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The therapeutic value of RDP58 in a mouse model of radiation-induced lung damage

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THE THERAPEUTIC VALUE OF RDP58 IN A MOUSE MODEL OF RADIATION-
INDUCED LUNG DAMAGE

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

By

Charles J. Sylvia, Jr.

December 2005

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ABSTRACT

THE THERAPEUTIC VALUE OF RDP58 IN A MOUSE MODEL OF RADIATION-INDUCED LUNG DAMAGE

by Charles J. Sylvia, Jr.

The goal of this project was to evaluate the efficacy of a novel anti-inflammatory peptide, RDP58 in a mouse model of radiation-induced lung damage. Classical indicators of lung damage were measured as dependant variables. These included protein concentration and cellular differentials of the bronchoalveolar lavage fluid (BAL), lung tissue histopathology, cytokine analysis and immunohistochemical staining. Together, these markers were used to assess the degree of lung damage caused by ionizing radiation and were used to assess the therapeutic efficacy of RDP58.

The test mice received 12 Gy of γ -radiation to the upper thorax. Irradiated mice were then given vehicle or RDP58 once per week. The RDP58 treated group showed improved survival at 21 days post-exposure ($P < 0.05$). No changes were noted with regard to lung tissue histopathology. Bronchoalveolar lavage (BAL) concentrations of TNF- α were significantly lower ($P < 0.05$) in the RDP58 treated group compared to the vehicle control on Day 4, but not on Days 7 and 14. Overall, RDP58 improved survival at 21 Days post-radiation exposure; however the mechanism by which this occurs is not clear nor could be correlated to the inflammatory processes in the lung.

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INTRODUCTION

The goal of this study was to assess the therapeutic protection offered by a novel anti-inflammatory compound, RDP58, in a mouse model of radiation-induced lung damage. RDP58 has been shown to act as an immunomodulator, thus interfering with the macrophage to T-cell interaction during the inflammatory process by inhibiting the secretion the cytokine TNF- α and the enzyme hemeoxygenase-1 (HO-1) [49]. It has also been postulated to decrease IL-12 secretion (likely through the modulation of TNF- α) [51]. TNF- α is a cytokine that has been implicated in many disease processes, such as septic shock, inflammation and radiation-induced lung damage. IL-12 is a signaling molecule secreted by macrophages that binds to its respective T-cell receptor and is largely responsible for T-cell immunity by its immunomodulatory activities on Th1 cells. IL-12 has been implicated as being an important cytokine modulating pathological Th1 type immune responses, such as in inflammatory bowel disease and multiple sclerosis.

It has been postulated that the inflammatory response following radiation exposure is in part the cause for the pathogenesis and fibrosis seen in the months following radiation exposure [1, 3, 4, 5]. Hence, the rationale for treatment in this experiment is to modulate the inflammatory response in an attempt to decrease the amount of lung damage caused by ionizing radiation. The clinical implication would be for patients undergoing radiation therapy in the treatment of lung cancer; with a reduction in the side effects associated with radiation treatment, namely pulmonary pneumonitis and late fibrosis.

The initial endpoints to establish efficacy were survival, lung histopathology and immunohistochemical staining at 21 days post-radiation exposure. To fully investigate the events leading to pulmonary lung damage and fibrosis, bronchoalveolar lavage (BAL) fluid analysis and lung tissue cytokine concentration were performed to help evaluate the molecular events surrounding this enigmatic process. The animals were divided into cohorts for each of the desired endpoints. These were Days 4, 7, and 14 for the *in vitro* cellular and biochemical evaluations and Day 21 for the histopathology and survival. Previous studies [8, 15, 34, 40, 46] have validated the following in-vitro measurements to assess lung damage in the mouse radiation model: protein content of the BAL fluid, cellular content of the BAL and cell differential counts of the BAL fluid. It was also decided to measure the cytokine levels of both TNF- α and IL-12 in lung tissue and BAL. The goal of testing cytokine concentration was two-fold: 1) to investigate the concentration of these cytokines following radiation exposure and 2) to assess how RDP58 can modulate these cytokine concentrations in order to help elucidate the mechanism of action and therapeutic potential. We assessed both histopathology and immunohistochemistry along with *in vitro* BAL and lung tissue cytokine measurements to fully evaluate the protective advantage of RDP58 in the mouse radiation-induced lung damage model.

