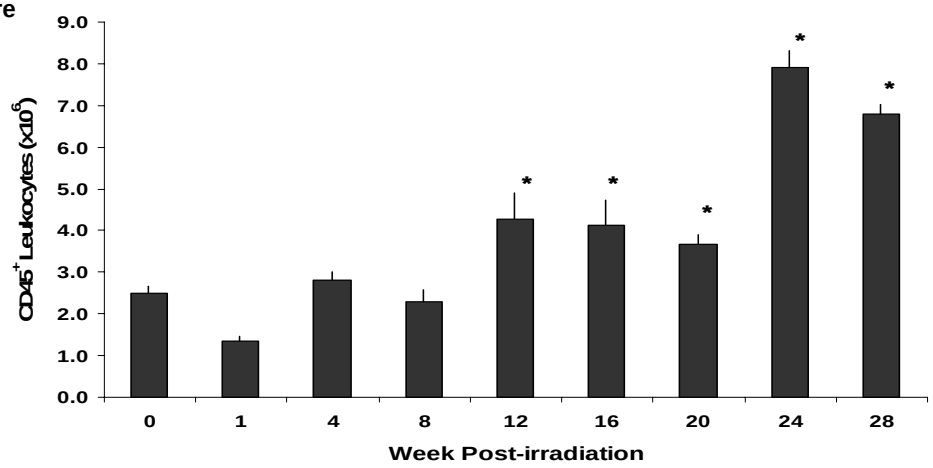


Figure 2.7B

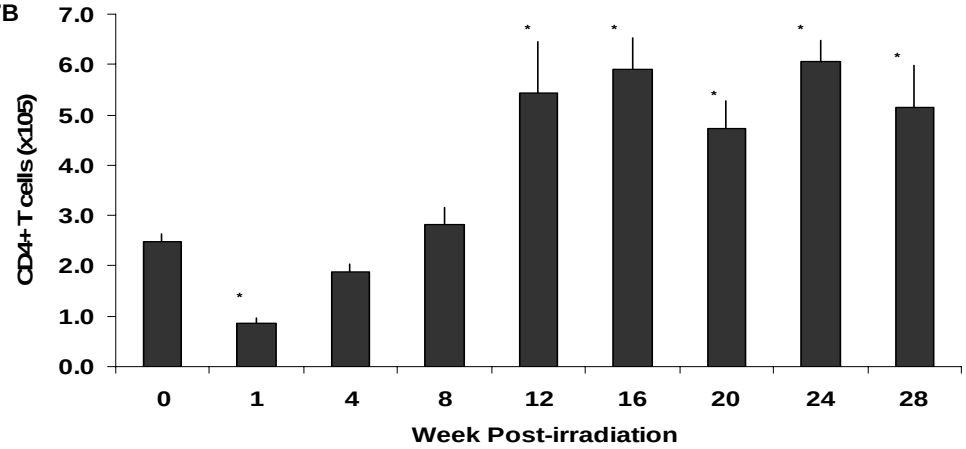
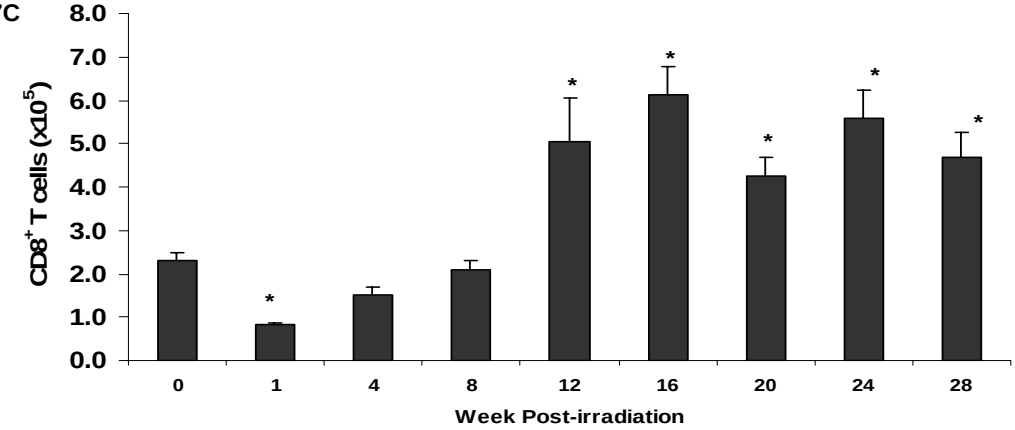


Figure 2.7C



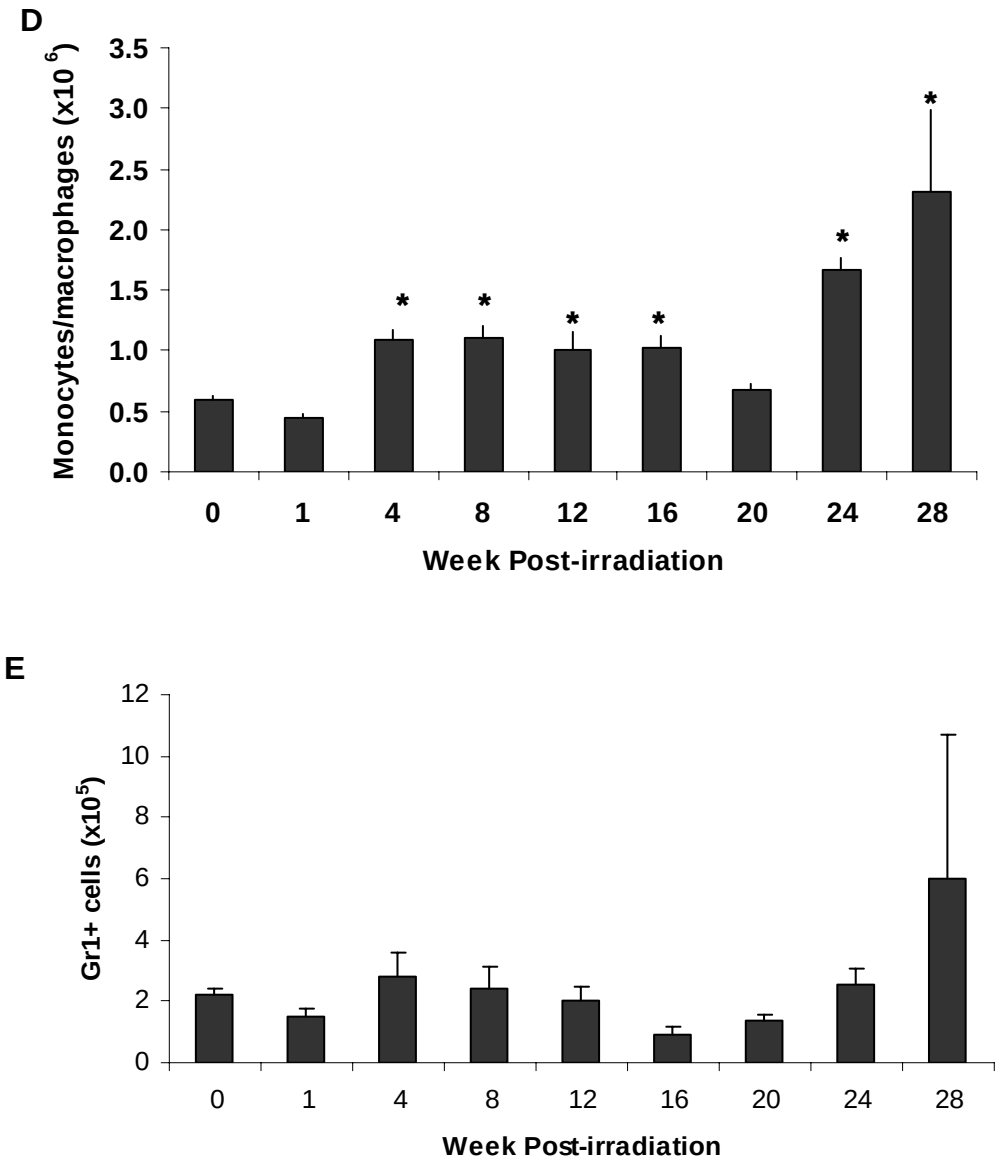


Figure 2.7 Analyses of the inflammatory cells in the lungs of female WT mice after thoracic irradiation. **A**. total leukocytes (CD45+); **B**. CD4+ T cells; **C**. CD8+ T cells; **D**. macrophage cells (F4/80+) ; **E**. neutrophils * $P < 0.05$, compared to the non-irradiated control mice.

28 weeks post-irradiation. Therefore, in our model we divide radiation-induced lung injury into two phases: pneumonitis (4-20 weeks) and fibrosis (>20weeks).

Summary of our model of radiation-induced lung injury

- Pneumonitis starts around 4-12 weeks actually 4-12 based.
- Marked infiltration of leukocytes, $CD4^+$ T cells, $CD8^+$ T cells and macrophages.
- Fibrosis becomes histologicaly apparent at 24-32 weeks with patchy foci of inflammatory cells and collagen deposition.
- Increased alveolar septal wall thickness; coincident with increased lung TGF-β protein.
- Decreased lung compliance and increased tissue elastance.

III. Macrophage inflammatory protein-1 alpha (MIP-1α/CCL3) is a mediator of radiation-induced lung injury

III.A. Abstract

Thoracic radiation is a common treatment for several types of cancer. Although patients often benefit from this treatment, it can also lead to pneumonitis and pulmonary fibrosis. Since MIP-1α (CCL3) functions in several types of pathogen-associated pneumonitis [138, 139], we reasoned that the cellular and molecular events linking thoracic radiation to pulmonary fibrosis might also involve CCL3. Thus, we used our model of a single dose thoracic radiation to study the role of CCL3 as a mediator of radiation lung injury.

Initially, we determined that CCL3 expression increased from baseline levels approximately 4 weeks after irradiation, declined to baseline levels by 8 weeks, and increased again at 20 weeks post-irradiation. This late increase in CCL3 expression coincided with a sharp increase in TGF-β1 expression, and preceded the death of these C57BL/6J mice from their radiation-induced injuries. Compared to wild type C57BL/6J mice, congenic, aged-matched CCL3-deficient mice had longer mean survival times, diminished inflammation, less collagen deposition and preserved lung function. In addition, CCL3-deficient mice did not display the increased TGF-β1 expression seen in C57BL/6J mice immediately prior to the onset of fibrosis. Taken together, these data demonstrate that CCL3 functions in the pneumonitis, TGF-β production and pulmonary fibrosis caused by thoracic radiation.

III.B. Introduction

Cell recruitment to injured tissue is an essential component of inflammation, which is largely orchestrated by a family of chemotactic cytokines known as chemokines [164, 165]. The chemokine CCL3 is secreted from various leukocytes, including T lymphocytes and activated macrophages, and acts to recruit CCR1-expressing and/or CCR5-expressing leukocytes, e.g., monocytes, dendritic cells, natural killer cells and T lymphocytes [140]. CCL3 can mediate pulmonary inflammation in response to a variety of pathogens, including influenza virus [166], *Cryptococcus neoformans* [167], *Apergillus fumigatus* [168], *schistosome mansoni* egg [147] and graft versus host disease (GVHD) [169]. Johntson reported a strong correlation between elevated CCL3 and development of late effects in radiation-induced fibrosis-sensitive mice [148]. Together, these observations suggest that CCL3 might be an important mediator in radiation-induced pneumonitis and fibrosis. Indeed, we show here that CCL3 expression increases in the lungs of irradiated mice, and that mice lacking this chemokine are protected from the marked pulmonary inflammation, fibrosis, and high mortality seen in irradiated wild type mice.

III.C. Materials and Methods

1. Cell Cultures

MRC5 lung fibroblast and SKLu-1 lung adenocarcinoma cell lines were irradiated with a single dose of 5 Gy. Culture supernatants were collected twelve hours later for CCL3 protein quantitation by ELISA (Quantikine^{TM}, R&D, Minneapolis, MN).

2. Animals

All animal studies were performed under approval by the IACUC at the University of North Carolina at Chapel Hill. Homozygous mice (C57BL6-Scya3^{tm1unc}), deficient in the

production of the chemokine CCL3, were generated by gene targeting [166]. Wild-type C57BL6 mice were purchased from Jackson Laboratories. All mice were housed in microisolator cages maintained in a specific pathogen-free environment. Lungs were harvested from mice after sedation with 2.5% Avertin, injection with 100 units of heparin to prevent clotting, and perfusion with 10 ml of PBS. Animals were then euthanized and lungs were harvested at various time points up to 32 weeks post-irradiation.

3. Irradiation

Anesthetized mice, 8-14 weeks of the age, were immobilized with a lead shield that protected the mouse body except for the thoracic cavity. Animals were irradiated with a dose of 14.5 Gy from a cesium source ((J. L. Shepherd & Associates, San Fernando, CA, USA) at a dose rate of 1.65 Gy/min.

4. ELISA analyses of TGF-β1

Quantitative ELISA measurements of CCL3 and active TGF-β1 protein levels from lung homogenates were determined using the Quantikine™ CCL3 and the Promega TGF-β1 Emax® Immunoassay systems according to the company protocols.

5. Quantification of Lung Hydroxyproline

After harvesting, lung tissue was acid digested and then hydroxyproline content was analyzed as described [170].

6. Flow Cytometry

Flow cytometric analyses of lung leukocytes were performed as described in Chapter II, using PE- or FITC-labeled monoclonal antibodies to murine CD3, CD4, CD8, CD19, B220, F4/80, and Gr-1(Pharmingen) and then analyzed on a FACScan with Cytomation software.

7. Histologic Analyses

Mice were sacrificed for histologic analyses at various times post-irradiation. After euthanasia, lungs were expanded and fixed by intra-tracheal instillation of 4% paraformaldehyde. Histologic sections were stained with hematoxylin and eosin for inflammation or Masson's Trichrome stains for interstitial and alveolar collagen. Lung sections were scored on a scale of 0-4+ for pneumonitis and fibrosis by a pathologist blinded to the sample genotype.

8. Antinuclear Antibody Assay

Serum specimens were prepared from control non-irradiated mice and irradiated mice at 32 weeks post-irradiation. Antinuclear antibody assays were performed according to the manufacturer's protocol (Alpha Diagnostic International Inc., San Antonio, TX).

9. Statistical Analyses

Differences between WT and $CCL3^{-/-}$ animals were examined for statistical significance by student's t-tests or by Mann-Whitney tests using SigmaStat software.

III.D. Results

1. Irradiation induces CCL3 expression in lung-derived cells in vitro

To determine whether CCL3 expression can be directly induced by radiation, we irradiated two lung-derived cell lines, MRC5 normal lung fibroblasts and SKLu-1 lung adenocarcinoma cells, with 5 Gy. Although not detectable (<1.5 pg/ml) prior to irradiation, CCL3 production increased significantly in both the MRC5 (5.22 pg/ml) and SKLu-1 (8.12 pg/ml) cell lines after irradiation (**Table 3.1**). The induction of CCL3 production in both normal and malignant lung-derived cell lines suggests that this chemokine might also be induced in the lungs of irradiated mice.

2. CCL3 was induced in the lungs of irradiated mice

We next investigated whether CCL3 was induced in vivo by thoracic irradiation. C57BL/6J mice were irradiated (14.5 Gy) and euthanized at various time points thereafter for analysis of CCL3 production in the lung (**Figure 3.1**). Surprisingly, CCL3 levels declined during the first few days post-irradiation (possibly due to the death of chemokine-producing cells) and by day 5 CCL3 levels was significantly lower (about 4-fold) than pre-irradiation levels. By two weeks CCL3 levels began recovering to pre-irradiation levels, followed by a peak at four weeks post-irradiation (2-fold increase). CCL3 levels then decreased over the 8-20 week period, except for the second peak at 20 wks. This biphasic pattern correlates well with the biphasic infiltration of inflammatory cells we noted over this time period (**Figure 2.7A**).

3. CCL3 -/- mice have improved survival after receiving thoracic radiation

We next examined the relevance of CCL3 expression to radiation-induced lung injury by comparing the responses of irradiated wild type and CCL3-deficient mice. WT and CCL3-deficient mice received a single dose (14.5 Gy) of thoracic irradiation. Approximately 52% of the WT mice receiving 14.5 Gy died by 32 weeks post-irradiation (**Figure 3.2**). In marked contrast, only 16% of the CCL3-deficient mice died following this dose of irradiation. These dramatic differences demonstrate that CCL3 functions in events leading to thoracic radiation-induced premature death.

4. CCL3-deficient mice have decreased fibrosis after lung irradiation

To determine whether the difference in mortality between WT and CCL3-deficient animals was related to fibrosis, mice were sacrificed at 32 weeks post-irradiation and their lungs were examined for fibrosis. The amount of collagen in the right lung was determined

Table 3.1 CCL3 Expression from Lung Cell Lines*****

Cell Line	Control	5 Gy Irradiation	TNF-α (+ control)
MRC5 Lung Fibroblast	**< 1.5 pg/ml**	**5.2 pg/ml**	**73.9 pg/ml**
SkLu Lung	**< 1.5 pg/ml**	**8.1 pg/ml**	**133.8 pg/ml**

*****Confluent monolayer of MRC5 normal lung fibroblasts and SkLu lung adenocarcinoma cells were irradiated with 5 Gy. Twelve hours later, cell supernatants were harvested and assayed for CCL3 content by ELISA.

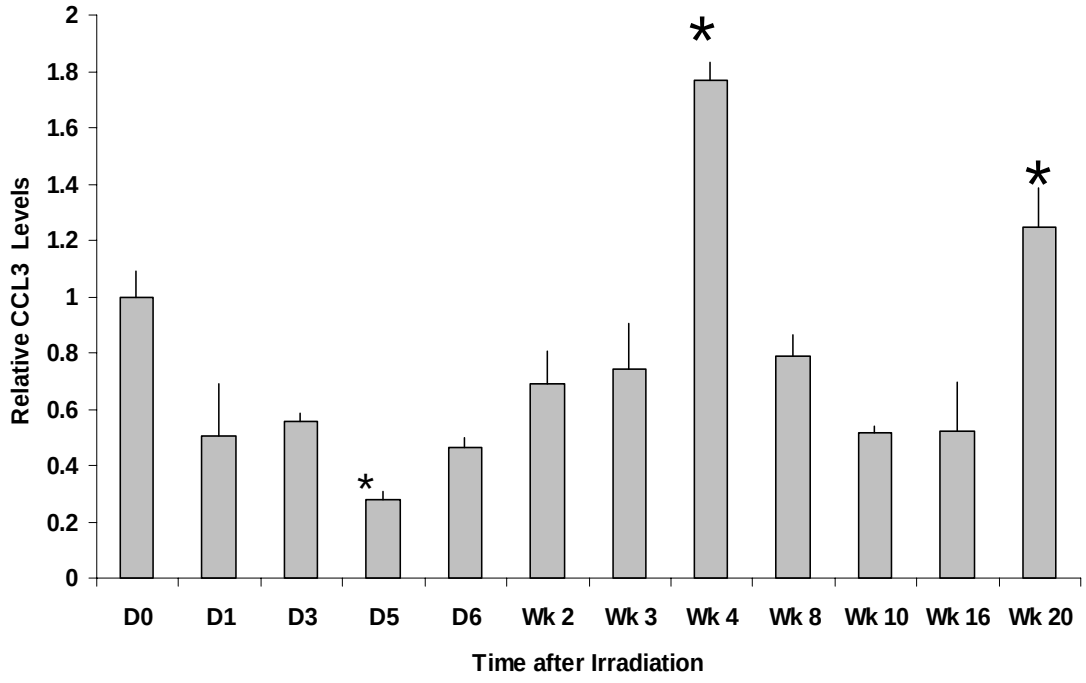


Figure 3.1 Thoracic irradiation-induced production of CCL3 *in vivo*. Wild-type mouse thoracic cavities were irradiated with 14.5 Gy. At the indicated times after irradiation, whole lungs were harvested for analysis of CCL3 content by ELISA. ***** p values were < 0.05 for the following time points compared to the sham controls: day 5, wk 4, and wk 20 (n = 3-6 mice per time point).

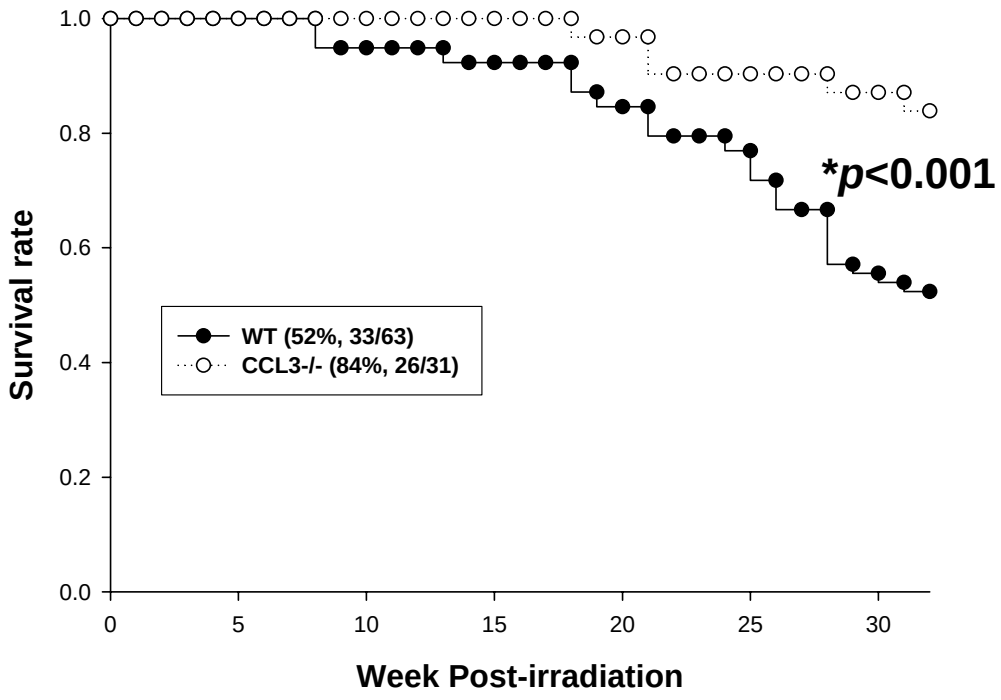


Figure 3.2 CCL3$^{-/-}$ mice have improved survival after thoracic radiation. WT and CCL3-deficient mice received 14.5 Gy thoracic irradiation and post-irradiation survival at 32 weeks was analyzed by Sigmaplot 8.0. *p<0.001, compared with irradiated WT mice.

by measurement of hydroxyproline content. As expected, there was a significant increase in lung hydroxyproline contents in WT mice after radiation (**Figure 3.3**). Lung hydroxyproline contents of irradiated CCL3-deficient mice were higher than in non-irradiated CCL3-deficient mice, but were significantly lower than irradiated WT mice. Hematoxylin and eosin and Masson's Trichrome staining of lung sections were used to examine lung sections for inflammation and fibrosis. As previously described for this model, marked alterations were observed in WT mice at 32 weeks following thoracic irradiation, i.e., inflammatory and fibrotic foci, increased septal cellularity and thickness, and increased collagen deposition (**Figure 3.4A**, **B** and **C**). In contrast, $CCL3^{-/-}$ mice have only rare tiny foci of inflammation or fibrosis.

5. $CCL3^{-/-}$ mice have decreased TGF-β1 production after thoracic irradiation

An association between TGF-β1 production and pulmonary fibrosis is well documented [171]. We therefore compared the levels of this protein in C57BL/6J and CCL3-deficient animals at various time points after 14.5 Gy of thoracic irradiation (**Figure 3.5**). It is notable that at 3 weeks post-irradiation, TGF-β1 protein levels were slightly increased to similar levels in both WT and CCL3-deficient animals, but returned to baseline levels shortly thereafter in WT mice and below baseline in CCL3-defficient mice. This data suggests that this early increase in TGF-β1 may be not crucial to the development of fibrosis in WT animals, although it did occur just prior to the initial influx of inflammatory cells. In contrast, at the time when fibrosis is beginning (> 16 wks), TGF-β1 levels progressively increase to above the baseline in the WT lungs, suggesting that late TGF-β1 production is a key mediator of the fibrosis phenotype seen in these animals. CCL3 has been reported to induce TGF-β production from macrophages which accumulated in the lungs of irradiated WT mice

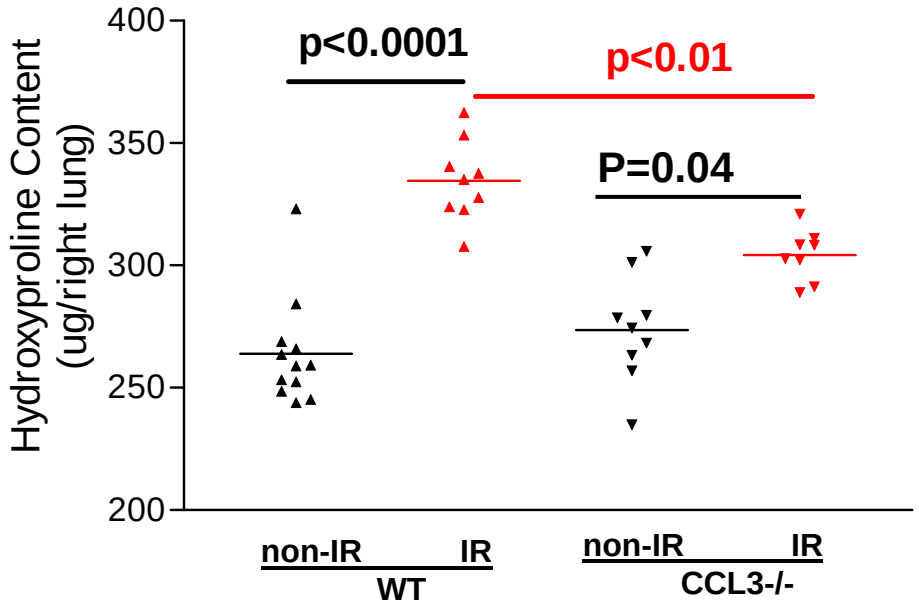


Figure 3.3 CCL3-deficient mice have much less lung fibrosis after thoracic irradiation. Right lungs from WT and CCL3-deficient mice were harvested 32 weeks after radiation and hydroxyproline assays were conducted. non-IR=non-irradiated control; IR=irradiated.