LITERATURE REVIEW

Radiation therapy is a common treatment for the control of lung cancer and malignancies of the chest; however, lung exposure to the radiation field is a major dose limiting consideration [1]. This is due to the relatively low regenerative capacity of the lung tissue. The earliest reports on the adverse effects of radiation on lung tissue and pleura date back to Hines in 1922 [2]. Radiation-induced lung damage has been reported by numerous authors [1, 3, 4], and includes two distinct clinical phases: pulmonary pneumonitis and fibrosis. These are divided into three main phases of the radiation response: 1) an early (acute) phase, latent period of pneumonitis that occurs within the first month, 2) an intermediate phase that consists of exudative pneumonitis that occurs from the third week to several months post exposure, and 3) a late phase, where fibrosis of the lung occurs after six months post-exposure. There are many theories surrounding the possible causes of the above clinical symptoms. The reader is referred to the excellent classical reviews by Gross [5], Coogler *et al.*, [1] and Morgan and Abid *et al.* [4] for a comprehensive description of radiation effects on the lung.

The physical damage caused by ionizing radiation occurs when energy from the radiation is transferred to the tissue or chemical entity it encounters. This energy transfer occurs by the interaction of the incoming photons with the electrons of the tissue (or chemicals) it encounters. This encounter occurs via three primary processes: 1) the photoelectric scattering process, 2) the Compton scattering process [also known as incoherent scattering process], 3) pair production [discussed below].

The photoelectric absorption process occurs when the incoming photon from the radiation source interacts with an orbital electron of the targeted atom. The incoming photon then disappears and a photoelectron is ejected from its orbital position, usually in the K, L, or M shells of the atom. The orbital position that is vacated from the outgoing photoelectron is then replaced by one of the electrons from an outer orbital. This shifting of electron orbital is accompanied by the release of a fluorescence photon, due to the shift in orbital binding energies of the vacated photoelectron and the energy of the electron filling the vacated orbital. A diagram of this process is shown in Figure 1. The incoming photon energy is equal to (Plank's constant) x (photon frequency), written as $E = h\nu$. If the photon interacts with an electron in an outer orbital, the energy of the photon must be equal to the binding energy (B.E.) of the electron that is being displaced. The kinetic energy of the photoelectron is equal to the energy of the incoming photon minus the binding energy of the electron displaced. The equation for this process is: photoelectron $KE = h\nu - B.E.$ This type of interaction is most likely to occur if the energy of the incoming photon is just greater than the binding energy of the electron with which it interacts. Due to this effect, when the attenuation coefficient (the measure of the probability of interaction) is plotted as a function of the photon energy, sharp peaks at the binding energies of the various orbital shells occur, with a strong dependence on the atomic number of the atom. The plot of this interaction is referred to as the photoelectric mass partial attenuation coefficient (also called partial cross section) commonly designated by the symbol (τ/ρ) . Again, the specific values for this will vary on the

specific media. This energy transfer gives the electron significant momentum to cause damage to biologic tissue by a variety of mechanisms discussed below.

Another process by which an incoming photon can interact with tissue is by a process known as Compton scattering. The Compton process is known as an incoherent scattering process. Coherent scattering occurs when a photon passes through a medium with no transfer of kinetic energy. This type of scattering is of very little interest in a biologic system. Incoherent scattering (Compton scattering) on the other hand occurs when an incoming photon interacts with a loosely bound electron in the media and a transfer of kinetic energy occurs. A visual diagram of this process is shown in Figure 2. The mathematical calculation of this process will not be addressed here but the key highlights follow: The incoming photon of energy ($h\nu$) interacts with a loosely bound electron of the tissue. The electron is then accelerated in recoil with a certain kinetic energy and momentum. The original photon then reappears as a scattered photon of lower energy. This process is almost independent of the atomic number. The cross section decreases as the incoming photon energy increases. The fraction of energy delivered to the tissue increases as the photon energy increases. This process is the most important for energy absorption in soft tissues for photon energies in the range of 100 KeV to 10 MeV.

Pair production occurs when a highly energetic photon (with energy greater than 1.02 MeV) enters matter and interacts with the nucleus of the atom. This mechanism is shown in Figure 3. The high energy photon is subjected to the strong field effects of the nucleus and disappears as a photon and is converted to an electron and a positron. The

photon is simply converted from a wave to a positive and negative electron. It is of note that these electrons are created *de novo* in the mass/energy conversion of the disappearing photon. Ironically, the energy transferred to the nucleus of the atom changes very little after this interaction, except for a very small gain of kinetic energy and momentum. The electron and positron emerge with a specific kinetic energy and momentum. The positron will lose energy by transferring it to the media in a similar fashion to an electron until it reaches the Fermi energy. It will interact with a free electron to produce two annihilation photons of 0.511 MeV in opposed directions. This release of 1.022 MeV of energy accounts for the energy equivalence for the two electrons that disappeared. The total energy transferred in this system is $(h\nu - 1.022 \text{ MeV})$. This process has a rapidly increasing cross section above the threshold energy (1.022 MeV). The two annihilation photons are generally considered to be energy that is not absorbed, as they may undergo further scattering before they leave the system.