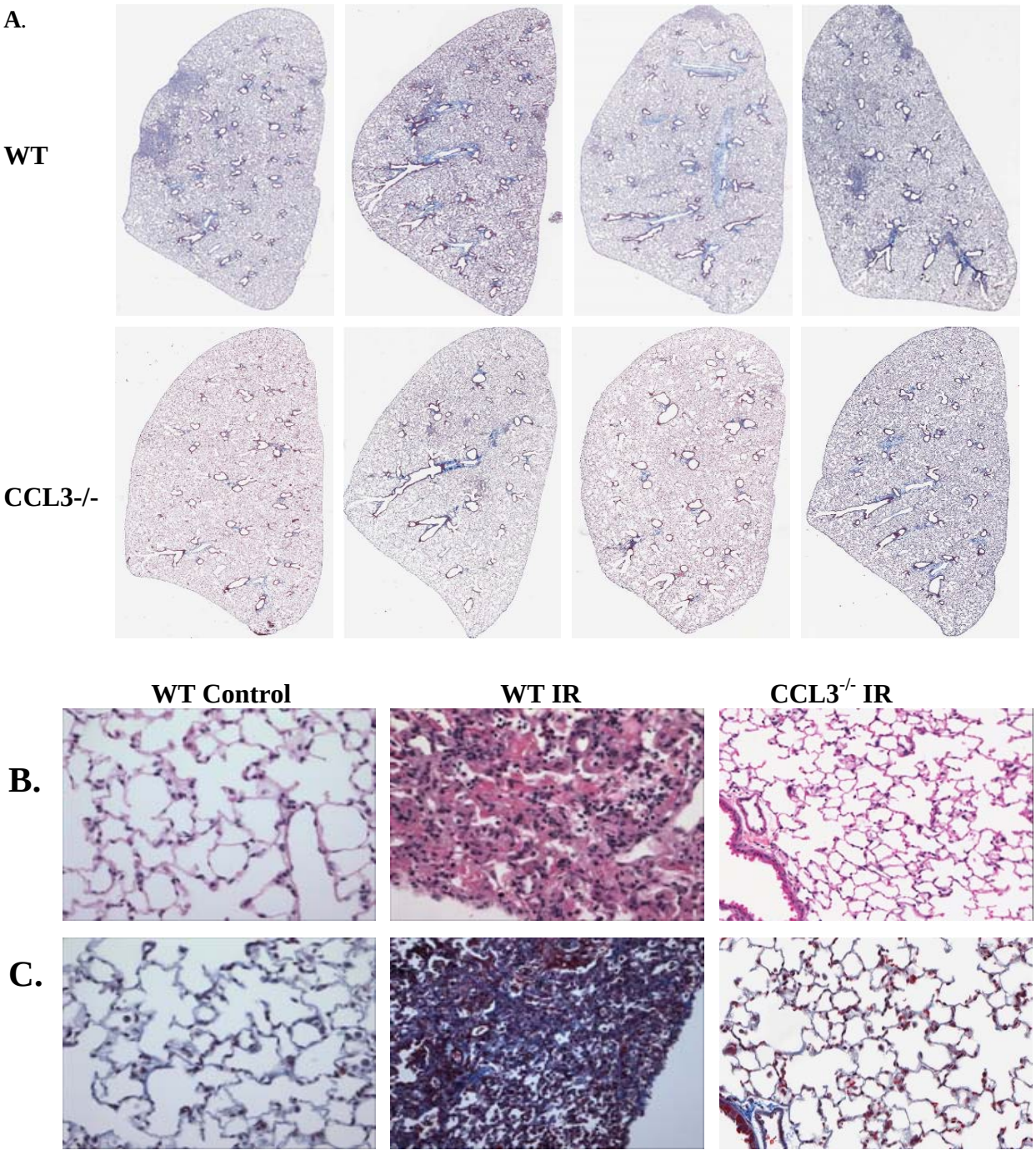


Figure 3.4 Histologic analyses showed that CCL3-deficient mice had less lung inflammation and fibrosis after irradiation. Lungs harvested 32 weeks post-irradiation and stained with Hematoxylin-eosin (H&E) or Masson's trichrome (MT). **A**. Whole sections of MT stained lungs. **B**. H&E stained section at 20x magnification. **C**. MT stained sections at 20x magnification. Inflammatory and fibrotic foci were seen in irradiated WT, but not in $CCL3^{-/-}$ mice.

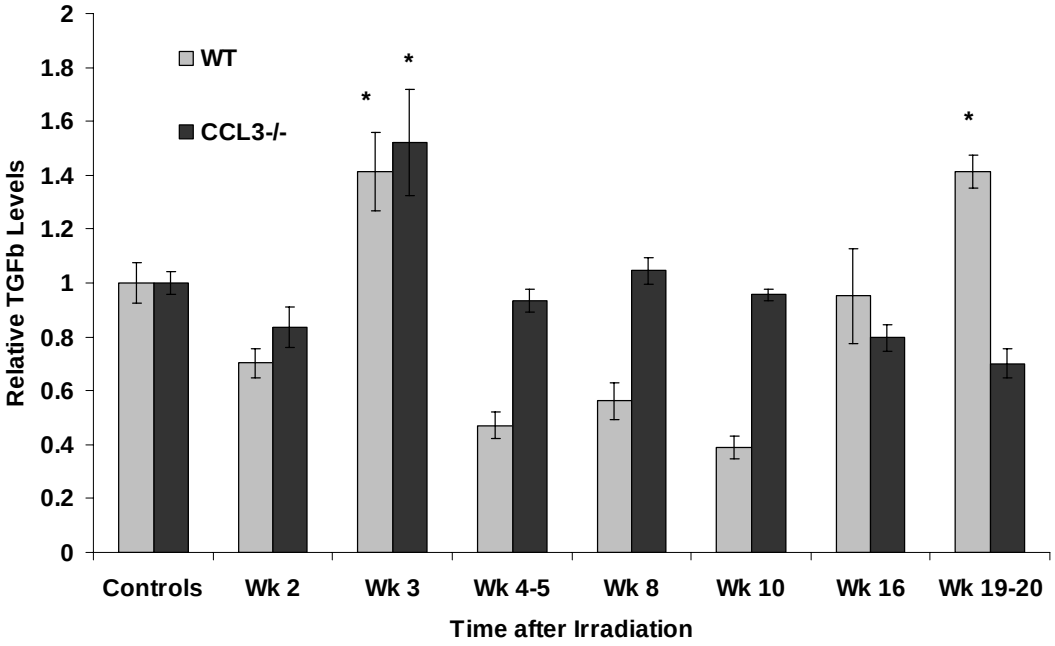


Figure 3.5 Quantitative analysis of lung totalTGF-*β1* protein by ELISA after irradiation. Each bar represents 3-5 animals of each genotype. Data from control (non-irradiated) mice of each genotype from various time points were pooled and are shown at day 0 (controls). * P values < 0.05 at wk 3 and wk 20 for the WT mice compared to the non-irradiated WT mice and at 3 wks for the CCL3-deficient mice compared to the non-irradiated CCL3-deficient mice.

during the fibrotic phase [172]. It probably explains why we observed low TGF-β1 levels at fibrotic phase in CCL3-deficient mice. In conclusion, TGF-β1 protein upregulation preceded the radiation-induced pulmonary fibrosis, suggesting a pathogenetic role in the development of radiation fibrosis.

6. *CCL3-deficient mice have decreased pneumonitis after lung irradiation*

CCL3 functions in several models of pulmonary inflammation [138, 139]. We therefore investigated whether the decreased mortality and collagen production seen in the irradiated CCL3-deficient mice was also associated with a decrease in radiation-induced pneumonitis. Mice were harvested up to 32 weeks post-irradiation and their lungs prepared and sectioned for histologic staining and scored in a blinded fashion for inflammation and fibrosis. Each section was assigned a score between 0 and 4, based on the extent of inflammation (**Table 3.2**). Irradiated CCL3-deficient mice have a statistically significant decrease in pneumonitis as well as fibrotic foci compared with irradiated WT mice. Thus, the decreased mortality and collagen production in CCL3-deficient mice is associated with the reduced inflammation.

7. Flow cytometric analyses of pulmonary inflammatory cell infiltration

To better understand the inflammatory events following thoracic irradiation, cells were prepared from the lungs of C57BL/6J and CCL3-deficient mice at various times post-irradiation and analyzed by flow cytometry. The prepared cells were stained with fluorescent-labeled antibodies against the cell surface markers CD3, CD4, CD8, CD19, B220, F4/80, and Gr-1.

The greatest differences between irradiated wild type and CCL3-deficient mice were the numbers of lung $CD4^+$ T cells and monocytes/macrophages (**Figure 3.6**). In both WT and CCL3-deficient mice, the number of $CD4^+$ T cells decreased during the first few weeks after

Table 3.2 Histologic analyses of lungs after irradiation

Genotype	Pneumonitis	Fibrosis
WT	**1.7 ± 0.33**	**++**
CCL3-/-	**0.27 ± 0.14***	**negligible**

* p = 0.001, compared to WT.

The extent of parenchymal inflammation was determined by averaging the scores (0 to 4, representing no, <25%, 25-50%, 50-75%, and >75% involvement) from 10 high power fields. Fibrosis was scored by examination of 10 high power fields for fibrotic foci as identified with Masson's1 Trichrome staining.

irradiation due to depletion of cells by radiation, and progressively increased to the basal level by 12 weeks. By 20 weeks, the number of lung $CD4^+$ T cells in irradiated WT mice was significantly greater than the basal level and remained elevated during the following period. In contrast, the number of lung $CD4^+$ T cells in irradiated CCL3-deficient mice was never higher than the basal level. Analyses of $F4/80^+$ macrophages showed a biphasic pattern but significant increases were only seen in irradiated WT mice at 12 and 32 weeks post-irradiation (**Figure 3.6D**). Consistently, we also saw numerous large foamy macrophages in histologic sections of WT lungs at 32 week post-irradiation, but not in CCL3-deficient mice (**Figure 3.6E**). The pattern of macrophage accumulation correlates with the production of TGF-β1 and indeed the second peak of macrophage infiltration occurs when the fibrosis phase starts.

Neutrophilic inflammation, clearly a hallmark of many acute lung injury models, does not appear to be a significant factor in this model since the patterns of neutrophil infiltration into the lung of irradiated WT and CCL3-deficient mice were similar (data not shown).

Taken together, these data suggest that macrophages and $CD4^+$ T lymphocytes may be the most important cellular mediators of radiation pneumonitis and fibrosis. Lastly, analyses of peripheral white blood cell counts did not reveal marked differences between C57BL/6J and CCL3-deficient mice, suggesting that CCL3 likely functioned locally in recruiting cells to the lung, rather than as a result of systemic inflammation.

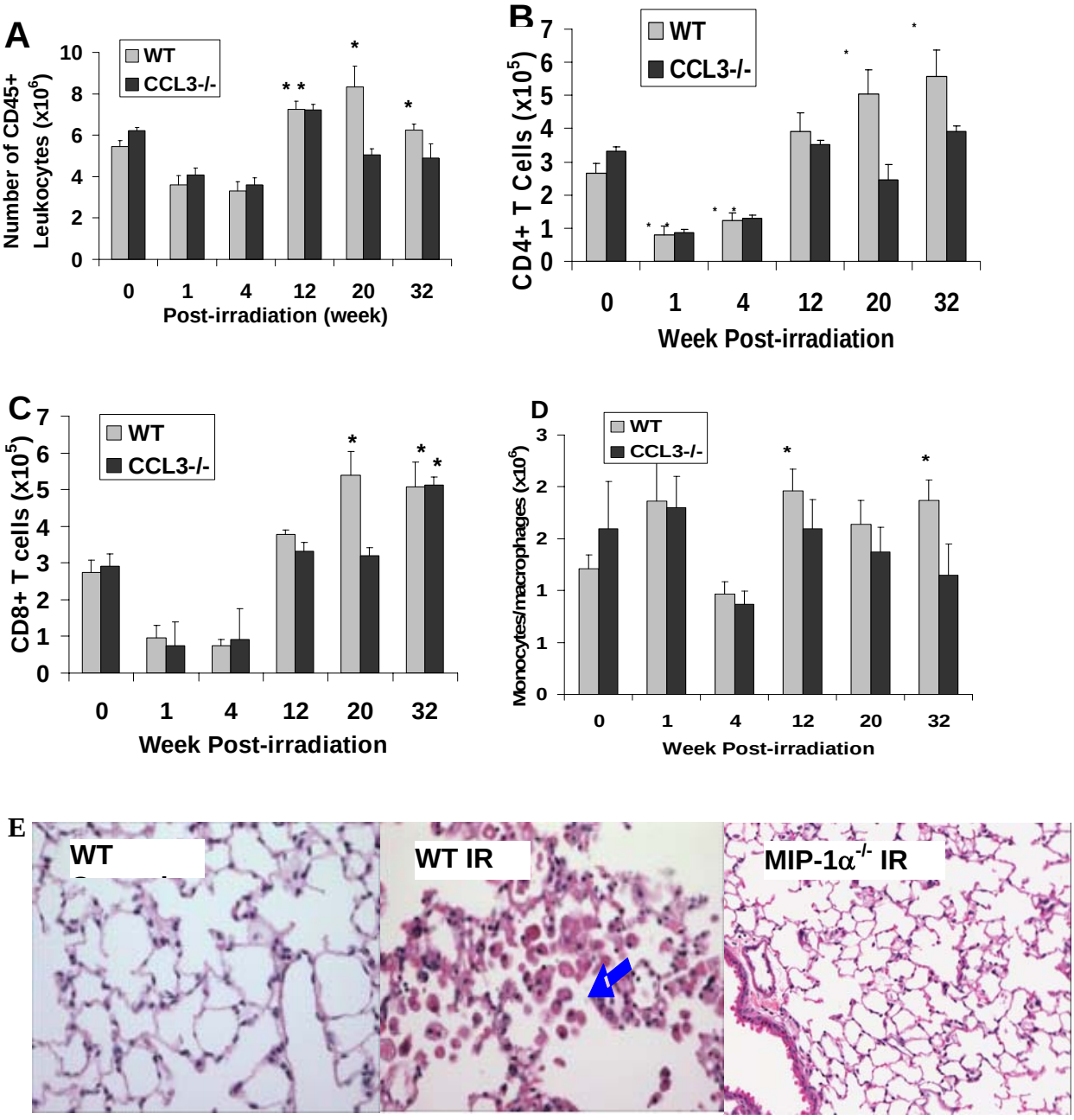


Figure 3.6 CCL3-deficient mice have fewer lung inflammatory cells after irradiation. The lungs were harvested and minced at 0,1,4,12,20 and 32 weeks post-irradiation. After enzymatic digestion, the cells were stained with FITC, PE, PerCP, or APC labeled monoclonal antibodies to murine CD45, CD3, CD4, CD8, and F4/80 (Pharmingen). **A**. $CD45^+$ leukocytes, **B**. $CD4^+$ T cells, **C**. $CD8^+$ T cells, **D**. Macrophage, and **E**. Arrow indicates increased foamy macrophages in H&E stained sections of irradiated WT mouse lungs, but not seen in nonirradiated WT and irradiated CCL3-deficient mice. Each bar represents 3-8 animals of each genotype.

8. Antinuclear autoantibody and B cell infiltration

We tested for the relevance of autoimmune phenomena in our model by analyzing serum levels of antinuclear autoantibodies (ANAs) by ELISA. Serum ANAs were elevated significantly at 32 weeks post-irradiation in WT mice, however, there was no change seen in irradiated CCL3-deficient mice (**Figure 3.7A**). In WT animals, there was also significant infiltration of B cells into lung at 12 and 20 weeks post-irradiation that, again, was not observed in CCL3-deficient mice (**Figure 3.7B**). Taken together, these data suggest that after the initial oxidative damage and cytokine induction, post-irradiation lung inflammation and fibrosis may occur at least in part as an autoimmune disease.

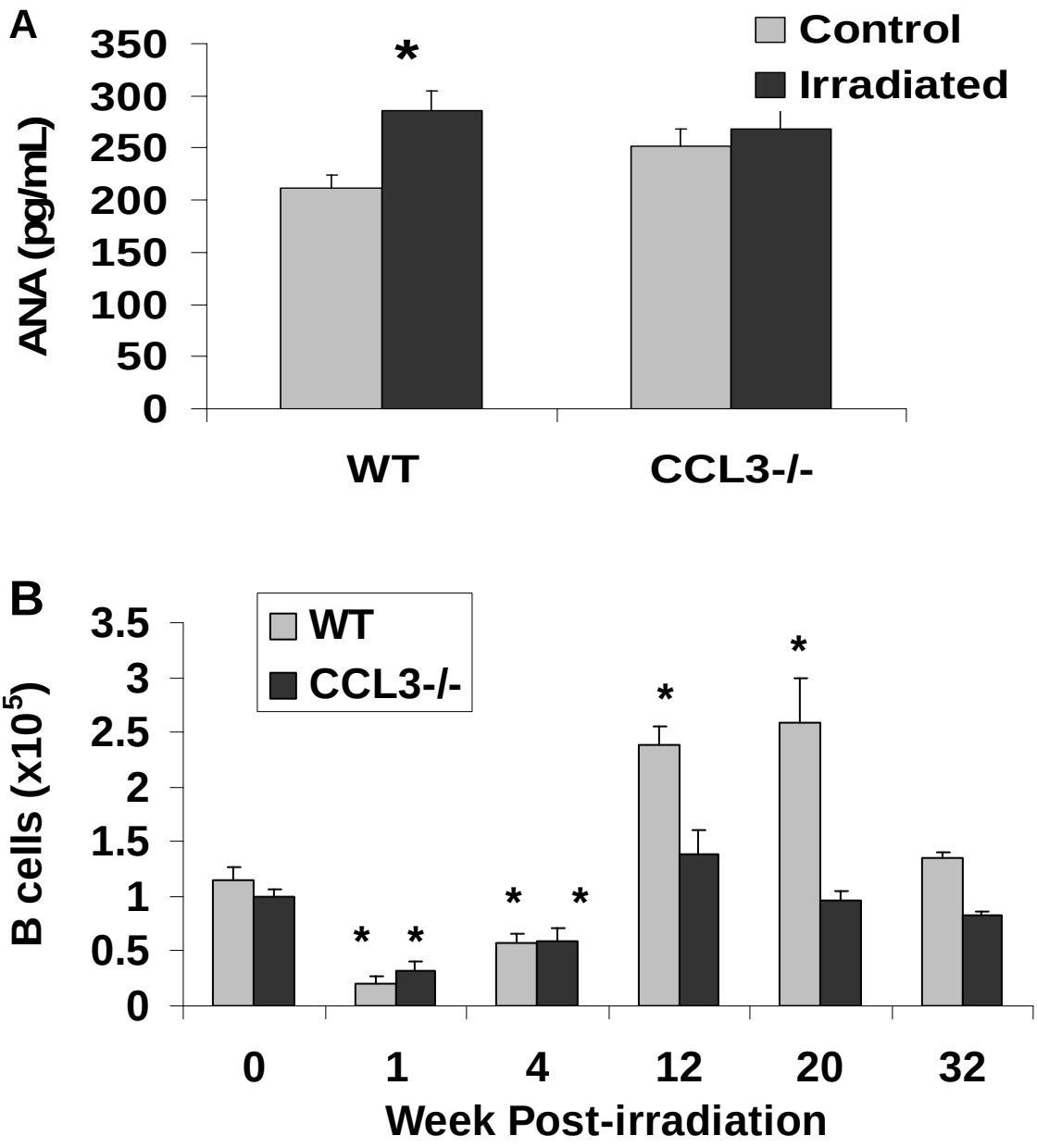


Figure 3.7 Evidence for autoimmune processes in radiation-induced lung injury. A. Serum antinuclear antibody measured by ELISA. **B**. B cell infiltration in lungs after irradiation. * $p < 0.05$, for irradiated mouse lung compared to nonirradiated control.

III.E. Discussion

The work reported here was begun to gain a better understanding of the cellular and molecular events leading from thoracic radiation to pulmonary fibrosis. We showed that thoracic radiation of mice induces the pulmonary expression of CCL3 and that mice lacking this chemokine have diminished inflammation, less collagen deposition and longer mean survival times than irradiated C57BL/6J mice. Our finding that radiation can also induce CCL3 production in cultured cell lines suggests chemokine production can be an immediate and locally residual response to radiation. In irradiated mice, pulmonary expression of CCL3 was highest at 4 weeks and 20 weeks post-irradiation, which precedes the influx of inflammatory cells and fibrotic stage. There was basically no increase over basal levels of macrophages, $CD4^+$ T cells, $CD8^+$ T cells and B cells in CCL3-deficient mice compared to WT mice, whereas WT mice showed significant increases in all of these types over basal levels beginning at 12 weeks post-irradiation. These findings suggest that CCL3 production is required for recruitment of macrophages, $CD4^+$ T cells, $CD8^+$ T cells and B cells into irradiated lungs.

In addition to the significant infiltrations of B cells in WT mice after thoracic irradiation, increased serum levels of antinuclear autoantibodies were seen in irradiated WT mice, but not in CCL3-deficient mice. These findings suggest autoimmunity may contribute to the development of radiation-induced lung injury.

Among the cytokines known to participate in fibrosis, TGF-β1 is probably the-best studied. TGF-β1 is upregulated in the lung parenchyma of patients with pulmonary fibrosis [173], increases the production of collagen and other extracellular matrix components [174-

176], and regulates the expression of other genes associated with ECM degradation and proteolysis. Indeed, a common feature among many models of pulmonary fibrosis is the upregulation of TGF-β1 expression in the lung. Our finding that the second increase in TGF-β1 expression requires CCL3, suggests that it either acts directly to induce production of TGF-β1 in resident lung cells, or recruits TGF-β1-expressing cells to the lung. One obvious candidate is the macrophage, which can be recruited by CCL3, and is among the wide variety of cells that can produce TGF-β1. Not surprisingly, the pattern of macrophage infiltration we saw in irradiated WT lung well correlates with the increased production of TGF-β1.

In summary, our results demonstrate that CCL3 production is induced by thoracic radiation, and that this production leads to enhanced pneumonitis, TGF-β1 production and ultimately, death of the irradiated mice from pulmonary fibrosis. Although additional studies are required to determine the impact of individual CCL3-dependent cellular and molecular events on fibrosis, our findings suggest that the CCL3 signaling pathways might lead to novel therapies to treat individuals at risk for radiation-induced pulmonary fibrosis.

IV. C-C chemokine receptor 1 mediates radiation-induced pulmonary injury

IV.A. Abstract

Radiation-induced lung injury is a common adverse effect in thoracic radiation-treated patients. We previously found that CCL3 deficiency significantly protects mice from developing radiation-induced pneumonitis and lung fibrosis. To determine which CCL3 receptor is crucial in mediating radiation-induced lung injury, C-C chemokine receptor 1 (CCR1)-deficient and C-C chemokine receptor 5 (CCR5)-deficient mice were irradiated with a single dose of 14.5 Gy to the thorax. Our present studies show that CCR1-deficient, but not CCR5-deficient, mice have radio-protected phenotype. In fact, the CCR5-deficient mice have worse pneumonitis and fibrosis than wild-type (WT) mice after thoracic radiation. In contrast, when compared to WT mice, irradiated CCR1-deficient mice have improved survival, virtually no pneumonitis or fibrosis, and preserved lung function. We also show that CCR1-deficient mice have less inflammatory infiltration after thoracic irradiation. Compared with irradiated WT mice, irradiated CCR1-deficient mice have lower levels of TGF-β1 protein and lower levels of mRNA expression of other profibrotic cytokines, IL-4 and IL-13. Furthermore, mice treated with CCR1 inhibitor (BX471) show similar protection from pulmonary fibrosis and preserved lung function after thoracic radiation. Therefore, the fact that genetically generated CCR1 deficiency and CCR1 blockade with a specific antagonist had the same effect on pulmonary inflammation and fibrosis after thoracic irradiation provides powerful evidence that CCR1 is a promising target for reducing radiation-induced injury.

III.B. Introduction

In spite of intensive research addressing radiation pneumopathy, there is no universally accepted reliable model. Compared with other lung fibrosis models such as bleomycin-induced fibrosis, radiation-induced lung fibrosis has some unique characteristics. Radiation-induced lung fibrosis has an insidious onset and is irreversible, which indicates that its pathogenesis is at least in part different from other lung fibrosis models. Therefore, evidence from other lung fibrosis models can not be simply extrapolated to radiation pneumopathy.

We have recently found that in comparison to WT mice, CCL3-deficient animals are significantly protected from developing radiation-induced lung pneumonitis and fibrosis. These findings suggest that chemokines and their receptors might represent excellent molecular therapeutic targets for reducing or eliminating radiation-induced pneumonitis and subsequent fibrosis. Here, we specifically studied the CCL3 receptors, CCR1 and CCR5, for their potential roles in development of radiation-induced lung injury. In this study, we demonstrated that CCR1, induced by thoracic irradiation, accompanied the recruitment and activation of lymphocytes and macrophages and the subsequent cytokine production in the development of radiation pneumonitis and fibrosis. Furthermore, we show that the CCR1 inhibitor, BX471, ameliorates the progressive pneumonitis and fibrosis induced by thoracic irradiation.

IV.C. Materials and Methods

1. Mice

All animal studies were performed under approval by the IACUC at the University of North Carolina at Chapel Hill. Wild-type C57BL6 mice were purchased from Jackson

Laboratories. CCR1$^{-/-}$ and CCR5$^{-/-}$ C57BL6 mice in were obtained from Dr. Nobuyo Maeda and bred according to DLAM policies. All mice were housed in microisolator cages maintained in a specific pathogen-free environment. All mice used in this study were male and age-matched.

2. Thoracic irradiation

Animals were irradiated with a singled dose of 14.5 Gy as described in chapter II.

3. Histopathology and morphometric analyses

After euthanasia, the lungs were inflated with 10% formalin via a tracheal cannula, removed from the thoracic cavity, fixed overnight in formalin and then paraffin embedded. Lung sections were stained with hematoxylin and eosin for routine histology and Masson's trichrome for evaluation of collagen deposition. Histologic data were quantitated by digital imaging of the hematoxylin and eosin stained sections. While blinded to genotype and treatment, we captured ten images (5x) of representative areas of each lung lobe and then analyzed each image for septal thickening due to increased inflammatory cell infiltration, extracellular matrix deposition, and fibroblasts proliferation. With the software ImageJ (National Institutes of Health), the threshold was set to count the number of pixels contained within areas of the digital images. The threshold number of pixels was then divided by the total number of pixels in the entire image and multiplied by 100 to generate a percentage of area affected by fibrosis and inflammation.

4. Hydroxyproline assay

Lung hydroxyproline content was measured as described in Chapter II.

5. Measurements of lung mechanics

Lung compliance and tissue elastance were evaluated as described Chapter II.

6. Flow Cytometry

Flow cytometric analyses of lung leukocytes were performed as described in Chapter II.

7. Gene expression analyses

Gene expression analyses of lung homogenates were performed as described in Chapter II, page 41.

8. ELISA Analyses

Quantitative ELISA measurement of TGF-β protein levels from lung extracts was performed using the Promega TGF-β1 Emax® Immunoassay systems according to the company protocols.

9. Administration of the CCR1 Inhibitor BX-471

BX-471 was dissolved in a 40% Cyclodextrin vehicle (Sigma). BX-471 treatment was initiated 4 weeks after irradiation based on our previous data showing that CCR1 gene expression started to increase at this time point. Irradiated WT mice received twice daily subcutaneous treatments with BX-471 for a total of 4 weeks at a dose of 30 mg/Kg of body weight. One group of irradiated mice was given BX-471 treatment for 4 weeks. A control group of irradiated mice were given vehicle only twice daily for 4 weeks. A second control group was the nonirradiated age-matched mice. Mice were closely monitored daily and were euthanized and analyzed at 32 weeks after irradiation.

10. Statistical Analyses

Two-tailed Student's t-tests were performed to compare results of hydroxyproline content, histological analysis, function test, flow cytometry analysis, and RT-PCR. A p value ≤ 0.05 was considered significant.

IV.D. Results

1. CCR1 and CCR5 expression in the lung after thoracic irradiation

To characterize the patterns of CCR1 and CCR5 expression in the lung after thoracic irradiation, WT mice were irradiated and their lungs were harvested at 1, 4, 12, 20 and 32 weeks following irradiation. CCR1 and CCR5 expression in the lung was analyzed by quantitative RT-PCR. CCR1 expression began to increase significantly at 4 weeks following irradiation, had a 5-

fold elevation at 20 weeks and remained elevated through 32 weeks (**Figure 4.1**). The expression of CCR5 was significantly increased at 12 weeks following irradiation and remained elevated through 32 weeks. It is noted that the increase in CCR1 expression at 4 and 20 weeks coincides very well with the increases in CCL3, suggesting that CCL3 may directly lead to CCR1-expressing cell accumulation.

2. CCR1-, but not CCR5-deficient, mice have a protected phenotype from radiation-induced lung injury

In order to determine whether CCR1-deficient or CCR5-deficient mice were protected from radiation-induced lung injury, these mice received thoracic irradiation at a single dose of 14.5 Gy and were monitored for up to 32 weeks. Premature death is defined as death before 32 weeks. In comparison with the WT mice, CCR1-deficient mice had a significantly improved survival rate (**Figure 4.2A**). In contrast, there was no difference in survival rate between WT and CCR5-deficient mice after radiation.

In order to assess lung fibrosis, the amount of collagen in the right lung was determined by measurement of hydroxyproline. As expected, there was a significant increase in the lung hydroxyproline content in the irradiated WT (**Figure 4.2B**); however, this increase was not

seen in irradiated CCR1$^{-/-}$ mice. In contrast, the lung hydroxyproline content of irradiated CCR5$^{-/-}$ mice was even higher (15%) than that seen in the irradiated WT mice. Hematoxylin and eosin and Masson's Trichrome staining of lung sections was used to assess lung inflammation and fibrosis. As previously described, we observed inflammatory and fibrotic foci, increased septal cellularity and thickness, inflammatory cell infiltration and increased collagen deposition in WT mice at 32 weeks post-irradiation (**Figure 4.3**). These histologic changes were even more profound in the irradiated CCR5$^{-/-}$ mice. In contrast, CCR1$^{-/-}$ mice had virtually no any foci of inflammation or fibrosis. The digital imaging analyses showing that when compared with non-irradiated controls, both the irradiated WT and CCR5$^{-/-}$, but not CCR1$^{-/-}$, mice had increased septal thickening is also consistent with the hydroxyproline data (**Figure 4.3D**).

3. CCR1-deficient mice have preserved lung function in response to thoracic irradiation

In order to evaluate alterations of lung mechanics in CCR1$^{-/-}$, and CCR5$^{-/-}$, we used a computer-controlled small-animal ventilator. Decreases in compliance and increases in elastance were expected in radiation-induced fibrotic lung. Consistent with this expectation, the irradiated WT mice showed a significant decrease in static compliance and a significant increase in tissue elastance compared with non-irradiated control mice (**Figure 4.4**). Similar changes in compliance and tissue elastance were observed in the irradiated CCR5$^{-/-}$ mice. However, the static compliance of irradiated CCR5$^{-/-}$ mice was significantly lower than that of the irradiated WT mice. Neither a decrease in static compliance nor an increase in tissue elastance was observed in the irradiated CCR1$^{-/-}$ mice. As expected, based on lung histology,

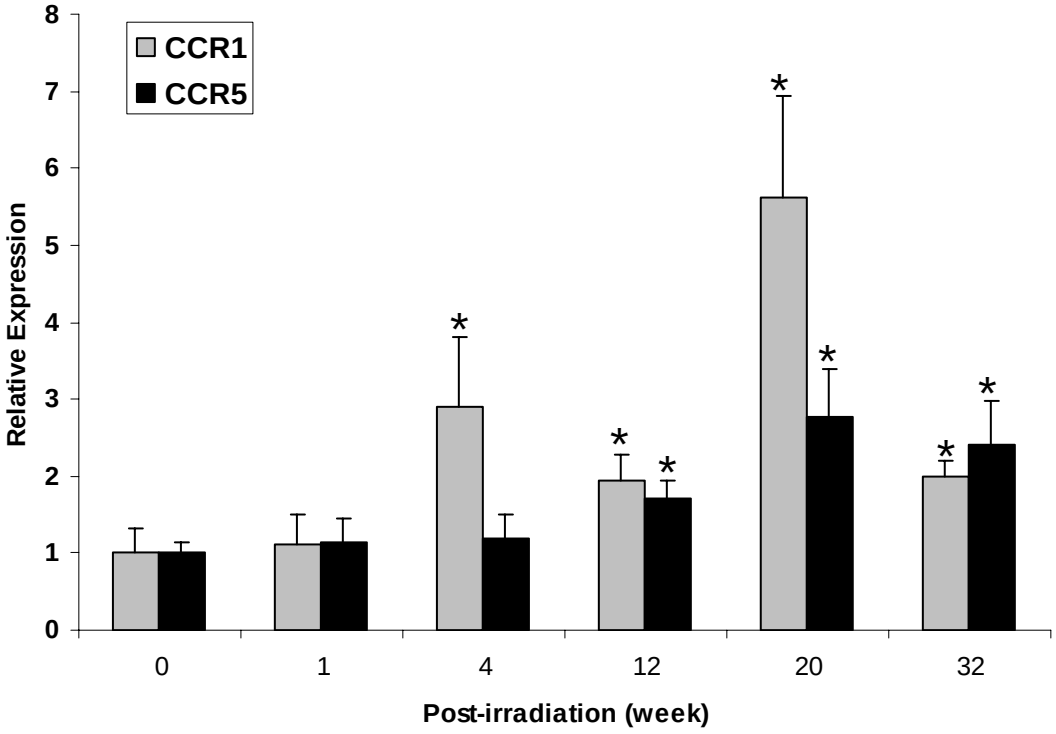


Figure 4.1 Increased CCR1 and CCR5 mRNA expression in the lung after thoracic irradiation. Lung total RNA from non-irradiated control and irradiated WT mice was analyzed for CCR1 and CCR5 mRNA expression by real-time RT-PCR. Levels of mRNA expression of CCR1 and CCR5 were expressed relative to Gusb mRNA expression as indicated in Materials and Methods. CCR1 and CCR5 mRNA expression normalized to that of the non-irradiated controls. Values shown represent means±SD from 5~9 mice per group. ***** $p < 0.05$, compared with WT non-irradiated control.

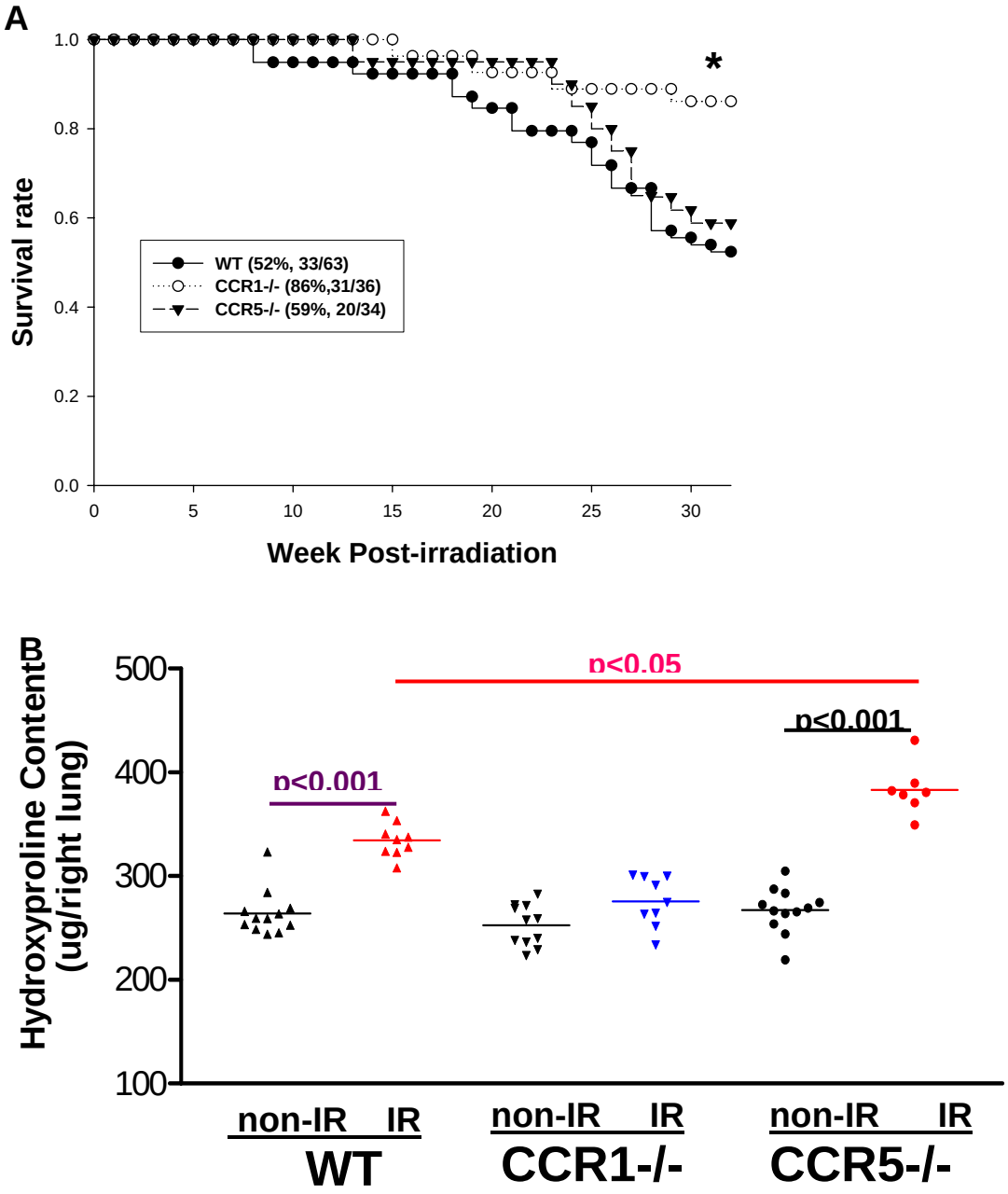


Figure 4.2 CCR1-, but not CCR5-deficient, mice have a radio-protected phenotype. **A.** Survival curves at 32 weeks post-irradiation by Sigmaplot 8.0 showed CCR1$^{-/-}$, but not CCR5$^{-/-}$ mice have better survival than irradiated WT mice. WT, CCR1-deficient, and CCR5-deficient mice received thoracic irradiation as a single dose of 14.5 Gy. * $p < 0.01$. **B.** Hydroxyproline content of right lungs of non-irradiated control and irradiated mice 32 weeks post-irradiation. nonIR, nonirradiated controls. IR, irradiated.

Figure4.3A

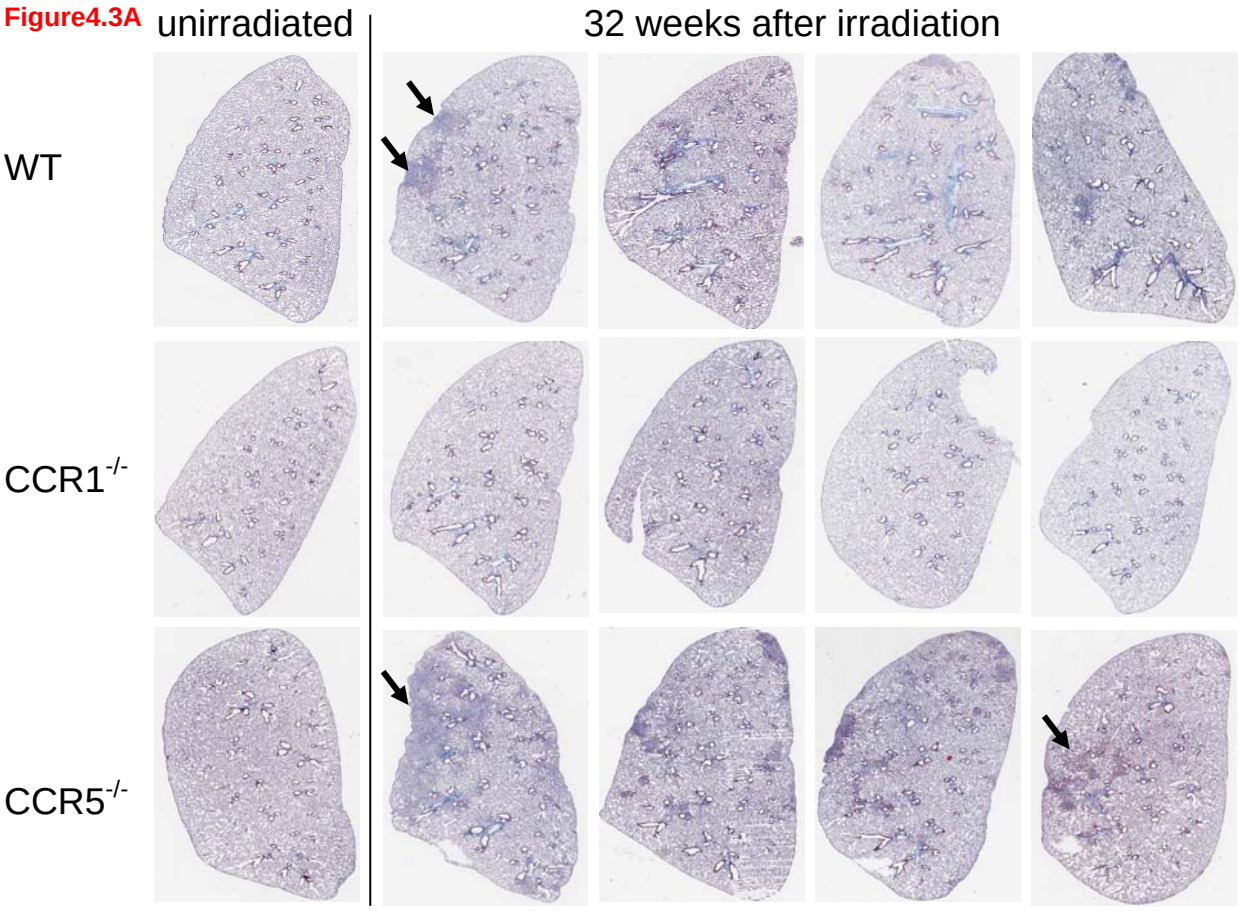


Figure 4.3B

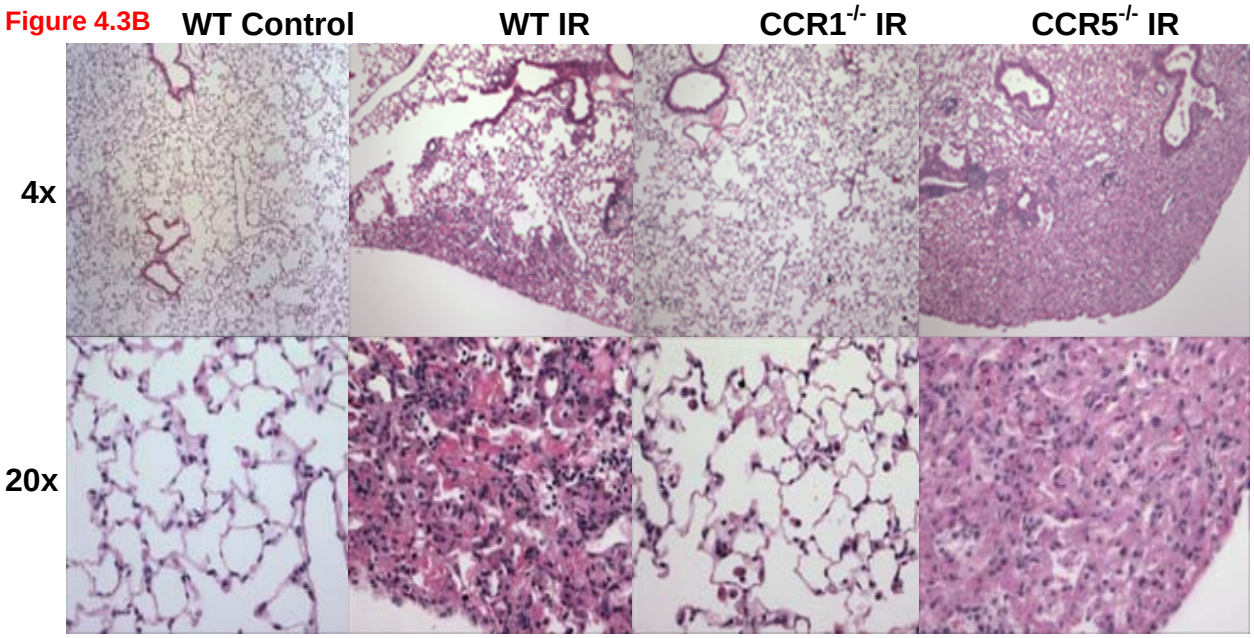
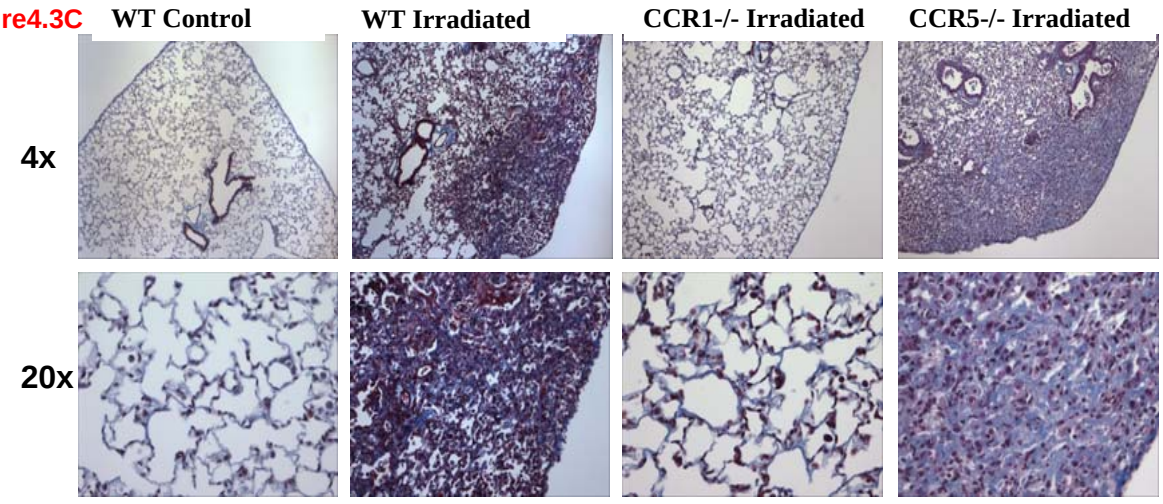


Figure4.3C



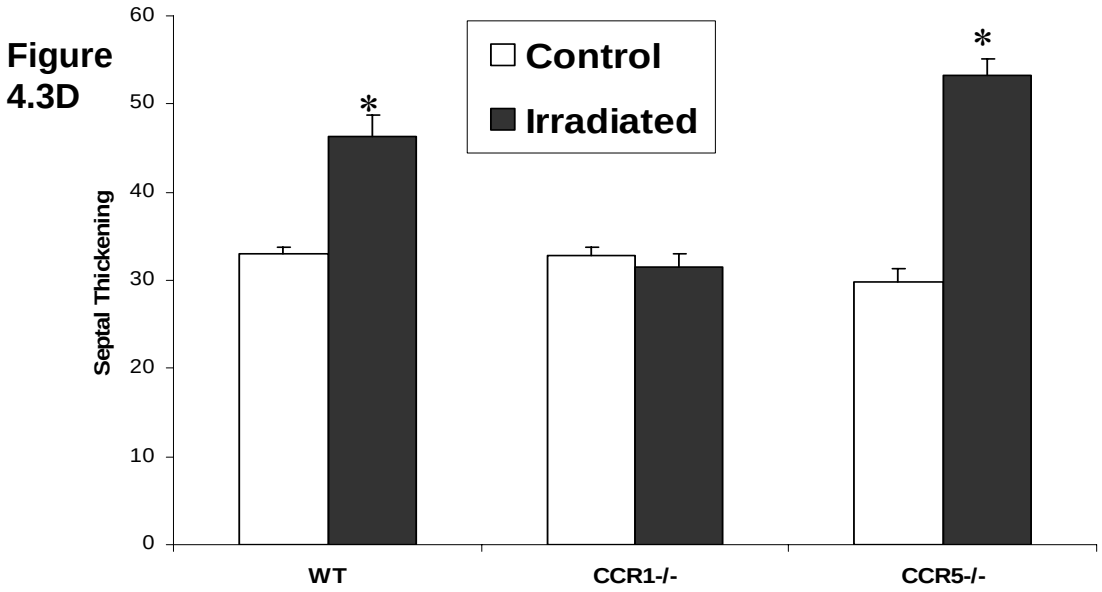


Figure 4.3 Histologic analyses show that CCR1-deficient, but not CCR5-deficient, mice have radio-protected phenotype. WT, CCR1-/- and CCR5 -/- mice received a single dose of 14.5 Gy thoracic irradiation. Lungs were harvested at 32 weeks post-irradiation and stained with Hematoxylin&eosin (H&E) or Masson's trichrome (MT). **A**. Whole sections of MT stained lungs: inflammatory and fibrotic foci in irradiated WT and $CCR5^{-/-}$ mice, but not in $CCR1^{-/-}$ mice; arrows indicate areas of inflammation and fibrosis. **B**. H&E staining: areas of inflammation and fibrosis are seen in the lungs of the irradiated WT and CCR5 -/- mice, but not CCR1 -/- mice. **C**. MT staining: Collagen deposition is seen in the lungs of irradiated WT and CCR5 -/- mice but not CCR1 -/- mice. **D**. Quantitative measurement of lung tissue abnormalities by morphometric analysis of lung sections. Ten 5x images of each lung section were analyzed to quantify septal thickening as an indication of lung inflammation and fibrosis. Values shown are means±SEM of 4-8 lung sections per group. ***** $p < 0.05$, compared with non-irradiated WT controls.

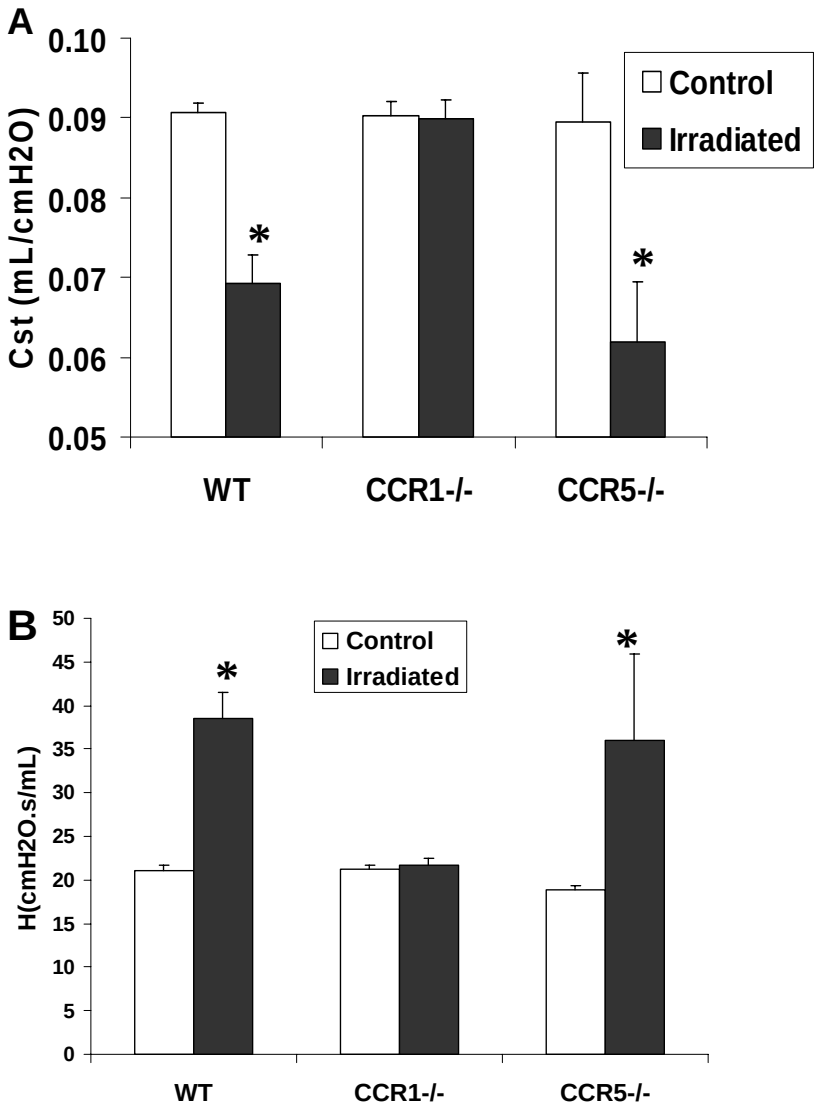


Figure 4.4 Analyses of lung mechanics demonstrate that CCR1 deficiency, but not CCR5 deficiency, confers protection from the decreased lung function seen after thoracic irradiation in WT mice. Lung mechanics were measured by FlexiVent in anesthetized, paralyzed, and mechanically ventilated mice at 32 weeks post thoracic irradiation. **A:** Static compliance (Cst) determined by fitting the Salazar-Knowles equation to pressure-volume curves. **B:** Tissue Elastance (H) determined by applying prime wave impedance values to the constant phase model. Values shown are the means±SEM of 6-9 mice per group. * $p < 0.05$, compared with non-irradiated controls of the same genotype.

these findings showed that CCR1$^{-/-}$ mice also have preserved lung function after thoracic irradiation.

4. CCR1-deficient mice have less inflammatory cell infiltration into the lung after thoracic irradiation

To better understand the inflammatory events following thoracic irradiation, subtypes of inflammatory cells were analyzed by flow cytometry. As demonstrated in **Figure 4.5A**, the CD45^{+} leukocytes in the irradiated WT lungs decreased within the first week after irradiation and gradually returned at 4 weeks post-irradiation. By 12 weeks post-irradiation, the total number of leukocytes was significantly higher than the basal level, remaining elevated during the pneumonitic phase (12-20 weeks). In contrast, the total lung leukocytes of irradiated CCR1$^{-/-}$ mice decreased at the first week post-irradiation and gradually returned to the basal level, but never surpassed the basal level. The changes of lung CD4^{+} lymphocytes and CD8^{+} lymphocytes after irradiation showed a similar pattern as total leukocytes (**Figure 4.5B&C**). Both lung CD4^{+} lymphocytes and CD8^{+} lymphocytes in the irradiated WT and CCR1$^{-/-}$ mice were significantly lower than the basal level at 1 and 4 weeks post-irradiation. In WT mice, both lung CD4^{+} T cells and CD8^{+} T cells increased at 12 weeks post-irradiation and remained elevated above the basal levels during the following period. At 12 weeks post-irradiation, both lung CD4^{+} T cells and CD8^{+} T cells in the irradiated CCR1$^{-/-}$ mice were close to the basal levels. CD4^{+} and CD8^{+} essentially never exceeded the basal levels except for CD4^{+} T cells at 20 weeks. The irradiated WT mice also had increased macrophages (F4/80^{+}) beginning at 12 and 32 weeks post-irradiation that was not observed in the irradiated CCR1$^{-/-}$ mice (**Figure 4.5D**). Consistently, we saw a number of large macrophages in histologic sections of WT mouse lungs at 32 weeks

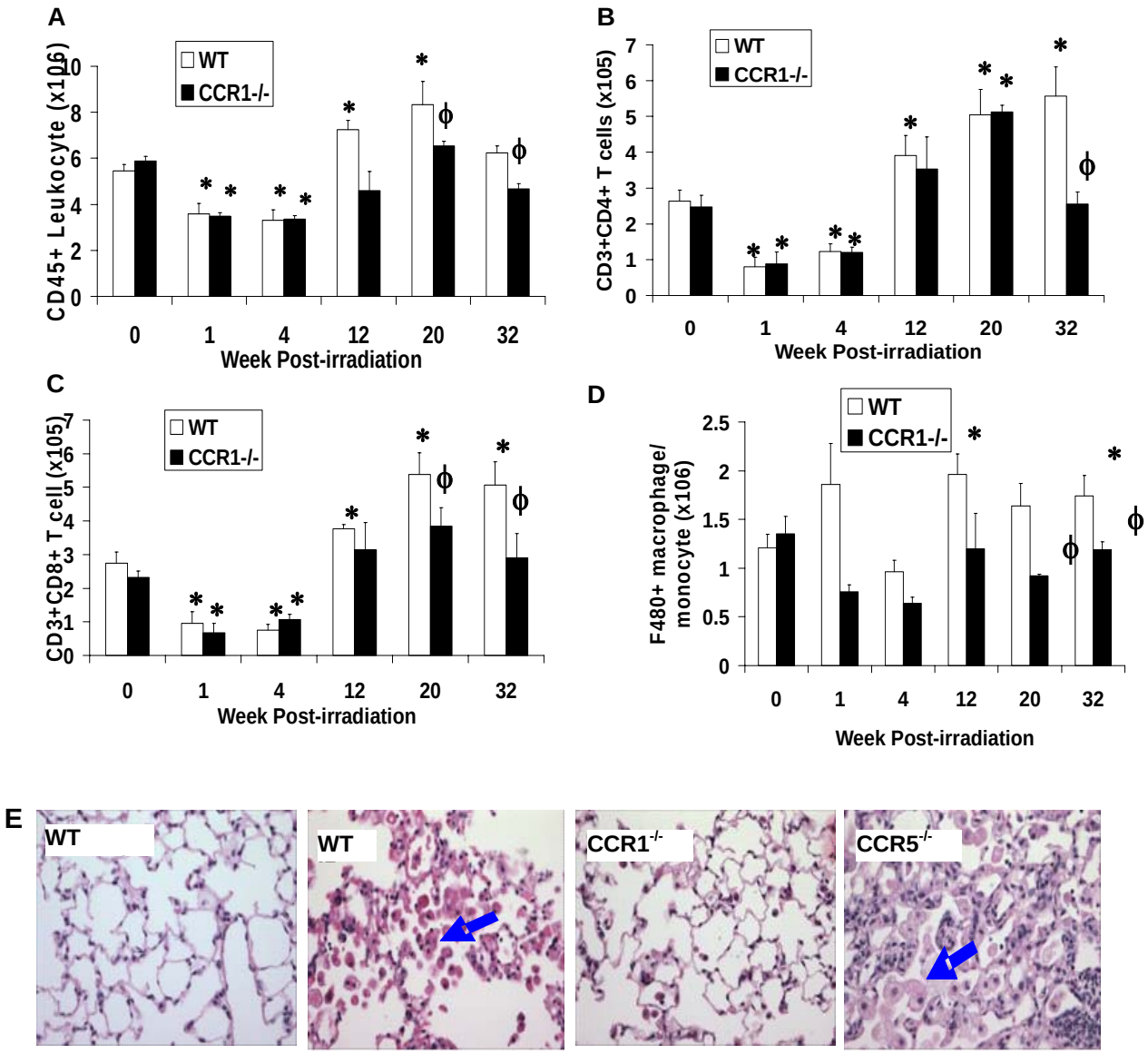


Figure 4.5 Infiltration of leukocytes into the lung post-irradiation. WT and CCR1-/- mice received a single dose of 14.5 Gy thoracic irradiation. Lungs and minced were harvested at 0, 1, 4, 12, 20 and 32 weeks post-irradiation. After enzymatic digestion, the cells were stained with FITC, PE, PerCP, or APC labeled monoclonal antibodies to murine CD45, CD3, CD4, CD8, and F4/80 (Pharmingen) and then analyzed on a FACScan with Cytomation software. **A**. Increased CD45+ leukocytes in the lungs of irradiated WT mice, but not CCR1-deficient mice. **B**. Fewer CD4+ T cells and CD8+ T cells accumulated in the lungs of CCR1-deficient mice than WT mice after thoracic irradiation. **C**. Increased macrophage/monocyte in the lungs of irradiated WT mice but not CCR1 deficient mice. **D**. Arrows indicate increased foamy macrophages in H&E staining of irradiated mouse lungs. * $p < 0.05$, compared with non-irradiated controls of the same genotype. ϕ $p \leq 0.05$, compared with irradiated WT at the same time point.

post-irradiation, but not in CCR1$^{-/-}$ mice (**Figure 5E**). Macrophages are considered an initiator of fibrosis, and thus, we saw a correlation between CCR1 expression and macrophage accumulation and fibrosis after irradiation. Furthermore, our data demonstrate that CCR1$^{-/-}$ mice are significantly protected lung from the cellular and histologic changes seen in the lungs of the irradiation in WT mice.

5. CCR1$^{-/-}$ mice have decreased TGFβ1 production after thoracic irradiation

An association between TGF-β1 production and pulmonary fibrosis has been well documented [34]. We therefore compared levels of this cytokine in C57BL/6J and CCR1-deficient animals at 32 weeks after 14.5 Gy of thoracic irradiation (**Figure 4.6**). The TGF-β1 levels were significantly increased in WT mice, but not in CCR1$^{-/-}$ mice. This increase in TGF-β1 levels was not seen in CCL3-deficient mice either as shown in chapter III (Figure 3.5).

6. T_H1 and T_H2 cytokine analyses after thoracic irradiation

A key role for proinflammatory cytokines is suggested by detecting, blocking, or augmenting cytokine expression in various experimental models of lung fibrosis [31, 32]. In order to investigate the role of T_H1 and T_H2 cytokines in the development of radiation induced lung injury, we used TaqMan gene expression assays to determine the expression of some crucial cytokines. Although there was no significant change in IFN-γ expression in the irradiated WT mice, there were significant increases seen in the irradiated CCR1$^{-/-}$ mice at 4 and 12 weeks (**Figure 4.7A**). A transient drop in IL-12p40 expression levels occurred in WT mice and CCR1$^{-/-}$ mice at 1 week post-irradiation, but IL-12p40 expression increased in WT mice and remained elevated through 32 weeks post-irradiation (**Figure 4.7B**). In contrast, IL-12p40 expression

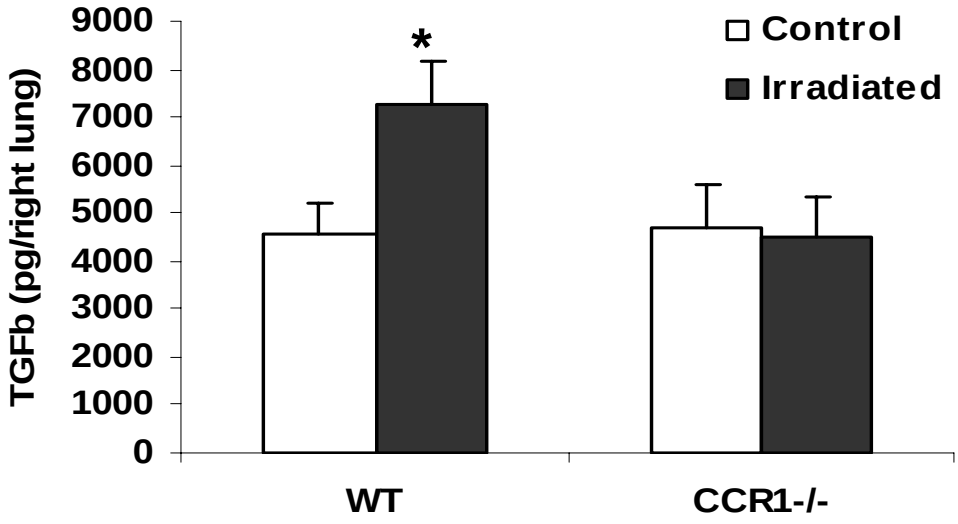


Figure 4.6 Increased TGF-β protein at 32 weeks post-irradiation in WT, but not in CCR1$^{-/-}$ mice. TGF-β protein in right lungs was measured by ELISA (Promega, Madison, WI). Values shown are means±SEM of 6-13 animals of each genotype. * P values < 0.05, compared to the nonirradiated controls of the same genotype mice.

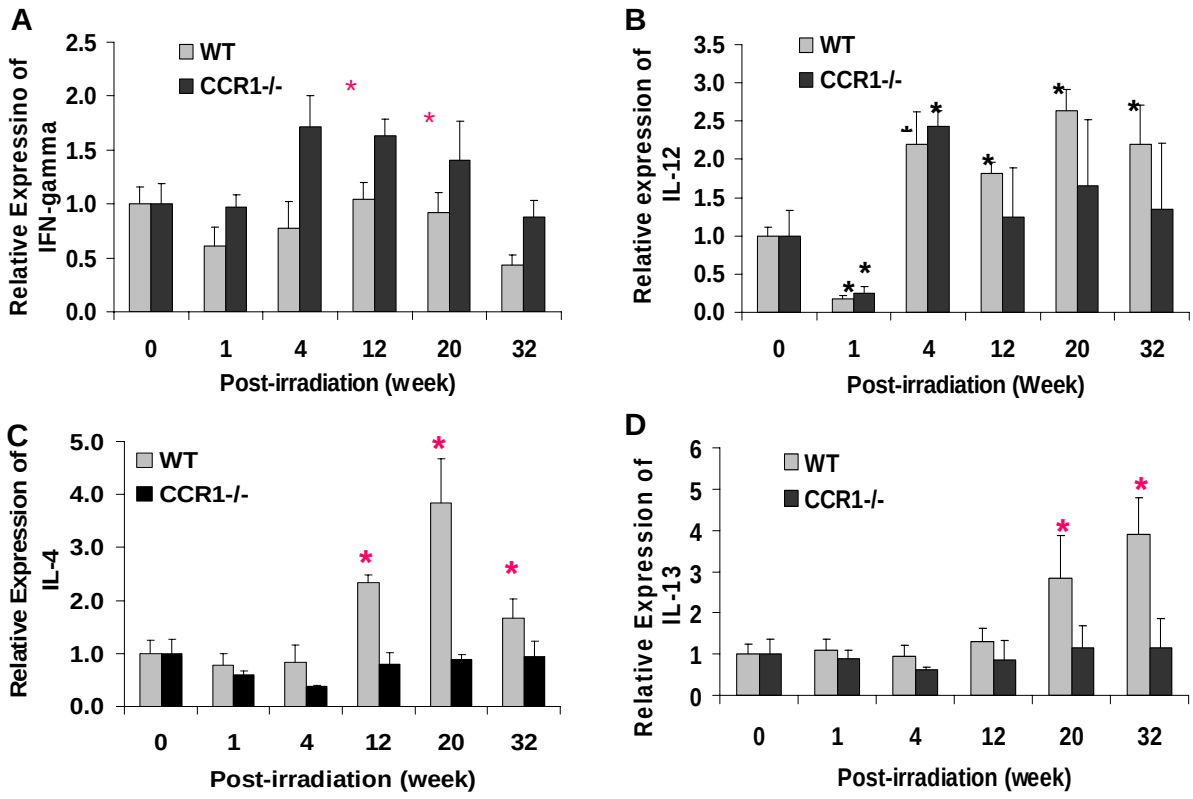


Figure 4.7 T_H1 and T_H2 cytokine gene expression. Total RNA isolated from the left lungs at 0, 1, 4, 12, 20, and 32 weeks post-irradiation was analyzed by quantitative real-time PCR using commercial TaqMan gene expression assays (Applied Biosystems). Data were analyzed using the 2-ΔΔCT method. Data presented as the fold change in gene expression normalized to an internal control gene, glucuronidase beta (Gusb) and relative to a calibrator sample (nonirradiated mouse). **A**. interferon gamma (IFN-γ), **B**.IL-12, **C**. IL-4, **D**. IL-13 expression. * $p < 0.05$, compared to nonirradiated controls of the same genotype.

increased only at 4 weeks post-irradiation in $CCR1^{-/-}$ mice and then decreased to the basal level. The pro-fibrotic cytokine IL-4 started to increase at 12 weeks, peaked at 20 weeks and remained elevated through 32 weeks post-irradiation in WT mice (**Figure 4.7C**). In contrast, $CCR1^{-/-}$ mice had no increase in IL-4 expression after irradiation. Expression of another important pro-fibrotic cytokine, IL-13, increased in the irradiated WT mice at 20 and 32 weeks (4-fold increase). In contrast, there was no increase of IL-13 expression in the irradiated $CCR1^{-/-}$ mice (**Figure 4.7D**). In summary, the irradiated WT mice had increased expressions of the profibrotic T_H2 cytokines IL-4, and IL-13 which were not seen in the irradiated $CCR1^{-/-}$ mice, while the irradiated $CCR1^{-/-}$ mice have slightly increased expression of antifibrotic T_H2 cytokines IFN-γ.

7. CCR1 inhibitor (BX471) attenuates radiation-induced lung injury

Our data showed that the genetic deficiency of CCR1 is radioprotective, suggesting that targeting this receptor with an antagonist might be therapeutically useful. Therefore, we tested whether blocking CCR1 signaling with a specific inhibitor (BX471) could protect WT mice from radiation-induced lung injury. BX471 is one of the most extensively studied CCR1 small molecular inhibitors. BX471 has previously been shown to ameliorate renal inflammation and fibrosis, chronic fungal asthma, and pancreatitis-associated lung injury in rodent models [93, 177, 178]. Thus, we tested BX471 in our model for its efficacy as a radioprotector.

Irradiated WT mice treated with twice daily injection of 30 mg/Kg BX471 from 3 weeks to 7 weeks post-irradiation had complete protection from radiation-induced lung fibrosis as shown by hydroxyproline assays and confirmed by histologic analyses of whole lung samples (**Figure 4.8A&B**). Furthermore, we show that when compared with mice receiving vehicle

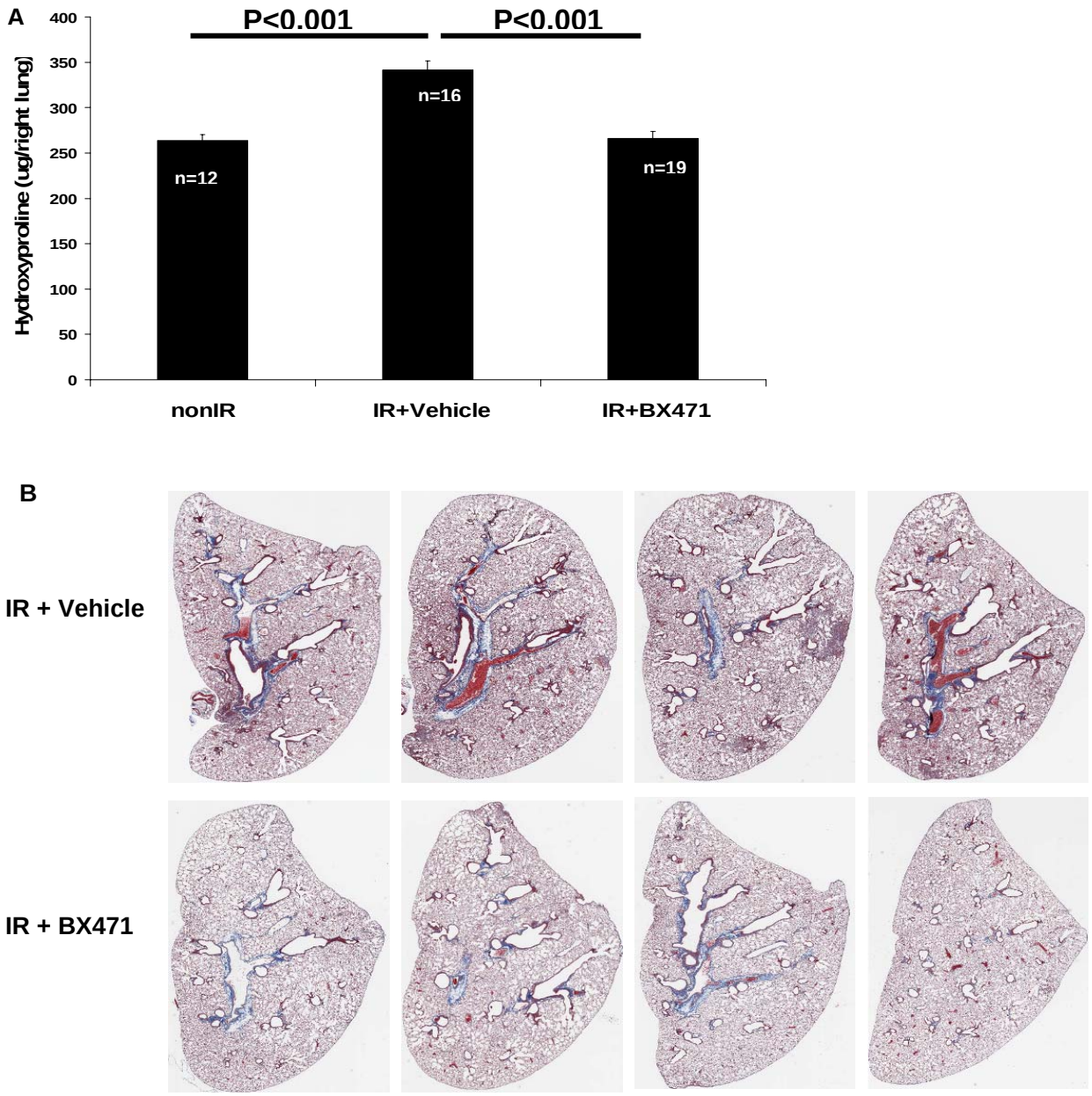


Figure 4.8 The CCR1 inhibitor BX471 reduces radiation-induced lung fibrosis. **A.** Quantitation of collagen in right lungs as determined by measurements of hydroxyproline at 32 weeks post-irradiation. nonIR=non-irradiated and non-treated; IR+vehicle=irradiated and treated with daily subcutaneous injection of vehicle (40% Cyclodextrin) from week 3 to week 7 post-irradiation; IR+BX471=irradiated and treated with daily subcutaneous injection of BX-471 at 30 mg/Kg of body weight from week 3 to week 7 post-irradiation. Values shown are the means±SEM. N= number of mice per group. There was no increase in the collagen content in the lungs of irradiated mice receiving BX471 as was seen in the vehicle-treated irradiated mice. **B.** Lungs of irradiated mice treated with BX471, but not vehicle-treated, are protected from inflammation and fibrosis.

only, irradiated WT mice receiving BX471 treatment had preserved lung function after thoracic irradiation (**Figure 4.9**). Not surprisingly, irradiated WT mice that received vehicle treatment had significantly diminished lung function as shown by a reduced lung compliance and increased lung tissue elastance. In contrast, irradiated WT mice receiving twice daily 30 mg/Kg BX-471 treatments had significantly higher compliance and lower tissue elastance than vehicle treated irradiated mice. Thus, BX471 did successfully reproduce the radioprotected phenotype we saw with mice having a genetic deficiency of CCR1.

IV.E. Discussion

We used mice with targeted mutants to study the roles of the CCL3 receptors, CCR1 and CCR5, in mediating radiation-induced lung injury. Our studies show that either the genetic deficiency or the blockade of CCR1 effectively reduces the inflammatory cell infiltration and subsequent lung fibrosis seen after thoracic irradiation. In contrast, lack of CCR5 exacerbates radiation lung injury.

1. Lack of CCR1 protects from radiation-induced lung injury

Chemokines are fundamental regulators of leukocyte homeostasis and inflammation, and their antagonism by small molecule chemokine receptor antagonists may be therapeutic important in human respiratory diseases. The CC and CCR chemokine families have important regulatory roles in fibrotic processes. In particular, CCL3 and CCL2 (monocyte chemoattractant protein 1, MCP-1), that are chemotactic for mononuclear, phagocytes, have been identified as essential pro-fibrotic mediators [140]. Blocking the function of CCL2 or CCL5 significantly decreased proteinuria as well as numbers of infiltrating leukocytes during crescentic nephritis [179]. CCR2-deficient mice produced similar results, confirming crucial

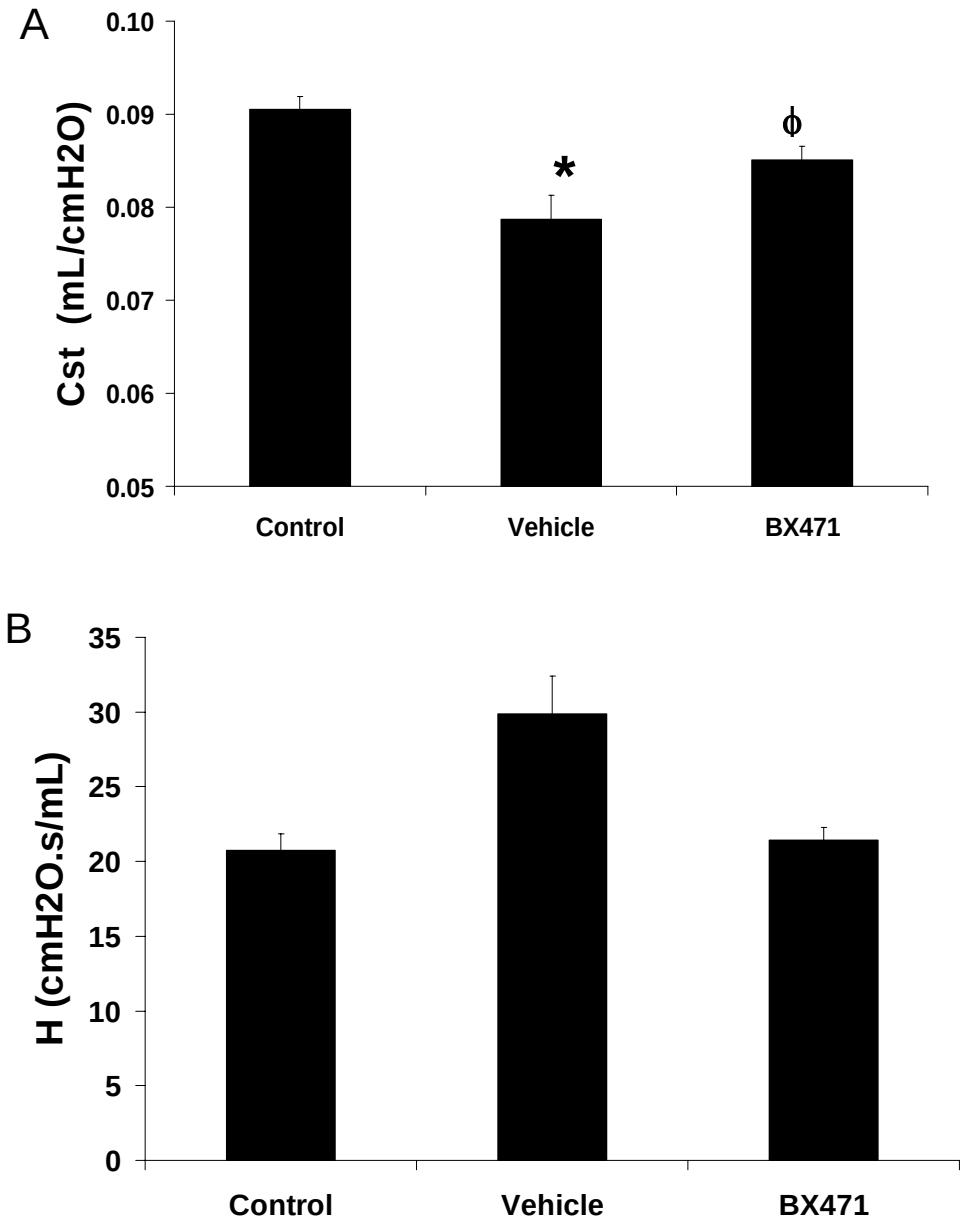


Figure 4.9 BX471 treated WT mice had preserved lung function after thoracic irradiation. Lung mechanics were measured by FlexiVent in anesthetized, paralyzed, and mechanically ventilated mice at 32 weeks post thoracic irradiation. **A:** Static compliance (Cst) determined by fitting the Salazar-Knowles equation to pressure-volume curves. **B:** Tissue Elastance (H) determined by applying prime wave impedance values to the constant phase model. Values are means±SE of 6-9 mice per group. Control=non irradiated, Vehicle=irradiated mice treated with vehicle from week 4 to 7 after irradiation, BX471=irradiated mice treated with BX471 from week 4 to 7 after irradiation *$p<0.05$ compared with non-irradiated control; ϕ $p<0.05$, compared with irradiated WT treated with vehicle.

roles for the CCL3- and CCL2-mediated signaling pathways in renal fibrogenesis [76, 144]. The mRNA expression of BLC, C10, IP-10, CCL2, CCL7 , CCL9, CCL5, CCR1, CCR2, CCR5 and CCR6 was elevated in fibrosis-sensitive (C57BL/6) mice at 26 weeks post-irradiation at the dose of 12.5 Gy thoracic radiation [16]. We also found that CCL3 is a mediator of radiation-induced lung injury and the lack of CCL3 attenuates lung fibrosis in our model.

The major receptor for CCL3, CCR1 is an important recruiter for leukocyte to sites of inflammation. CCR1 has been implicated as an attractive therapeutic target for modulating leukocyte-mediated tissue damage. For example, anti-CCR1 antibodies significantly reduced the accumulation of inflammatory cells and collagen deposition, with dramatic improvement in survival after bleomycin lung injury [146]. CCR1 mediates leukocyte recruitment and subsequent renal fibrosis after unilateral ureteral obstruction, the CCR1 inhibitor BX471 significantly reduced leukocyte recruitment, especially macrophages, and, thus diminished renal fibrosis [180].

Our studies show that in WT mice CCR1 gene expression began to significantly increase by 4 weeks following irradiation and remained elevated through 32 weeks. Coincident with this increase in CCR1 expression, numerous leukocytes were recruited into the lung. Ultimately, collagen deposition and lung fibrosis ensued. In contrast, both $CCR1^{-/-}$ and BX471-treated mice were protected from these changes with resultant improved survival and preserved lung function. Thus, our data indicate that deficiency of CCR1 precludes both pneumonitis and subsequent fibrosis, suggesting a crucial linkage of these processes. Furthermore, only short term treatment (4 weeks was as effective as 8 weeks) with a specific

CCR1 inhibitor during the early pneumonitic phase can prevent the entire spectrum of long-term sequalae, including fibrosis, loss of lung function and premature death.

2. Lack of CCR5 exacerbates radiation-induced lung injury

CCL3 binds and signals through both CCR1 and CCR5. Both receptors are co-expressed on macrophages and T cells. In contrast to CCR1, whose absence or blockade resulted in significant protection from radiation lung injury, the lack of CCR5 exacerbated lung fibrosis and function damage. CCR5-deficient mice have marked reduction in T cell recruitment into the lung from *Aspergillus fungigatus* conidia-induced asthma and into the brain after *Cryptococcus neoformans* infection [181, 182]; however, in the absence of CCR5, greater number of primed T lymphocytes were recruited into the lung in *Mycobacterium tuberculosis* infected mice [183]. CCR5-deficient mice had increased infiltration by $CD4^+$ and $NK1.1^+$ lymphocytes in a murine model of colitis using dextran sodium sulfate [184]. We also observed increased leukocyte recruitment in the lungs of irradiated $CCR5^{-/-}$ mice in our model (data not shown). CCR5 mediates lymphocyte trafficking, whereas macrophage recruitment is CCR5-independent [185]. Histologically many macrophages were seen in the lungs of irradiated CCR5-deficient mice; however, we have not yet quantified the subsets of recruited inflammatory cells.

CCR5 is highly expressed on T_H1 cells and may be critical for the promotion of T_H1-type immune responses in mice. CCR5-deficient mice had a decrease in T_H1, and an increase in T_H2, cytokine expression in a murine colitis model [184]. We also observed increased expression of the T_H2 cytokine, IL-4, in irradiated CCR5-deficient mice (data not shown), suggesting a role of T_H2 cells in their worsened phenotype. CCR5 also regulates the homing of T regulatory cells (T-reg) and CCR5 deficiency prevented T-reg cells from reaching the

site of *Leishmania* major infection, thereby altering the course of the disease [186]. Together, these findings suggest that, in comparison with WT mice, $CCR5^{-/-}$ mice may have fewer T-reg cells, and increased T_H2 immune response in the lung after thoracic irradiation with the resultant exacerbated lung injury.

One of CCR5 ligands is CCL3. The fact that $CCR5^{-/-}$ mice have worse radiation lung fibrosis compared to WT mice, may explain that CCL3 deficiency attenuates radiation lung fibrosis but CCR1 deficiency abolishes radiation lung fibrosis.

3. Clear differences in the levels and timing of cytokine gene expression in the lungs of irradiated WT and $CCR1^{-/-}$ mice

TGF-β is a central cytokine in fibrotic processes as it induces lung fibroblast transcription and synthesis of collagen and various other components of the extracellular matrix, e.g., fibronectin, glycosaminoglycans, and proteoglycans [35]. Patients with higher plasma TGF-ß levels before, or during, thoracic radiation, have a higher risk of developing pneumonitis [37, 38]. In rat model of thoracic irradiation TGF-β in BAL fluid was up-regulated and peaks between 3 and 6 weeks, coincident with the initial influx of inflammatory cells in BAL fluid, but preceding histologically discernable pulmonary fibrosis, suggesting a pathogenetic role of TGF-β in the development of radiation fibrosis [39]. Consistently, our studies showed increased TGF-β1 levels in the lungs of irradiated WT mice that developed lung fibrosis. In contrast, increased TGF-β1 levels were not seen in irradiated $CCR1^{-/-}$ and $CCL3^{-/-}$mice that did not have fibrosis. Our interpretation is that macrophages are the major source of TGF-β1 as CCR1-/- mice have minimal macrophage infiltration compared to WT mice after thoracic irradiation; however, we have not yet specifically examined the role of recruited fibrocytes in our model.

$CD4^+$ T cells are classified as T_H1 and T_H2 cells according to their cytokine production [49, 50]. The main T_H1 cytokines are IFN-γ and IL-12, whereas T_H2 cytokines include interleukin-4 (IL-4), IL-5 and IL-13. T_H2 cytokines are generally activators of fibroblasts and may promote extracellular matrix deposition and remodeling [52]. T_H1 cytokines, particularly IFN-γ, appear to have antifibrotic effects by inhibiting myofibroblasts [54, 55]. A shift from a T_H1 to a T_H2 cytokine profile is likely to be a key event in the progression of inflammation to fibrosis in IPF [53]. Although increased lung IL-4 expression was found in hemi-thoracicaly radiated rats [62], it was still unknown whether radiation-induced lung fibrosis may be T_H1 or T_H2-mediated. Our present study showed that the radiation-induced lung injury increases cytokine expression and thus enhances lung inflammation. The irradiated WT mice had significantly increased IL-12 expression by 4 weeks post-irradiation after an initial transient drop, whereas there was no significant change in IFN-γ expression. In contrast, expression of the T_H2 cytokine IL-4 started to increase at 12 weeks post-irradiation, peaking at 20 weeks, and IL-13 expression, one of main pro-fibrotic mediators, increased at 20 weeks and continued increasing to 32 weeks. Consistent with these increases in profibrotic cytokines, histologic evidence of lung fibrosis starts around 20 weeks after thoracic irradiation. In contrast, neither IL-4 nor IL-13 expression increased in the radioprotected $CCR1^{-/-}$ mice after thoracic irradiation. Furthermore, irradiated CCR1-/- mice showed increased IFN-γ expression at 4 weeks and 12 weeks. Together, these data implicate T_H2 cells in mediating fibrosis. Interestingly, the lack of CCR1 has been shown to enhance T_H1 response in nephrotoxic nephritis in the mouse, including titers of antigen-specific IgG2a antibody, delayed-type hypersensitivity responses, and production of IFN-γ and TNF-α [187]. On the other hand, $CCR5^{-/-}$ mice have even worse radiation-induced fibrosis than WT, which

supports that T_H2 mediation of radiation-induced fibrosis as CCR5 is preferentially expressed in T_H1 cells. In summary, our findings suggest that a shift towards a T_H2 cytokine pattern occurs around 12 to 20 weeks after thoracic irradiation that may directly affect the overall pattern of proliferation and/or extracellular matrix gene expression of lung fibroblasts, resulting in development of fibrosis. Further study is needed to more completely address this issue, such as using T_H1 or T_H2 knock-out animals or possibly adoptive transfer of these T_H subsets into protected mice.

4. The CCR1 inhibitor, BX471 effectively reduces radiation-induced lung inflammation and fibrosis

A number of pharmaceutical companies have identified small molecule CCR1 antagonists [177]. Among them, BX471 is one of most extensively studied CCR1 small molecular inhibitors. BX471 has been shown to ameliorate renal inflammation and fibrosis [180], chronic fungal asthma [78], and pancreatitis-associated lung injury in rodent models [79]. BX471 reduced leukocyte recruitment in unilateral ureteral obstruction model [188]. In our murine model of radiation-induced lung injury, 4 week-treatment with BX471 significantly eliminates lung injury and preserved lung function. This finding was consistent with the beneficial effects we saw with genetically-induced CCR1 deficiency. Our studies clearly suggested a key role for CCR1 is to recruit macrophages and T cells into the lung after thoracic irradiation. Currently, BX471 has been tested in clinical phase I and II trial in patients with psoriasis and in phase II trial in patients with multiple sclerosis. Although these clinical trials did not show significant benefit, we hypothesize that using BX471 for preventing radiation lung injury may be a more feasible target than using BX471 for dampening well-established autoimmune diseases.

In summary, CCR1 plays an important role in mediating the infiltration of inflammatory cells into the lung after thoracic irradiation. Lack of CCR1 reduced lymphocyte and monocyte/macrophage infiltration, the expression of profibrotic cytokines, IL-4, IL-13 and TGF-β, and the subsequent lung fibrosis after thoracic radiation. Moreover, the blockade with the CCR1 antagonist BX471 reproduced these protective effects in radiation-induced lung injury. Cumulatively, these observations demonstrate that CCR1 blockade may offer a new therapeutic strategy to ameliorate radiation-induced pneumonitis and fibrosis.

IV.E. The Effects of BX471 on tumor cells (ongoing study)

Chemokines and their receptors in cancer have well-established roles in cancer. Chemokines are involved in almost all aspects of tumor establishment and progression: they provide directional cues for migration/metastasis, shape the tumor microenvironment and provide survival and/or growth signals [105, 106, 189]. In chronic inflammation, leukocyte recruitment, led by chemokines and chemokine receptors, may contribute to tumor growth and spread by inducing the expression of growth or survival factors and matrix metalloproteases. Additionally, chemokines are involved in tumor angiogenesis and in the homing of tumor cells to the sentinel lymph nodes and subsequent metastasis.

Notably, CC-chemokines have implicated in regulating the progression of many tumors such as breast cancer, melanoma, lung cancer, colorectal carcinoma, multiple myeloma, prostate cancer and ovarian cancer [106]. The chemokine ligand-receptor pair, CCL3/CCR1 facilitates tumor growth and metastasis in oral squamous cell carcinoma[190], hepatocellular carcinoma cell[191], solid ovarian tumors [192], myeloma and breast cancer.

Increased CCL3 expression is believed to play an important role in the development of lytic bone lesions in multiple myeloma and treatment with a monoclonal rat anti-mouse CCL3 antibody reduced both paraprotein levels and lytic lesions in mice inoculated with myeloma cells [193]. Thus, an additional benefit of CCR1 inhibition may include some antitumor effects. For example, the CCR1&5 antagonist, Met-CCL5 inhibits experimental breast tumor growth [194]. Similarly, the CCR1 antagonist BX471 reduces osteolytic lesions by 40% in myeloma 5T2MM mice [195].

We have shown that BX-471 effectively protects mice from radiation-induced lung injury. Clinically, many cancer patients who receive thoracic radiotherapy suffer from radiation-induced lung injury. In order to potentially use BX-471 for these patients, we should first understand the effects of BX-471 on tumors. Therefore, we plan to evaluate whether BX-471 affects tumor radiosenstivity and/or enhances tumor growth/metastasis. As Lewis lung carcinoma cells (LLC) are immunologically compatible with C57BL/6 mice, we have chosen this tumor cell line for these studies.

For tumor radiosenstivity experiments, Lewis lung carcinoma cells will be subcutaneously injected into mouse chest walls. When the transplanted tumors become palpable (about 6mm), one group of mice will receive thoracic radiation at a single dose of 14.5 Gy, a second group will receive 30 mg/kg BX-471 twice daily for 2-4 weeks, and the third group will receive both treatments. Mice will be observed daily and euthanized for analyses when tumor sizes reach 2 cm or by 4 weeks post-irradiation.

To evaluate the effects of BX-471 on tumor metastasis, one cohort of mice will be treated with 30 mg/kg BX-471 twice daily for 1 week and the other cohort are treated with

vehicle (40% cyclodextrin/saline). After 1 week, Lewis lung carcinoma cells will be injected into tail veins while treatment with BX471 or vehicle continues. After 3 weeks post LLC cell transfer, mice will be euthanized and lungs harvested for the number and average size of tumor metastases.

These studies will add to our current data as a basis for clinical trials of BX-471 in cancer patients undergoing thoracic radiotherapy.

V. The $T_H1/T_H2/T_H17$ paradigm and Radiation-induced Lung injury

V.A. Introduction

Radiation pneumopathy is an important dose-limiting complication of radiotherapy for which the pathogenesis is only partially understood, though initially, inflammation and pneumonitis predominate. One feature of radiation induced pulmonary injury is infiltration of inflammatory cells. As shown in our studies, and others, a variety of inflammatory cells are recruited into the lung after irradiation [15, 196]; however, specific functions for inflammatory cell subtypes remain poorly defined.

Radiation lung fibrosis is thought to develop through a number of nonexclusive mechanisms. One described mechanism is that damage to specific cells (i.e., epithelium or interstitial fibroblasts) stimulates them to alter their expression of inflammatory and chemotactic cytokines, which in turn act to recruit and activate inflammatory cells. Once recruited into the disturbed microenvironment, the stimulated inflammatory cells produce other mediators, thereby initiating a complex ‘‘cytokine cascade’’, and ultimately, leading to an overt, functionally significant, pneumonitis. In addition, during this persistent chronic inflammation, the activated cells, both inflammatory and parenchymal, can produce mediators that directly affect the overall pattern of proliferation and/or matrix gene expression of lung parenchymal fibroblasts, leading to fibrosis.

Several experimental models have shown the importance of $CD4^+$ T cells in the progression of fibrosis, including radiation lung injury [34]. Depletion of $CD4^+$, but not $CD8^+$, T cells reduced the production of TGF-β and lung fibrosis in bleomycin-treated T-bet-

deficient mice [197] and reduced the postirradiation parenchymal thickening in irradiated rat lungs [62]. Our studies show clear differences in the lung inflammatory cell content, including $CD4^+$ T cells, $CD8^+$ T cells and macrophages, after thoracic irradiation in radiosensitive animals (WT) and radioprotected animals ($CCL3^{-/-}$, $CCR1^{-/-}$).

Naive $CD4^+$ T helper cells can differentiate into one of three lineages of effector T helper cells, i.e., T_H1, T_H2 or T_H17 cells. T_H1 cells produce mainly pro-inflammatory cytokines IL-1, IL-2, IL-12, IL-15, IL-18, IFN-γ and lymphotoxin (TNF-β), while T_H2 cells are the source of IL-4, IL-5, IL-6, and IL-13. T_H1 cytokines IL-2, IFN-γ, and TNF-β activate cytotoxic T cells (Tc) and macrophages to stimulate cellular immunity and inflammation. The T_H2 cytokines IL-4, IL-5, IL-6, and IL-10 stimulate antibody production by B cells and mediate anti-parasite immunity and allergic diseases. T_H17 cells express IL-17, IL-17F, IL-21 and IL-22 (and IL-26 in humans) and act to regulate inflammatory responses [51]. The presence of IL-12 [signaling through signal transduction and activator of transcription (STAT)-4] skews towards T_H1, IL-4 (signaling through STAT-6) towards T_H2, and IL-6 and TGF-β towards T_H17 differentiation (signaling through STAT-3) [49, 50]. In addition, T_H1 and T_H2 type cytokines, notably IL-2, IFN-γ, and IL-4, inhibit T_H17 differentiation.

In general, fibrogenesis is more strongly associated with the T_H2 $CD4^+$ T cell response involving IL-4, IL-5, and IL-13 [34]. T_H2 cytokines have direct profibrotic effects, and also promote the expression and activation of the fibrogenic cytokine TGF-β1. For example, a predominant T_H2 cytokine (IL-4 and IL-5) profile is revealed in idiopathic pulmonary fibrosis (IPF) by immunostaining and in situ hybridization of open lung biopsy specimen. A shift from the T_H1 to T_H2 cytokine profile is a likely key event in the progression of inflammation to fibrosis in IPF [47, 53]. Alternatively, T_H1 cytokines, in

particular IFN-γ, have antifibrotic effects by inhibiting myofibroblasts [54, 55]. Although changes in both T_H1 cytokines and T_H2 cytokines have been observed in several studies of radiation lung injury, our results suggest that radiation lung fibrosis is more likely mediated by T_H2 cytokines (see chapter IV). However, same further study is needed to confirm these findings.

IL-17 is produced primarily but not exclusively, by $CD4^+$ and $CD8^+$ αβ T cells; however, γδ T cells can also produce IL-17. In the lung, NKT cells may be another source of IL-17. Thus, IL-17 production may be regulated by different cells and different cytokines in different organs [51]. T_H17 cells have distinct biological functions from other T_H cells. T_H17 cells are reportedly key players in several autoimmune and inflammatory diseases, i.e. rheumatoid arthritis, asthma, systemic lupus erythematosus, colitis and allograft rejection [198]. Overexpression of IL-17 in the lungs induces the production of various cytokines, chemokines and adhesion molecules, resulting in increased leukocyte infiltration [199, 200]. Together, IL-17 and TNF promote pro-inflammatory T_H1 cytokine gene expression. On the other hand, IL-17F-deficient mice have enhanced T_H2 cytokine production, while IL-17-deficient mice show a reduced T_H2 response [201].

T_H17 cells are enriched in the lung and GI tract, and are considered important in mucosal host defenses and other pulmonary diseases. A particular IL-17F mutation that antagonizes the activity of wild-type IL-17F was associated with airway inflammatory diseases, like human asthma and chronic obstructive pulmonary disease [202]. Both IL-17 and IL-17F are induced in a dose- and time-dependent fashion in *K. pneumoniae* infection and was associated with neutrophil emigration into the lung [203]. IL-23, and the downstream cytokines IL-17A and IL-17F, may be involved in the proinflammatory cytokine

network involved with the pathogenesis of cystic fibrosis [204]. IL-17 might also play an indirect role in airway remodeling in asthma by regulating local airway inflammation and profibrotic cytokine production [205]. In a rat model of bleomycin-induced lung injury, γδ T cells were the predominant source of IL-17. In this model, γδ T cell knockout mice showed more severe interstitial inflammation, but a significantly reduced cellular infiltration into the airways, reduced IL-6 and IL-17 expression in the lung, and a significant delay in epithelial repair (collagen deposition) [206]. Increased IL-17 production was observed in tissue fibrosis after chronic 2,4,6-trinitrobenzene sulfonic acid-induced colitis [207]. Lastly, IL-17 may also contribute to stromal fibrosis in nasal polyp disease [208]. The potential dynamics of T_H17 cell population and their interaction with other T helper cells have not yet been studied in radiation fibrosis.

A further subset of T cells, termed T regulatory (T_{-reg}) cells, have immunosuppressive functions and a cytokine profile distinct from that of either T_H1 or T_H2 cells. T_{Reg} cells are able to inhibit the development of T_H2 cell responses, as well as T_H1 cell responses, by producing high levels of IL-10 and TGF-β. T_{Reg} cells also modulate or suppress inflammatory responses from T_H17 cells, which are often present at the interface between the external and internal environments and mediate a number of autoimmune diseases. Although TGF-β induces Foxp3 in naive T cells, thereby generating Treg cells, TGF-β and IL-6 together drive the generation of T_H17 cells. Thus, the induction of T_H17 cells is dichotomously related to the induction of $Foxp3^+$ Treg cells: IL-6 is one of the switch factors that re-direct T cell differentiation from T-reg pathway into the T_H17 pathway. Treg cells were implicated in bleomycin-induced lung fibrosis [41]; however, studies of Treg cell functions in radiation-induced lung injury are thus far lacking.

We hypothesize that in response to radiation, the early proinflammatory cytokine expression, regulated by different populations of $CD4^+$ T helper cells, may directly or indirectly promote the development of radiation lung fibrosis. The aim of this study was to define the functions of $CD4^+$ T lymphocyte subsets in the pathogenesis of post-irradiation pneumonitis and fibrosis.

V.B. Materials and Methods

1. Mice

All animal studies were performed under approval by the IACUC at the University of North Carolina at Chapel Hill. Wild-type, $CD4^{-/-}$ and $CD8^{-/-}$ C57BL6mice were purchased from Jackson Laboratories. IL-10/IL-12 double knockout C57BL6 mice (IL-$10/12^{-/-}$) [68] were obtained from Taconic Farms (Germantown, NY). IL-17 receptor A knockout mice (IL-$17RA^{-/-}$) were obtained from Dr. Jay Kolls in NIEHS. All strains of mice were bred according to DLAM policies and housed in microisolator cages maintained in a specific pathogen-free environment. All mice except those used in lung cytokine protein assays were male.

2. Thoracic irradiation

Animals were irradiated with a singled dose of 14.5 Gy as described in chapter II.

3. Hydroxyproline (HYP) assay

Hydroxyproline assay were performed as described in chapter II.

4. Cytokine protein analyses

Irradiated female mouse lungs were homogenized in 1 ml of a protease inhibitor cocktail (ϕ complete protease inhibitors cocktail, Roche), then incubated on ice for 30 min and centrifuged at 1,4000rpm for 15 min at 4°C. Supernatants were aliquoted and stored at –70

°C before performing cytokine protein assays on the Luminex 100 by using a Bio-plex mouse cytokine 23-Plex Panel (catalog # 171-F11241, BIO-RAD, Hercules, CA) according to manufacturer's manual. Bio-plex cytokine assay kits simultaneously measure IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α.

5. Gene expression analyses

Gene expression analyses were performed as described in chapter II.

6. Histologic analyses

Histologic analyses were performed as described in chapter II.

7. Measurements of lung mechanics

Measurements of lung mechanics were performed as described in Chapter II.

8. *Antibodies and flow cytometry*

Antibodies with the following specificities were purchased from eBiosciences (San Diego, CA): anti-CD4 (RM 4.5), CD8 (53-6-7), IFN-γ (XMG1.2), IL-17F (eBio 18F10), F480 and Foxp3. Antibodies to IL-17A (TC11-18H10.1) and TNF-α (MP6-XT22) were purchased from Biolegend (San Diego, CA). Acquisition was performed on a FACSCalibur using CellQuest software (BD Biosciences; San Jose, CA). Analyses were performed using FlowJo software (Treestar Inc., Ashland, OR). Mice were euthanized at several time points post-irradiation and lung leukocyte isolation was performed as previously described. Isolated cells were stimulated with PMA, ionomycin, and brefeldin A. Intracellular cytokine staining was performed using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's recommendations.

9. In vitro suppression assays

Treg-mediated suppression was measured by a previously described protocol [209]. Briefly, allogeneic stimulators were prepared from spleen cell suspensions of B6D2 mice, depleted of T cells using anti-CD90 magnetic beads (Miltenyi Biotech), and irradiated with 2100 cGy from a ^{137}Cs source. Stimulators (1×10^5) were incubated with responders (WT CD4+CD25- T cells, 1×10^5) and various ratios of Tregs from WT or CCR5-/- donors. Cultures were incubated for 5 days at 37°C and 1 μCi (0.037 MBq) ^{3}H-thymidine was added for the last 16 hours of culture. Incorporation of ^{3}H-thymidine was assessed.

V.C. Results

1. CD4-deficient mice show partially radioprotective phenotype

Our previous studies show that the radiation injury-sensitive WT mice have greater lung infiltration of $CD4^+$ and $CD8^+$ T cells than the radioprotected $CCR1^{-/-}$ and $CCL3^{-/-}$ mice. To determine whether $CD4^+$ or $CD8^+$ T cells contribute to the pathogenesis of radiation-induced lung injury, $CD4^{-/-}$and $CD8^{-/-}$mice received thoracic irradiation. We showed that, overall, CD4-deficient mice had better survival at 32 weeks than WT after thoracic irradiation (76% vs 52%, p=0.054) (**Figure 5.1A**), while the survival rate for $CD8^{-/-}$ mice was close to that of WT mice. We quantified collagen deposition in lung by hydroxyproline (HYP) assays. All of the irradiated WT, $CD4^{-/-}$ and $CD8^{-/-}$mice had increased lung HYP content compared with non-irradiated controls of the same genotype (**Figure 5.1B**); however, lung HYP in irradiated $CD4^{-/-}$ mice was significantly lower than that seen in irradiated WT mice. In contrast, $CD8^{-/-}$ mice had much higher lung HYP than irradiated WT mice. Consistent with lung HYP results, histologic analyses demonstrated that $CD4^{-/-}$ mice had less radiation-induced lung fibrosis than WT, while $CD8^{-/-}$ mice were worse (**Figure 5.2**). Lung function analyses showed that

both irradiated WT and CD8$^{-/-}$ mice had a decreased compliance and an increased tissue elastance, while CD4$^{-/-}$ mice had preserved lung functions (**Figure 5.3**).

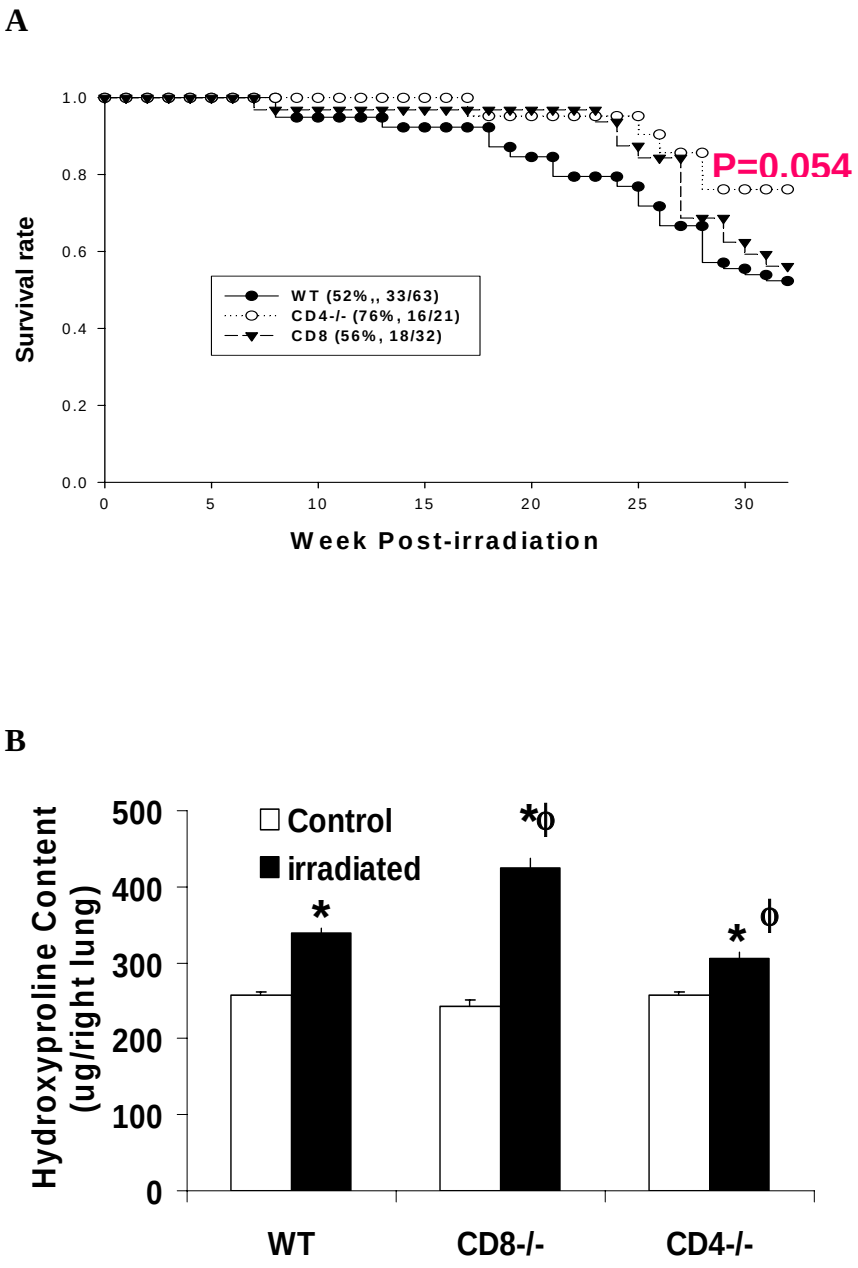


Figure 5.1 CD4- deficient, but not CD8-deficient, mice have a partially protected phenotype from radiation-induced lung injury. **A.** Survival curves at 32 weeks post-irradiation by Sigmaplot 8.0 showed that CD4$^{-/-}$ but not CD8$^{-/-}$ mice have improved survival compared to

WT. WT, CD4-deficient, and CD8-deficient animals received a single dose (14.5Gy) of thoracic irradiation. p=0.054, $CD4^{-/-}$ vs WT. **B.** Hydroxyproline content of the right lungs of non-irradiated control and irradiated mice 32 weeks post-irradiation. Values shown are the means±SEM of 6-9 mice per group. ***** $p < 0.05$, compared with non-irradiated controls of the same genotype; ϕ $p<0.01$, compared with irradiated WT.

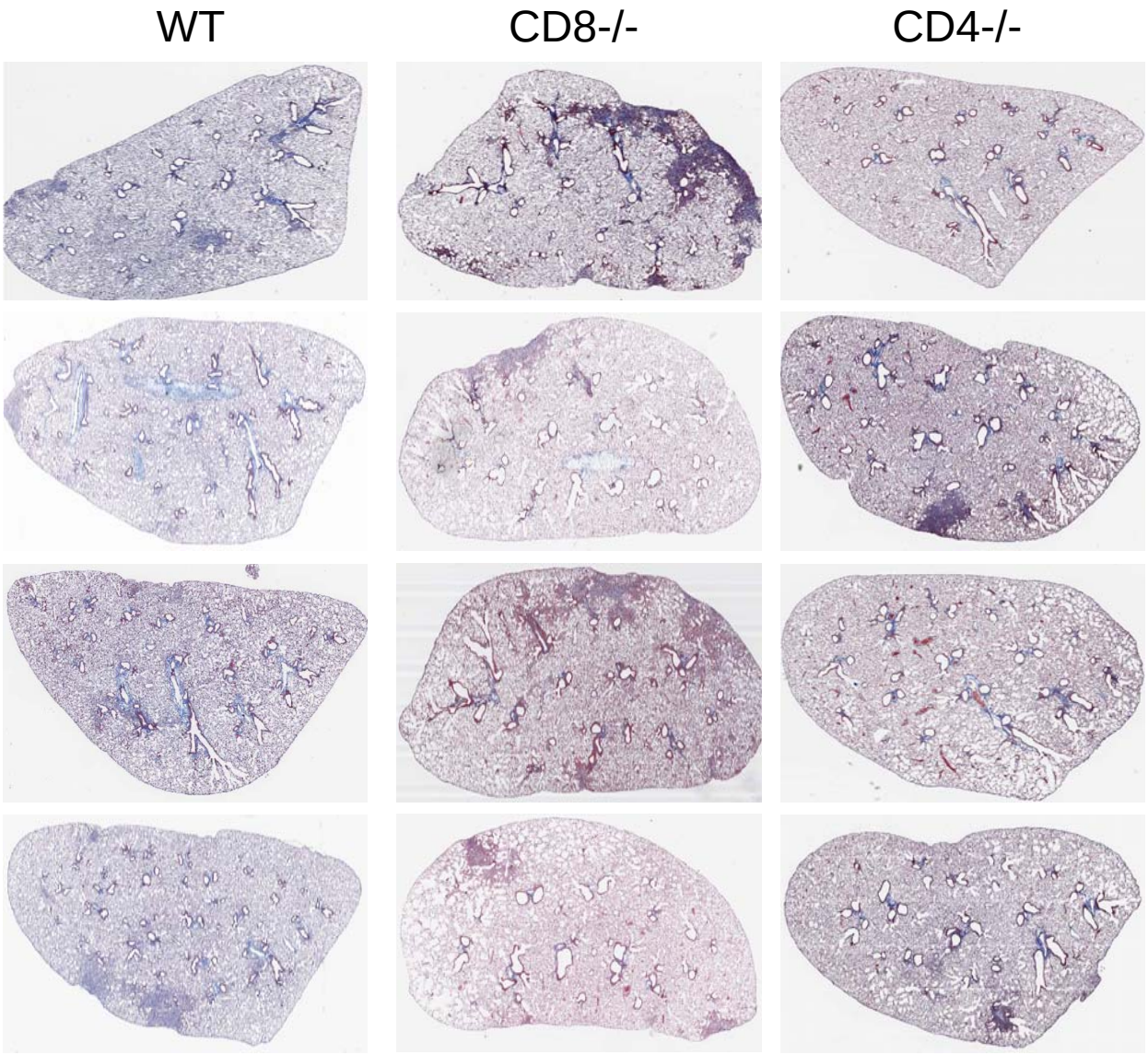
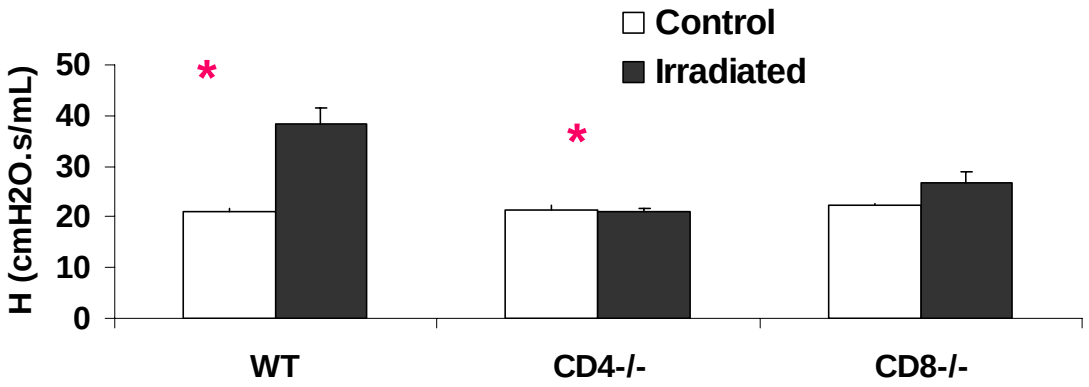


Figure 5.2 Histologic analyses show that CD4-, but not CD8-deficient, mice have a partially protective phenotype from radiation-induced lung inflammation and fibrosis. WT, $CD4^{-/-}$ and $CD8^{-/-}$ mice received a single dose (14.5Gy) of thoracic irradiation. Lungs were harvested at 32 weeks post-irradiation and stained with Masson's trichrome (MT). Whole sections of MT stained lungs demonstrate less inflammatory and fibrotic foci in irradiated $CD4^{-/-}$ mice.

A



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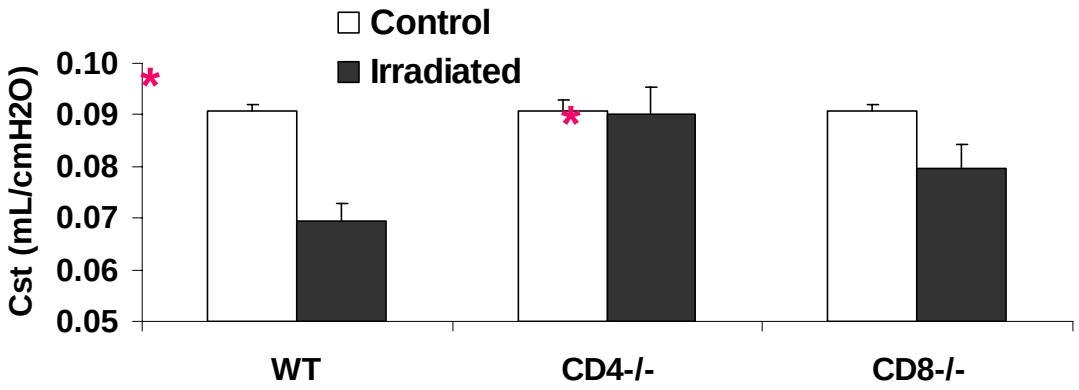


Figure 5.3 Analyses of lung mechanics demonstrate that irradiated WT and $CD8^{-/-}$ mice have decreased lung compliance and increased tissue resistance, but irradiated $CD4^{-/-}$ mice have preserved lung function. Lung mechanics were measured in anesthetized, paralyzed, and mechanically ventilated mice 32 weeks post thoracic irradiation. **A.** Static compliance (Cst) was determined by fitting the Salazar-Knowles equation to pressure-volume curves. **B.** Tissue Elastance (H) was determined by applying prime wave impedance values to the constant phase model. Values shown are the means±SEM of 6-9 mice per group. ***** $p < 0.05$, compared with corresponding genotype non-irradiated control.

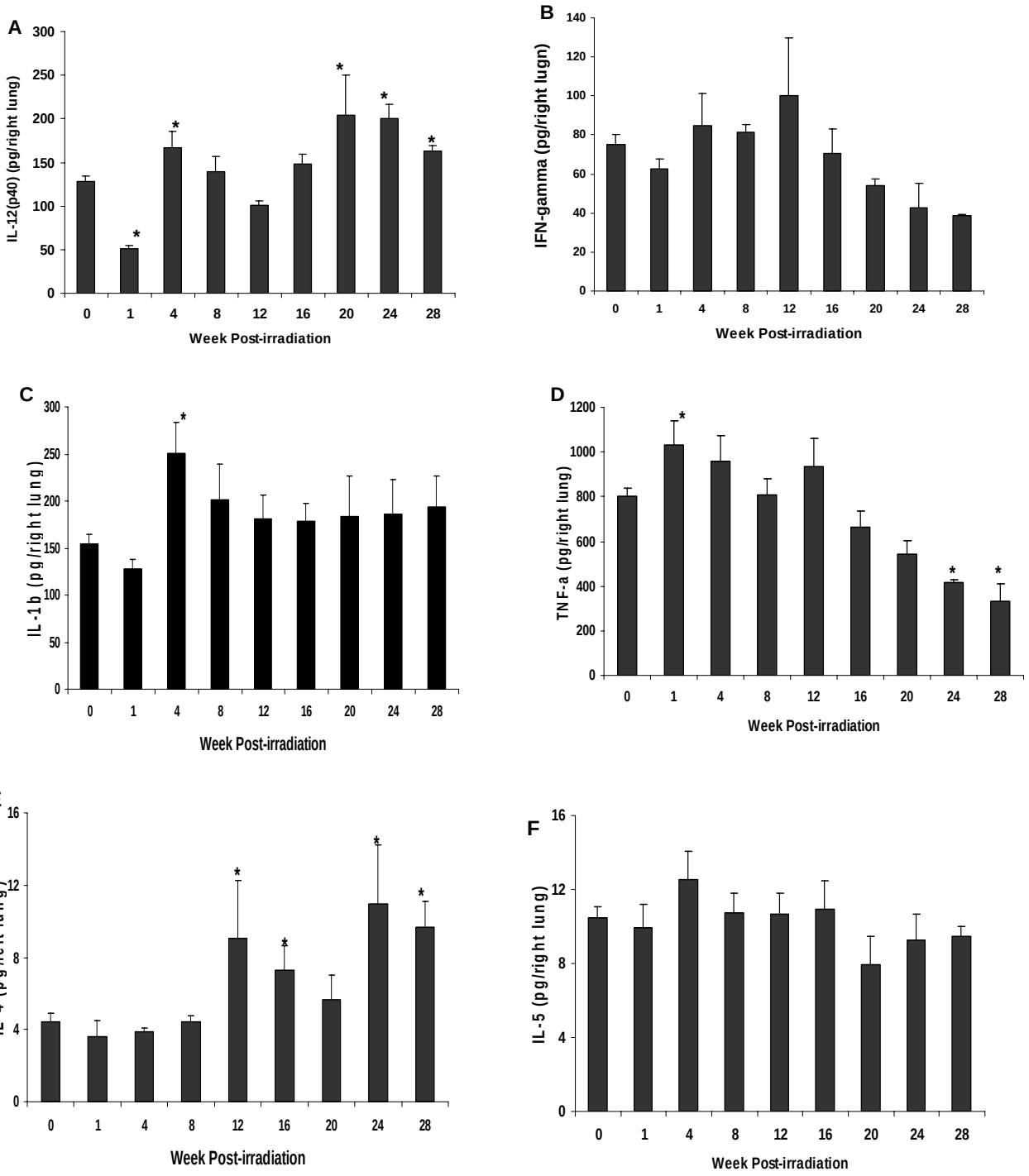
2. Cytokine protein assays of irradiated WT mouse lungs

In order to understand what molecular mediators contribute to radiation pneumopathy and fibrosis, we analyzed the lungs from irradiated female mice using Bio-Rad Plex cytokine protein assays. We examined these samples for their expression of IFN-γ, IL-12, IL-1β, TNF-α as representative the $T_H1$cytokines, and IL-4, IL-5, IL-6, IL-13 as representative the T_H2 cytokines. As shown in **Figure 5.4B**, the T_H1 cytokine IFN-γ did not significantly change after this radiation treatment. Both IL-1β and TNF-α increased within 1 week but then returned to the basal levels (**Figure 5.4C&D**). Although the expression of IL-12, which is required for T_H1 cell differentiation, increased at 20-28 weeks post-irradiation (**Figure 5.4A**), none of the T_H1 cytokines (IFN-γ, IL-1β and TNF-α) increased at this later phase. In contrast, the profibrotic T_H2 cytokines, IL-4 significantly increased at 12, 16, 24 and 28 weeks (**Figure 5.4E**) and IL-13 level increased at 28 weeks (**Figure 5.4G**). Lastly, the T_H2 cytokine IL-6, which is required for T_H17 differentiation, also significantly increased at 4, 8 and 24 weeks (**Figure 5.4H**). We also found that protein levels of IL-17 were elevated at 4 weeks and 16-28 weeks post-irradiation (**Figure 5.4I**).. Taken together, these data support our hypothesis for a crucial role of T_H2 cytokines in radiation pneumopathy.

3. IL-10/12$^{-/-}$ mice develop severe lung fibrosis after thoracic irradiation

As shown in chapter V, compared to the radioprotected CCR1-deficient mice, irradiated WT mice had higher mRNA expressions of the T_H2 cytokines, IL-4 and IL-13. We also demonstrated elevated protein levels of IL-4 and IL-13 in irradiated WT female mice. Both

IL-4 and IL-13 has been shown to be profibrotic. These findings suggest that radiation-induced lung fibrosis may be mediated by T_H2 cytokines. To validate this hypothesis, IL-10/12-double knockout mice were thoracically irradiated. The IL-10/12$^{-/-}$ mice did show



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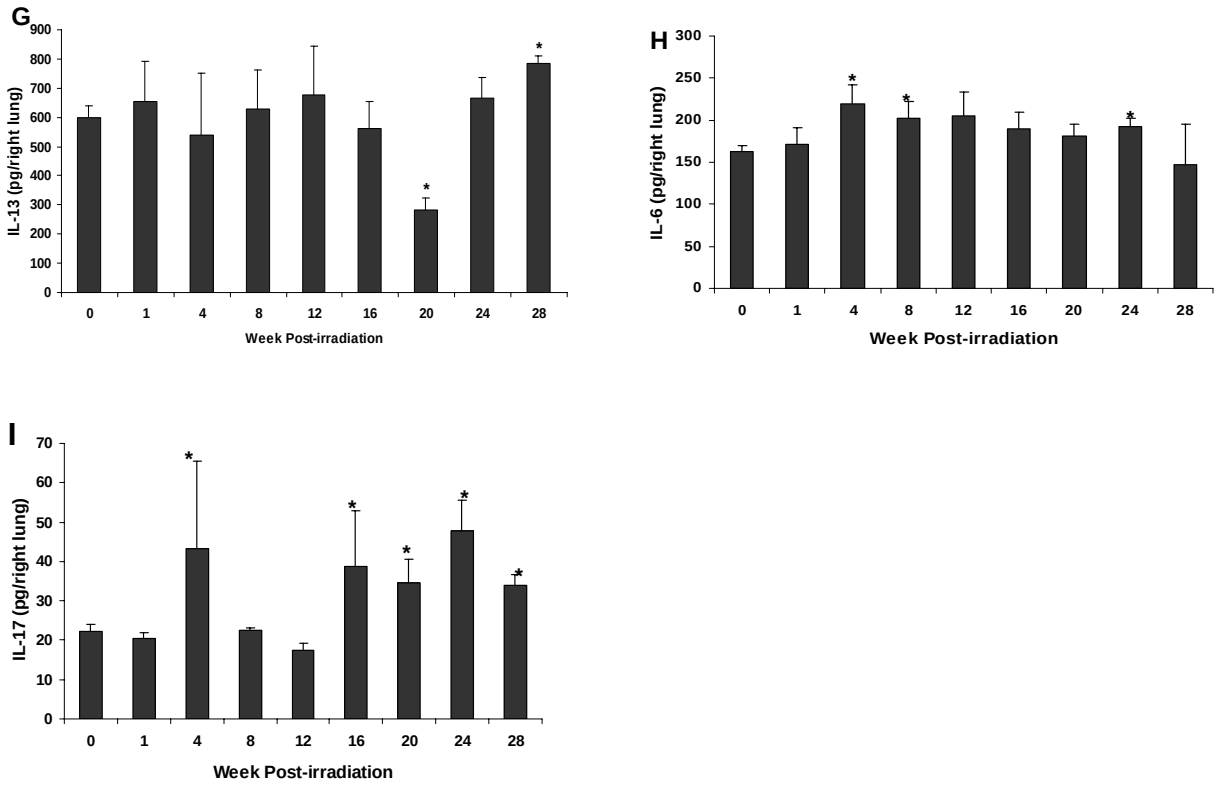


Figure 5.4 Cytokine analyses of irradiated female WT mouse lung homogenates. IL-12 (**A**), INF-γ (**B**), IL-1(**C**), TNF-α (**D**); IL-4 (**E**), IL-5 (**F**), IL-13 (**G**), IL-6 (**H**), IL-17 (**I**). Values shown are the means±SEM of 6-9 mice per group. ***** $p < 0.05$, compared with nonirradiated controls.

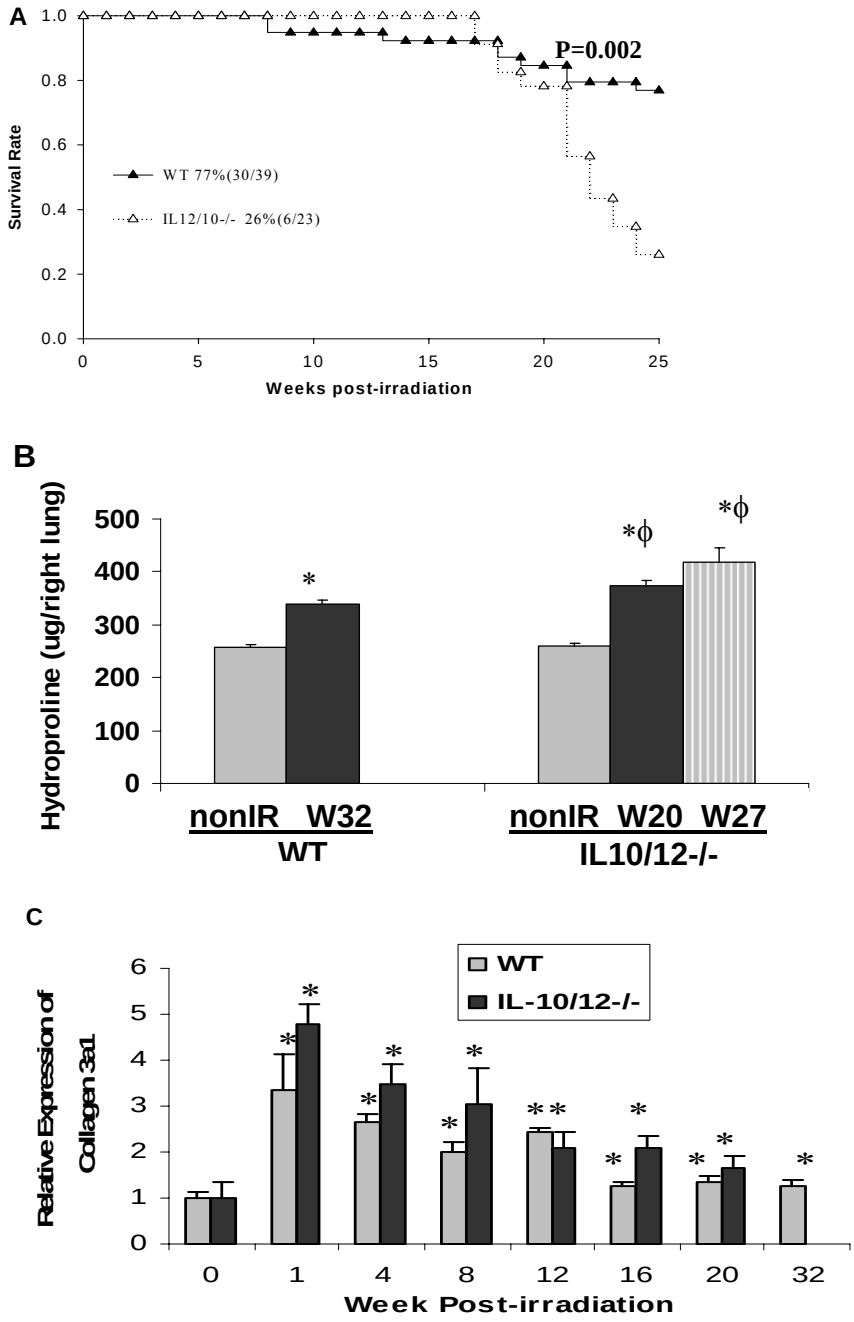


Figure 5.5 IL-10/12-double-deficient mice developed much worse radiation pneumopathy. WT and IL-10/12-double-deficient mice received thoracic irradiation at dose of 14.5 Gy. (**A**) Survival curves at 25 weeks post-irradiation by Sigmaplot 8.0 showed IL-10/12-double-deficient mice have much worse survival. (**B**) Hydroxyproline assays show that the irradiated IL-10/12-/- mice have both much worse and earlier onset of pulmonary fibrosis. (**C**) Gene expression of collagen 3α1 increased after thoracic irradiation. ***** $p < 0.05$, compared with non-irradiated controls of the same genotype.

enhanced T_H2 responses (increased IL-4 and IL-13 expression) and severe lung fibrosis. In our model of radiation-induced lung injury, irradiated IL-10/12$^{-/-}$ mice have markedly increased premature death at 25 weeks post-irradiation compared to irradiated WT (26% vs 79%, p=0.02) (**Figure 5.5A**). Moreover, the irradiated IL-10/12$^{-/-}$ mice have much worse and earlier onset of pulmonary fibrosis (**Figure 5.5B**). By 20 weeks post-irradiation the irradiated IL-10/12$^{-/-}$ mice already had significantly greater lung hydroxyproline content not only compared to non-irradiated control IL-10/12$^{-/-}$ mice, but also compared to the irradiated WT mice at 32 weeks post-irradiation. Collagen 3α1 gene expression increased at 1 week post-irradiation and remained elevated in the following period in both irradiated WT and IL-10/12$^{-/-}$ mice (**Figure 5.5C**). As expected, histologic images show that irradiated IL-10/12$^{-/-}$ mice had diffuse inflammation and fibrosis compared to only focal inflammation and fibrosis in WT mice (**Figure 5.6**). Consistent with our hydroxyproline and histologic analyses, lung function analyses showed decreased lung compliance and increased tissue elastance in the irradiated IL-10/12$^{-/-}$ mice at 20 weeks post-irradiation (**Figure 5.7**).Changes in lung compliance and tissue elastance were similar to what we saw in irradiated WT mice at 32 weeks post-irradiation. While irradiated WT mice at 20 weeks post-irradiation already had some decreased lung compliance and increased tissue elastance compared to WT control mice, their lung function was better than IL-10/12$^{-/-}$ mice at the same time point.

In summary, in our model of radiation-induced lung injury, IL-10/12$^{-/-}$ mice have much worse and earlier onset of fibrosis, and greater mortality after thoracic radiation, again supporting T_H2 cells as mediators of radiation lung injury.

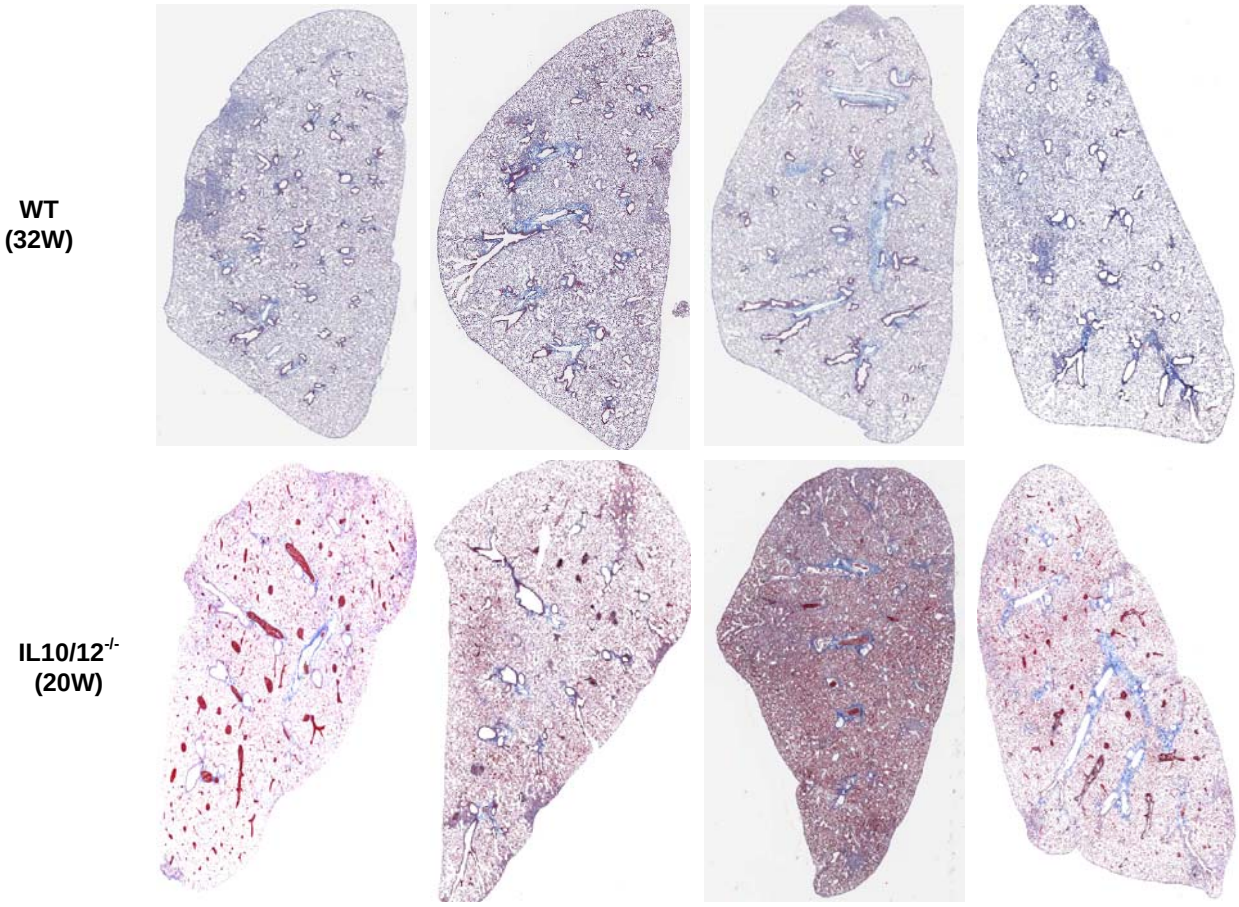


Figure 5.6 Histologic analyses show that the IL-10/12$^{-/-}$ mice have even worse radiation-induced lung injury than irradiated WT mice. WT and IL-10/12$^{-/-}$ mice were thoracically irradiated with a single dose of 14.5 Gy. The lungs from WT and IL-10/12$^{-/-}$ mice were harvested at 32 and 20 weeks post-irradiation, respectively, and stained with Masson's trichrome (MT). Whole sections of MT-stained lungs show worse inflammatory and fibrotic foci in IL-10/12$^{-/-}$ mice.

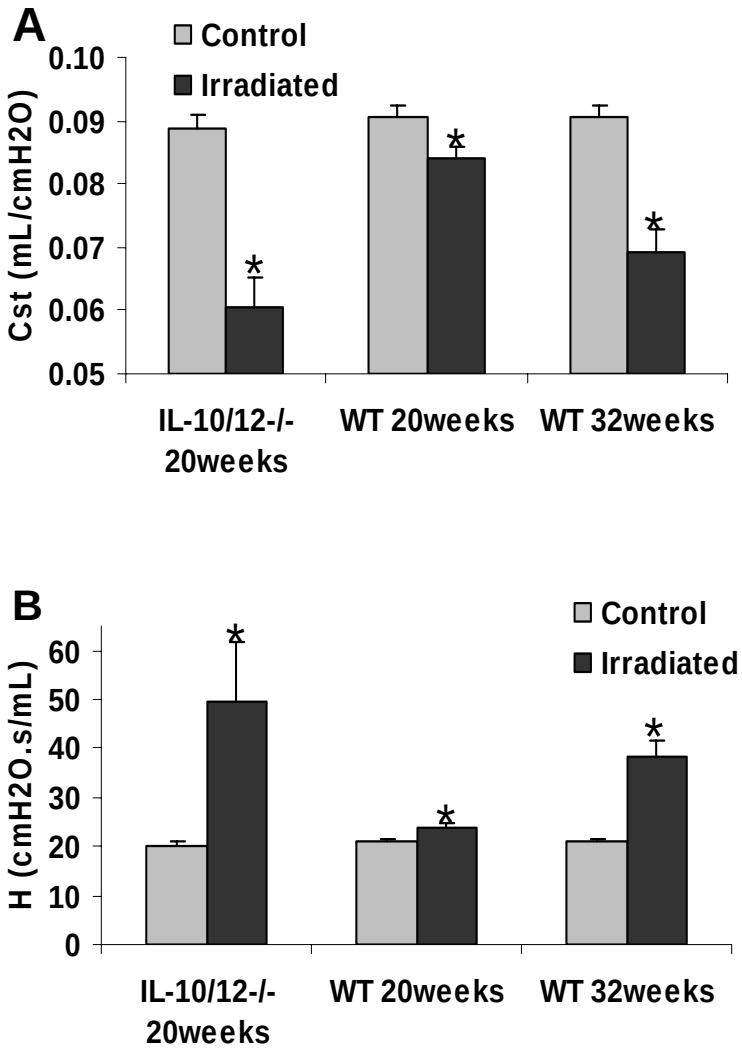


Figure 5.7 Analyses of lung mechanics demonstrate that IL-10/12-/- mice have even greater loss of lung function than irradiated WT mice. Lung mechanics were measured in anesthetized, paralyzed, and mechanically ventilated mice at 32 weeks post thoracic irradiation. **A:** Static compliance (Cst) was determined by fitting the Salazar-Knowles equation to pressure-volume curves. **B:** Tissue Elastance (H) was determined by applying prime wave impedance values to the constant phase model. *****$p<0.05$ compared with corresponding age-matched controls.

4. Inflammatory cells infiltrates differ in IL-10/12$^{-/-}$ and WT mice after irradiation

Flow cytometric analyses showed that the IL-10/12$^{-/-}$ mice had increased CD4^{+} T cells at 20 weeks post-irradiation (**Figure 5.8A**), while CD4^{+} T cells in WT mice increased at 20 and 32. The monocyte/macrophage accumulation occurs later: in WT (32 weeks), but IL-10/12$^{-/-}$ mice already had marked increases at 20 weeks (**Figure 5.8B**). Consistent with this finding, the histologic images confirm that the irradiated IL-10/12$^{-/-}$ mice had numerous foamy macrophages accumulating in their lungs as early as 20 weeks (**Figure 5.8C**). In summary, these findings from the relatively T_H1-deficient and T_H2-enhanced IL-10/12$^{-/-}$ mice support our other findings that T_H2 cells are crucial mediators of radiation pneumonitis and fibrosis.

5. *Cytokine gene expression in IL-10/12$^{-/-}$ and WT mice after thoracic irradiation*

To investigate the possible mechanisms for the pathologic differences seen in the lungs of irradiated IL-10/12$^{-/-}$ and WT mice, we analyzed T_H1, T_H2, and T_H17 cytokine gene expression by quantitative RT-PCR. As expected, the IL-10/12$^{-/-}$ mice, which showed a T_H2-skew response in murine *Schistoma* fibrosis model, had very low mRNA expression of the T_H1 cytokine IFN-γ both before and after irradiation. Furthermore, the mRNA expression of the T_H1 cytokines, IL-1β and TNF-α were relatively lower in IL-10/12$^{-/-}$ mice than in WT mice (**Figure 5.9A**). The T_H2 cytokines, IL-4 and IL-13 were elevated after irradiation in IL-10/12$^{-/-}$ mice, and these elevations occurred earlier than in WT mice (**Figure 5.9B**). However, we found striking differences in T_H17 cytokine profiles between IL-10/12$^{-/-}$ mice and WT mice after irradiation (**Figure 5.9C**). Irradiated IL-10/12$^{-/-}$ mice displayed an earlier onset of

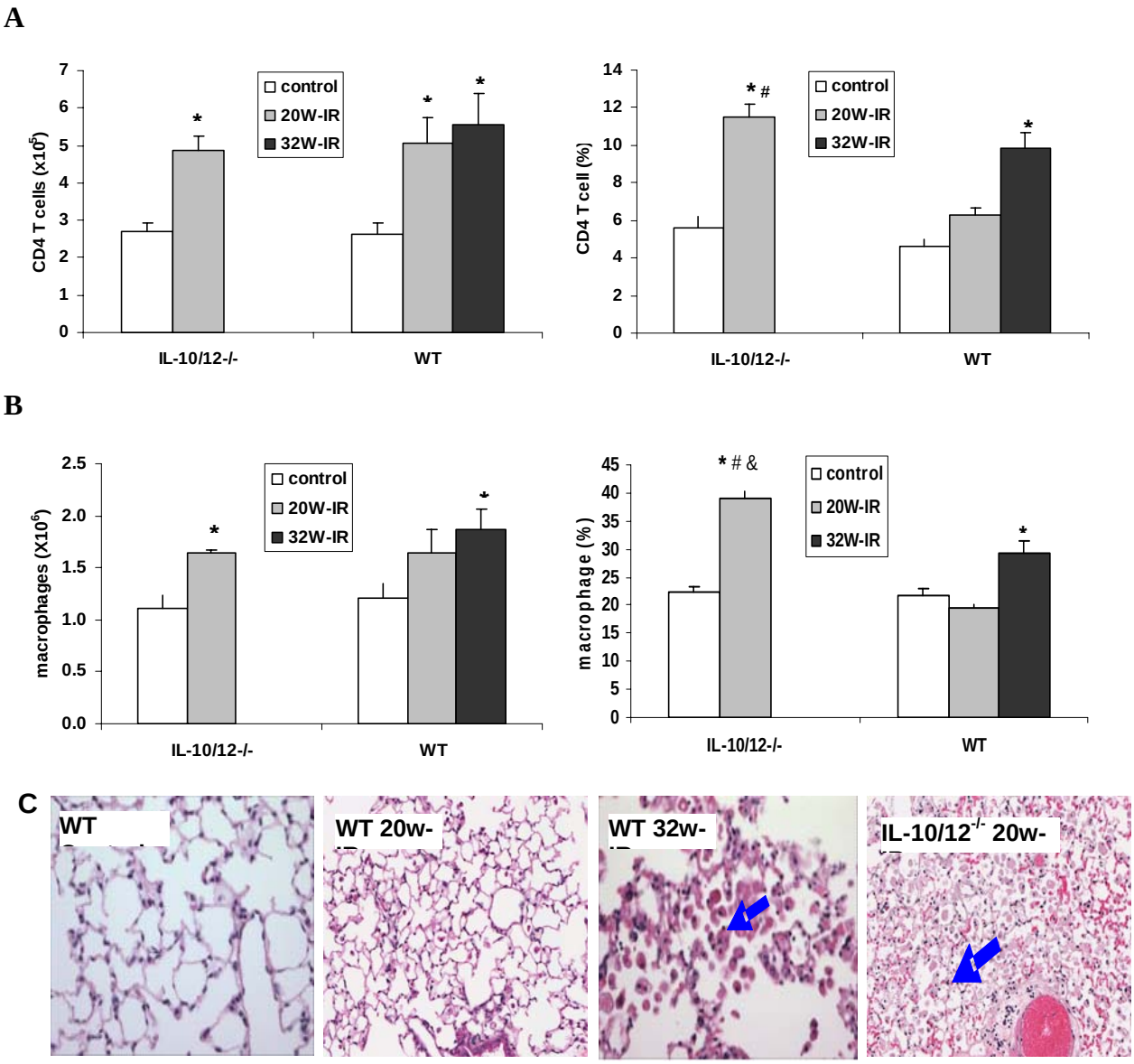
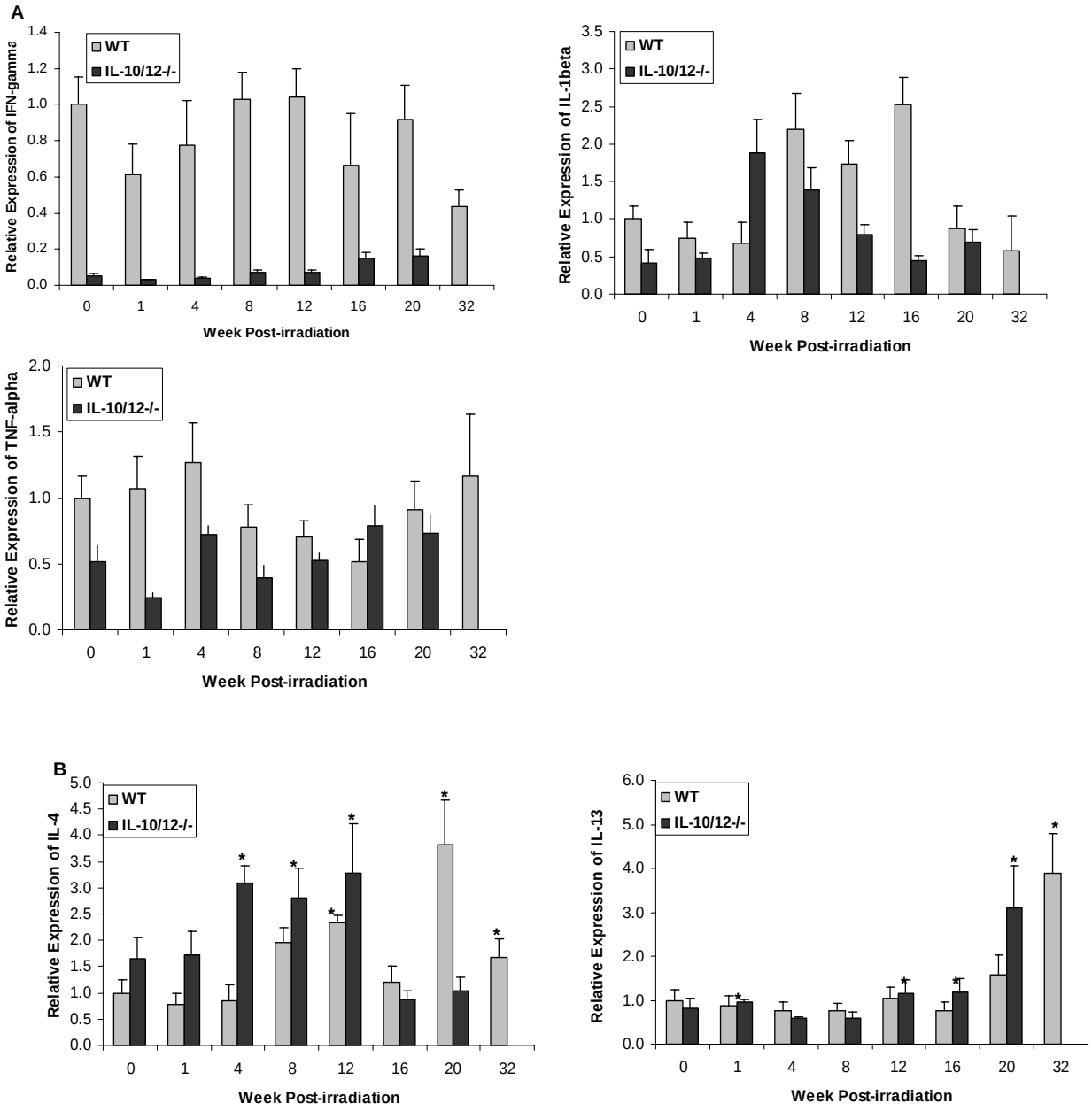


Figure 5.8 Inflammatory cell infiltrates in the lungs of irradiated WT and IL-10/12$^{-/-}$ mice. **A**. Greater infiltration of CD4^{+} T cells into the lungs of IL-10/12-/- mice than WT mice after thoracic irradiation. **B**. Macrophage/monocytes accumulation was increased in the lungs of irradiated IL-10/12-/- compared to WT mice. **C**. Arrows indicate foamy macrophages in H&E staining of irradiated mouse lungs. Control = non-irradiated; 20W-IR = 20 weeks post-irradiation; 32W-IR = 32 weeks post-irradiation. Values shown are the means±SEM of 3-6 mice per group.*$p<0.05$, compared to control; # $p<0.05$, compared to WT at same time point; & $p<0.05$, compared to WT at 32 weeks.



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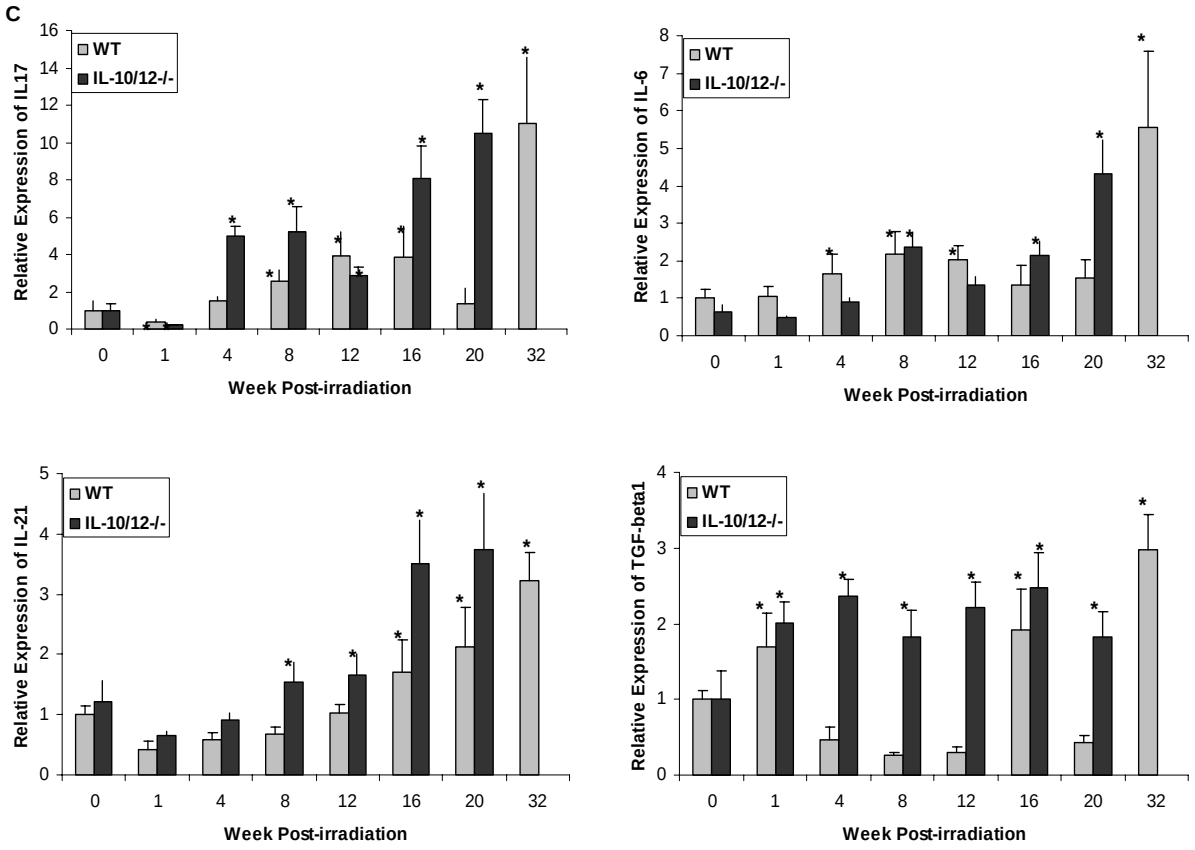

Figure 5.9 mRNA expression of T_H1, T_H2 and T_H17 cytokines in the lung homogenates from the irradiated WT and IL-10/12-deficient mice. Total RNA was isolated from the left lungs at 0, 1, 4, 8, 12, 16, 20, and 32 weeks post-irradiation for analysis by quantitative real-time PCR. Data are presented as the fold change in gene expression normalized to an internal control gene (Gus) and relative to a calibrator sample (non-irradiated WT mouse). **A.** T_H1 cytokines interferon gamma (IFN-γ), IL-1β, and TNF-α; **B**. T_H2 cytokines IL-4 and IL-13; **C**. T_H17 related cytokines IL-17, IL-6, IL-21 and TGF-β1. *$p < 0.05$, compared to nonirradiated controls of the same genotype. Data for IL-10/12-deficient mice not shown because no mouse of this genotype survived past 27 weeks.

increased IL-17 expression than in WT mice, increasing significantly at 4 weeks and approaching 10-fold higher levels by 20 weeks with associated very low IFN-γ levels. In contrast, IL-17 gene expression in irradiated WT mice increased at 8-16 weeks, returned to basal level at 20 weeks and again increased 10-fold by 32 weeks. Another T_H17 cytokine, IL-21, also showed an earlier onset of elevation in irradiated IL-10/12$^{-/-}$ mice. TGF-β1 and the T_H2 cytokine, IL-6, both of which are required for T_H17 differentiation, also had a similar pattern of elevations in irradiated IL-10/12$^{-/-}$ mice. It is notable that IL-4 expression, which suppresses T_H17 differentiation, was lower in irradiated IL-10/12$^{-/-}$ mice than in WT mice at 20 weeks. Moreover, increased IL-17 expression was not observed in radiation fibrosis-resistant neither CCL3$^{-/-}$ nor CCR1$^{-/-}$ mice (**Figure 5.10**). Together, these data support T_H2 and T_H17 cells as mediators in radiation lung fibrosis.

Lastly, we investigated whether there were differences in Treg cells, which suppress immune responses in our model. Although Foxp3 expression in irradiated IL-10/12$^{-/-}$ mice was relatively higher than WT mice, Foxp3$^+$ Treg cells in IL-10/12$^{-/-}$ mice may not function normally since IL-10 is one of cytokines Treg cells secrete in their roles as suppressors of immune responses. Thus, we tested Treg function in IL-10/12$^{-/-}$ mice in suppression assays, but found that they functioned normally (**Figure 5.11**).

6. Increased IL-17 producing CD4$^+$ T cells in irradiated IL-10/12$^{-/-}$ mice

To characterize the IL-17 producing cells present in the lung of irradiated mice, IL-17 intracellular staining was performed. We found that in contrast to CD8$^+$ T cells, lung CD4$^+$ T cells represent the critical T cell subset expressing a polarized T_H17 cytokine response.

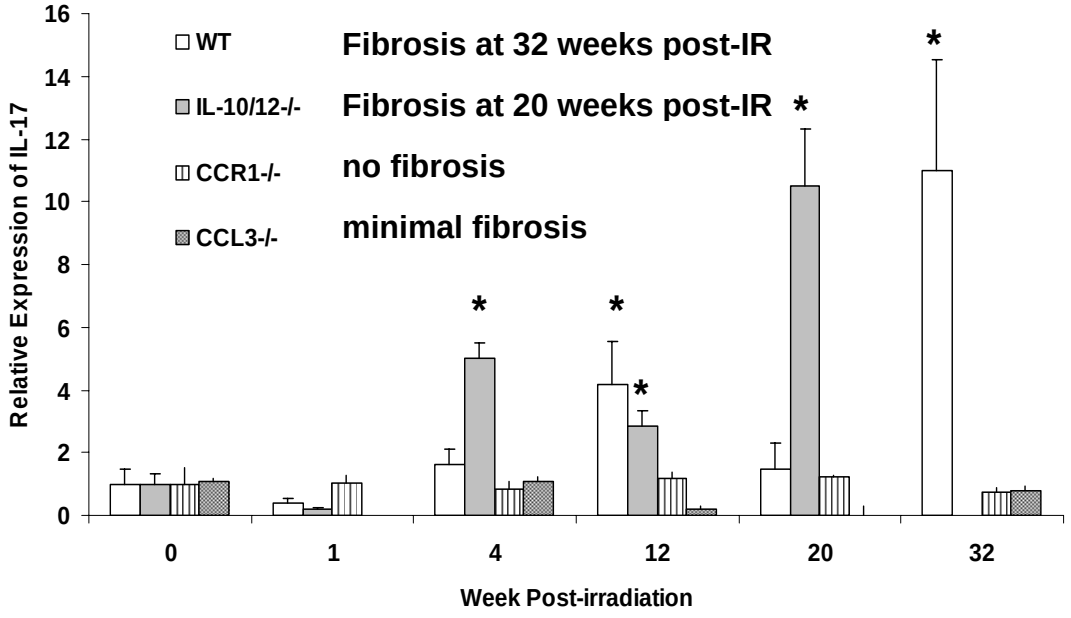

Figure 5.10 IL-17 expression in the lung homogenates from the irradiated WT, CCL3-deficient, CCR1-deficient and IL-10/12-deficient mice. Total RNA was isolated from the left lungs at 0, 1, 4, 12, 20, and 32 weeks post-irradiation for analysis by quantitative real-time PCR. Data are presented as the fold change in gene expression normalized to an internal control gene (Gusb) and relative to a calibrator sample (non-irradiated WT mouse). *$p < 0.05$, compared to nonirradiated controls of the same genotype.

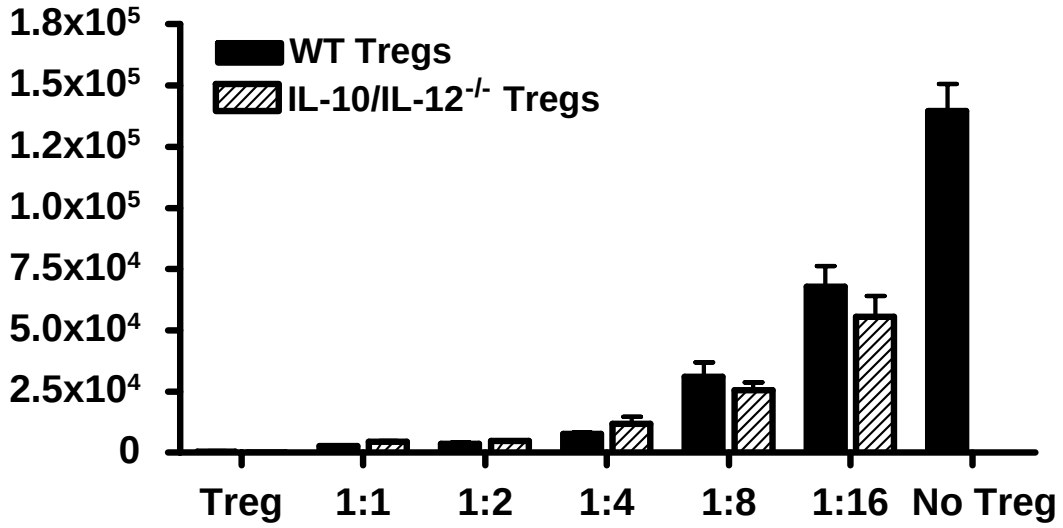

Figure 5.11 Treg cells in IL-10/-deficient mice have normal suppression function by in vitro Treg suppression assay. Stimulators (1 × 105) were incubated with responders (WT CD4+CD25- T cells, 1 × 105) and various ratios of Tregs from WT or IL-10/-deficient mice. Cultures were incubated for 3 days at 37°C and 1 μCi 3H-thymidine was added for the last 16 hours of culture. Incorporation of 3H-thymidine was assessed.

Consistent with our gene expression data, there was 3-4 fold increase of IL-17 producing $CD4^+$ T cells in IL-10/12-deficient mice at 20 weeks after irradiation (**Figure 5.12**). In contrast, the percentage of IL-17 producing $CD4^+$ T cells in WT mice did not change at this time point. Taken together, these data support a role for T_H17 cells in promoting radiation lung injury in WT and IL-$10/12^{-/-}$ mice, while absence of T_H17 cells in irradiated $CCR1^{-/-}$ mice is associated with the radioprotected phenotype.

We plan to do IL-17 intracellular staining on WT mice at 32 weeks post-irradiation as we expect WT mice also have increased IL-17 producing $CD4^+$ T cells by that time point.

7. Study on IL-17 receptor A-deficient mice (ongoing)

To confirm the role of IL-17 signaling pathway in radiation-induced lung injury, *IL*-17 receptor A-deficient mice (IL-$17RA^{-/-}$) were treated in our radiation model. Preliminary data show that IL-$17RA^{-/-}$ mice have much improved survival compared to WT mice after radiation (100% vs 62% at 28 weeks, $p<0.05$).

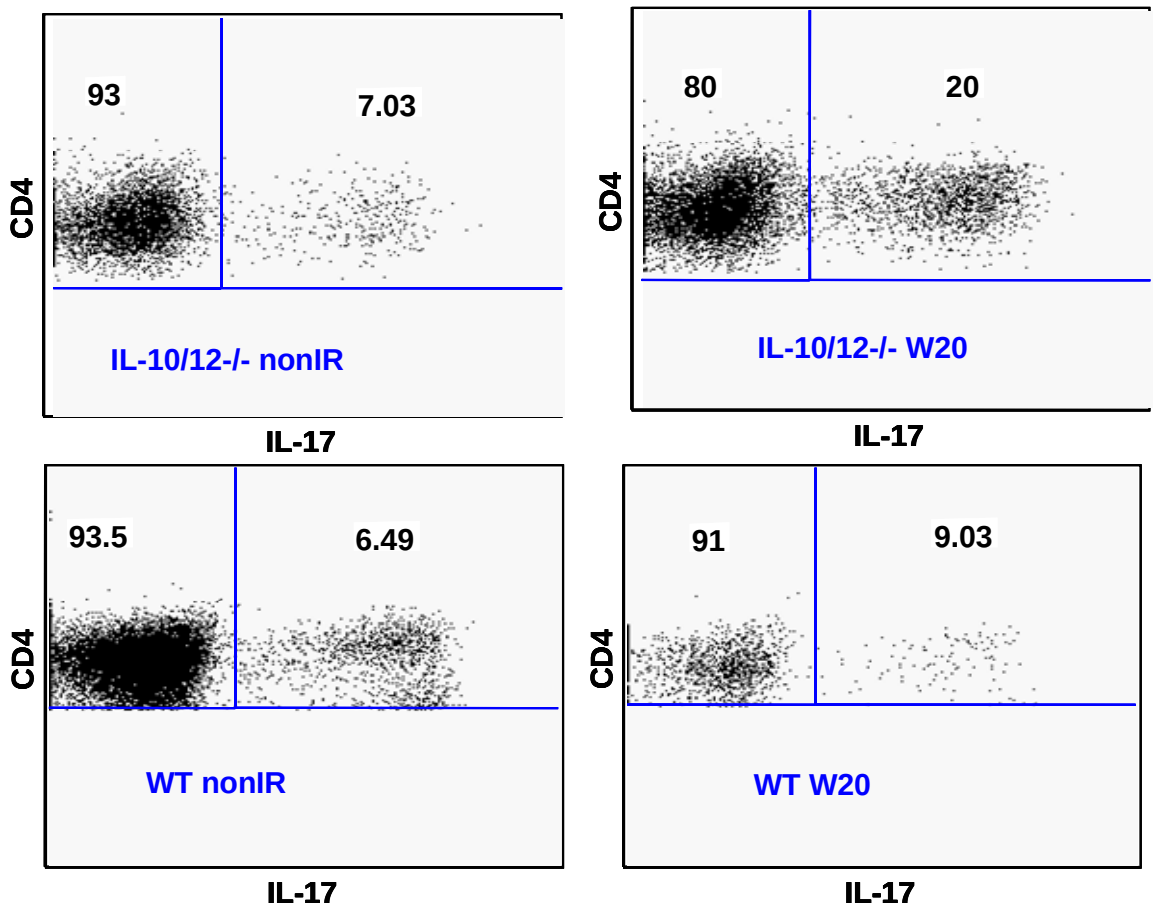

Figure 5.12 Accumulation of IL-17-producing $CD4^+$ T cells in IL-10/12-deficient mice at 20 weeks after irradiation. Lung leukocytes were isolated from irradiated mice. Isolated cells were stimulated with PMA, ionomycin, and Brefeldin, and then, Intracellular cytokine IL-17 staining was performed. Flow cytometric analyses of T_H17 cells from (**A**) IL-10/12-deficient mice, (**B**) WT mice. The percentages of IL-17 CD4+ T cells are shown in the upper right quadrant of each density plot.

V.D. Discussion

A variety of inflammatory cells are recruited into the lungs after thoracic irradiation, however, specific roles for specific subtypes of inflammatory cells have thus far been poorly understood in the pathogenesis of radiation-induced lung injury, particularly as regards fibrosis. Evidence from several other models of lung fibrosis suggests that T cells contribute significantly to the development of pulmonary fibrosis. Our previous studies show that the radiation fibrosis-resistant CCL3-deficient and CCR1-deficient mice have much less infiltration of $CD4^+$ and $CD8^+$ T cells into the lung after thoracic irradiation, suggesting that these cells may, at least part, mediate radiation pneumonitis and fibrosis.

The present studies, using CD4-deficient and CD8-deficient mice in our model, showed that in the absence of $CD4^+$ T cells, irradiated mice had improved survival, reduced pulmonary fibrosis and preserved lung function, whereas the absence of $CD8^+$ T lymphocytes exacerbated radiation-induced pulmonary fibrosis. These findings suggest that the recruitment of $CD4^+$ T cells to the lung is a critical step in the immune and fibrotic response to radiation.

Naive $CD4^+$ T helper cells can differentiate into one of three lineages of effector T helper cells - T_H1, T_H2 or T_H17 cells, each of which produces characteristic cytokines. Evidence from the bleomycin model of lung fibrosis and other models of solid organ fibrosis suggests that T_H2 cytokines, e.g., IL-4 and IL-13, promote fibrosis by stimulating fibroblast secretion of extracellular matrix proteins, types I and III collagen and fibronectin, while T_H1 cytokines, e.g., IFN-γ, attenuate these fibrotic processes. Our prior studies showed that, in contrast to WT mice, the fibrosis-resistant CCR1-deficient mice had slightly increased expression of the T_H1 cytokine IFN-γ and significantly reduced expression of T_H2 cytokines

after thoracic radiation. Cytokine profile analyses from irradiated WT lungs also showed increased protein levels of the T_H2 cytokines IL-4 and IL-13. Moreover, IL-10/12-deficient mice, which display a T_H2-skewed response in murine model of hepatic and lung fibrosis [210], had even more severe and earlier onset of radiation pneumopathy than WT mice. IL-10/12-deficient mice died from radiation lung injury much earlier than WT: no IL-10/12-deficient mice survived after 27 weeks, while 68% WT mice survived at this point. IL-10/12-deficient mice developed diffuse pulmonary fibrosis much earlier after irradiation (12 weeks earlier than WT). The earlier onset of elevation of IL-4 and IL-13 in these mice suggests that these cytokines and T_H2 cells contribute to the earlier development of fibrosis.

Interestingly, we found increased IL-17 expression in the lungs of both irradiated WT and IL-10/12-deficient mice, but not in the radioprotected CCR1-deficient mice. Consistent with the earlier onset of radiation injury we saw in these mice, IL-10/12-deficient mice also had an earlier onset of increased IL-17 expression after irradiation than WT mice. At an early stage after irradiation (8-16 weeks), WT mice showed some T_H1 responses by increasing IL-1β expression that later diminished (after 20 weeks). IL-4 expression was also increased at 8-20 weeks but also decreased later (32 weeks). In contrast, IL-13 expression increased gradually to a maximum 4-fold increase over basal levels at 32 weeks. Thus, the T_H2 cytokine IL-13 may be more important than IL-4 in the development of radiation-induced lung fibrosis. IL-17 expression increased slightly at some points before 20 weeks but returned to normal levels until achieving a maximum 10-fold increase at 32 weeks. In summary, the trends of cytokine changes we saw after thoracic irradiation in WT mice indicate a transition from T_H1 responses to T_H2 and T_H17 responses occurs around 20 weeks (**Figure 5.13**). The irradiated IL-10/12-deficient mice showed a similar correlated with the

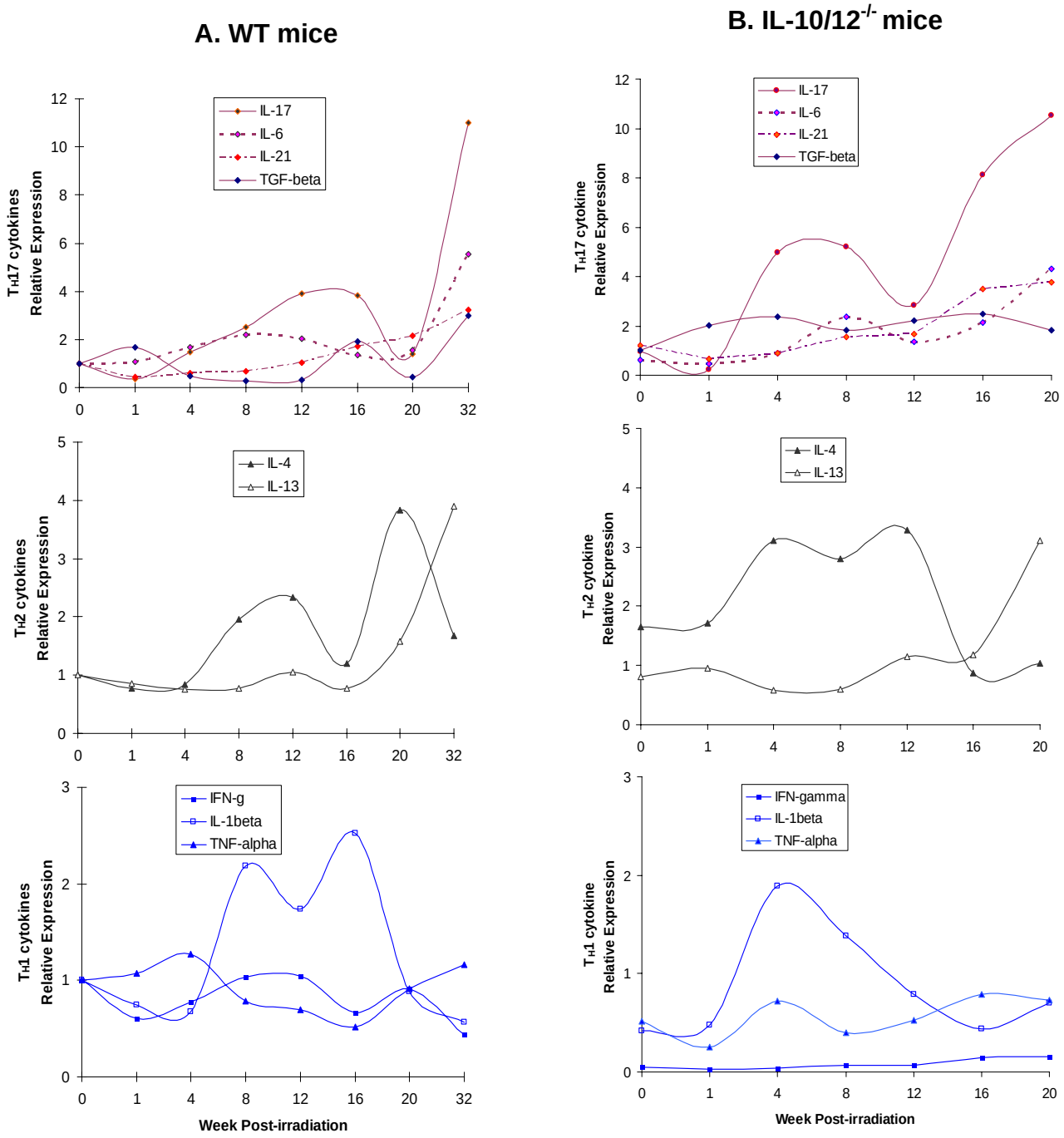

Figure 5.13 Gene expression patterns of T_H1, T_H2 and T_H17 cytokines in the lungs of irradiated WT and IL-10/12$^{-/-}$ mice. The fold changes of cytokine expression were generated by comparing with WT non-irradiated mice. (**A**) Fold change in mRNA expression of cytokines in WT mice. (**B**) Fold change in mRNA expression of cytokines in IL-10/12-deficient mice.

pattern, but had an earlier shift toward T_H2 and T_H17 responses (at 12-16 weeks), that again, earlier onset of lung fibrosis.

Consistent with our cytokine gene expression data, the intracellular cytokine staining data also showed that the frequency of IL-17 producing $CD4^+$ T cells in the lungs of IL-10/12-deficient mice increased several fold at 20 weeks post-irradiation: this was not seen in irradiated WT mice at the same time point. To further investigate the importance of the IL-17 signaling pathway in radiation-induced lung fibrosis, we have now irradiated the IL-17 receptor A-deficient mice. The preliminary results show that IL-17RA-deficient mice have much improved survival after thoracic irradiation (100% survived to 28 weeks). We expect to find significant protection from radiation-induced lung inflammation and fibrosis in IL-17RA-deficient mice.

The mechanisms by which IL-10/12-deficient mice have an earlier shift toward T_H17 phenotype in the lung after irradiation is yet unknown. One possibility is that Treg cells in IL-$10/12^{-/-}$ mice may not function normally as in the WT mice since IL-10 is one of cytokines Treg cells secrete to suppress immune response. However, comparison of Treg cell function of WT and IL-$10/12^{-/-}$ mice in *in vitro* suppression assays suggest that Treg function is adequate in IL-$10/12^{-/-}$ mice.

T_H17 cells play a critical role in sustaining inflammatory responses. Recently they have been suggested to be involved in the pathogenesis of fibrosis [206, 208]. Our findings support the critical role of T_H17 cells in radiation-induced lung fibrosis. The irradiated IL-10/12-deficient mice, with extensive T_H17 cells expansion in the lungs after thoracic irradiation, also had markedly increased accumulation of macrophages and collagen deposition in the lungs. IL-17 also can directly mediate the accumulation of macrophages.

For example, an anti-mouse IL-17 mAb reduced the accumulation of macrophages in allergen-induced airway inflammation [205, 211]. IL-17 also induces the migration of blood monocytes from sensitized and allergen-challenged mice inflammation [205, 211]. The mRNA expression of MMP-9 by airway neutrophils and macrophages in vivo was down-regulated by anti-IL-17. Il-17 also increased the survival of macrophages in a concentration-dependent manner. This IL-17-based survival advantage may result from increased FAS antigen expression selectively in bronchoalvelar macrophages, as FAS-antigen functions in the major extrinsic pathway of apoptosis [205, 211]. Thus, the increased $CD4^+$ T_H17 cells in lungs after irradiation may promote the accumulation and survival of macrophages, which are also major source of TGF-β, and which, in turn, acts on fibroblasts to increase collagen synthesis and cause fibrosis.

Of interest, significant number of IL-17 producing cells were neither CD4 nor CD8 negative, but Thy1.2 positive in irradiated mouse lungs. It is worth defining what these cells are, since CD4-deficient mice only showed partial protection from radiation-induced lung injury, suggesting that other IL-17-producing cells, like δγ T cells, may potentially involve in the development of radiation lung injury.

In summary, we found that the imbalance of T_H1, T_H2 or T_H17 cells is important to the development of lung inflammation and fibrosis induced by thoracic irradiation. Our data are the first to indicate an important role of $CD4^+$ T_H17 cells in radiation-induced lung injury. $CD4^+$ T_H17 cells may promote macrophage recruitment and survival and thus exacerbate lung fibrosis. Studies are ongoing to confirm the necessity of the IL-17 signaling pathway in the development of radiation-induced lung injury and further examine the mechanisms by which $CD4^+$ T_H17 cells promote radiation-induced lung fibrosis.

VI. Summary and Future Directions

Radiation-induced lung injury affects many patients undergoing thoracic irradiation. Dissection of the cellular and molecular mechanisms mediating radiation-induced lung inflammation and fibrosis is the key to revealing potential novel therapeutic targets.

Our studies, for the first time, demonstrate the critical roles of chemokines and their receptors in the development of radiation-induced lung injury and that a specific inhibitor of a chemokine receptor can block the infiltration of leukocytes and thereby ameliorate the progressive pneumonitis and fibrosis.

We found that radiation-induced increases in the expression of the CC chemokine, CCL3, mediated the recruitment of lymphocytes and macrophages, and thereby contributed to the development of radiation-induced lung inflammation and fibrosis. Furthermore, we demonstrated that the lack of a CCL3 receptor, CCR1, is protective from radiation-induced lung inflammation and fibrosis. Lastly, we have shown that CCR1 blockade with a specific antagonist, BX471, conferred the same protective effect as the genetic deletion of the receptor. The radioprotection we saw with CCR1 deficiency results from reduced lung infiltration by lymphocytes and monocytes/macrophages and an associated reduction of the profibrotic cytokines, TGF-β, IL-4 and IL-13. Cumulatively, these observations, for the first time, demonstrate that CCR1 blockade may offer a promising therapeutic strategy to reduce radiation-induced lung injury. However, since thoracic radiation treatment is given for patients with tumors, it is also crucial to understand the effects of CCR1 deficiency on tumor

growth and metastasis before using CCR1 antagonists for preventing radiation lung injury in patients. Further study on the effects of BX471 on tumor growth and metastases are ongoing.

Our research is the also first to demonstrate that the imbalance of T_H1, T_H2 or T_H17 cells is important for the development of radiation-induced lung inflammation and fibrosis. Cytokine analyses of whole lung homogenates at various time points after irradiation showed a predominant T_H2 and T_H17 phenotypes in lungs of irradiated mice. A shift toward T_H2 and T_H17 responses correlated with the development of radiation lung fibrosis. Our findings (chapter V) indicate the involvement of $CD4^+$ T_H17 cells in radiation fibrosis. Confirmatory studies of thoracic irradiation using mice with IL-17 receptor A-deficiency are ongoing. Together, these studies will clarify the role of the IL-17 signaling pathways in the pathogenesis of radiation lung injury. Further studies are also needed to determine the mechanisms by which IL-17 mediates radiation lung injury. Additionally, we should identify the role of other IL-17 producing cells, such as gammadelta T cells and NKT cells, in our model.

In contrast to the radioprotected CCL3-deficient and CCR1-deficient mice, we found marked accumulation of macrophages in the lungs of the radiation injury-sensitive WT and IL-10/12-deficient mice, suggesting that macrophages are also important in the development of radiation lung inflammation and fibrosis. A new transgenic mouse (CD11b-DTR) in which macrophages could be selectively depleted offers a powerful new tool to study macrophages [72]. This transgenic mouse has a diphtheria toxin (DT) inducible system that transiently depletes macrophages. The transgene insert contains a fusion product involving simian diphtheria toxin receptor and green fluorescent protein under the control of the human *ITGAM* (integrin alpha M) promoter (CD11b). Therefore, macrophages could be specifically

ablated by a simple intravenous injection of DT at specific time intervals after irradiation to determine their role in radiation lung injury.

VII. References

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