The energy transfer from the high kinetic energy electron generated by the processes mentioned above can elicit both direct and indirect effects on biologic tissue. The electrons that are scattered for example can generate free radicals that are highly reactive (an indirect effect). These can produce chemical changes including the breakage of chemical bonds in DNA, and macromolecules including lipids leading to biologic effects. Some of these chemical changes are reversible; but if oxygen molecules are present these can react with free radicals producing organic peroxides, thus “fixing” the chemical lesion in a non-restorable form. For this reason, ionizing radiation produces more biological damage when the tissue is oxygenated.

When radiation acts in a direct manner, the high kinetic energy electrons cause damage to DNA or macromolecules such as proteins and polysaccharides. This damage is more extensive but can be repaired to a limited extent by intracellular DNA repair enzymes or other repair mechanisms. It can also cause DNA double strand breaks to such an extent that the cell dies. Due to this effect, and the kinetics of cellular repair, a single large dose of radiation is more damaging than the same total dose delivered in several fractions [5].

The major indirect effect involves the interaction of radiation with water molecules causing radiolysis. On a time scale, this happens rapidly, in the order of 10^{-16} to 10^{-11} seconds. The products of this radiolysis reaction include the reactive products of $\text{OH}\cdot$, $\text{H}\cdot$, and e^-_{eq} (aqueous or hydrated electron). The water molecules surround the ionization electrons as they lose kinetic energy by multiple interactions with the surrounding environment and approach the Fermi energy. Once these electrons slow down where their kinetic energy has fallen to 100 eV or below and they have not been captured by interactions with other species (hydrogen ion or water), they attract the permanent dipoles of several water molecules to form the hydrated electron, e^-_{eq} . This entity has a lifetime of about 2×10^{-4} seconds in neutral water [6]. A diagram of the radiolysis of water can be seen in Figure 4. These free radicals and reactive oxygen species then proceed to act with biological tissue causing damage to either cellular macromolecules or to DNA [6]. This mechanism is of particular importance, since the body contains about 60 % water by weight. A summary linking the physical-time course of these events of radiation exposure to the biological events is shown in Figure 5.

The damage that occurs to DNA from both direct and indirect action is not due to the susceptibility of the DNA molecule, but more to its critical role in cell survival and replication. There is very limited redundancy in the genome and when damage occurs, it may lead to the loss of genetic coding vital to cell function and survival. Damage sustained by DNA can be of several types 1) functional groups on purine and pyrimidine bases may be irreversibly altered, leading to incorrect nucleotide pairing, 2) damage may be so extensive as to cause loss of functional groups from the DNA molecule at apurine and apyrimidine sites, 3) a radical transfer mechanism in which a radical is formed in a base site and is transferred to the sugar-phosphate backbone, leading to the loss of the base and chain scission, 4) damage to the deoxyribose-phosphate backbone may cause the breakage of a single strand, referred to as single-strand breaks (SSB's), and 5) damage to the deoxyribose-phosphate backbone in two or more nearby locations can lead to breakage of the molecule, referred to as double-strand breaks (DSB's). Of the mechanisms listed above, the most important mechanisms of damage resulting in cell death appear to be attacks on the sugar-phosphate backbone leading to SSB's and DSB's [6]. This is because these breaks may or may not be repaired by DNA repair enzymes. If the damage is irreparable, then the cell may die by either necrosis or apoptosis. The damage caused by these strand breaks is widely expressed when the cell enters mitosis, where major chromosomal aberrations result in anaphase arrest or non-viable daughter cells [5]. This can arise when the chromatids are unable to separate during mitosis, leading to anaphase arrest, ring forms, dicentrics, bridges and fragments. Unequal, asymmetric distribution of DNA during mitosis may occur resulting in aneuploidy and

cell death [7]. Some cells will die at the first post-irradiation mitosis, while others may successfully undergo one or two divisions before dying at the third or fourth post-irradiation mitosis [1, 8]. It is a well known observation in radiation biology that the response of a tissue is contingent upon the level of cell killing [6].

The most important factors determining the extent of radiation injury to tissue are its ability to repair and repopulate the affected tissue with new cells after the damage. This repopulation must involve both the dividing stem cells and the non-dividing, functionally mature cells. The former will begin to die when they attempt their first or second post-irradiation divisions. While the non-dividing differentiated cells in a tissue remain relatively unaffected by radiation and will continue to function and die at their normal rate, they will not be efficiently replaced because of the damage to the stem cell compartment [1, 9]. The time course of the repair is closely related to the efficiency of the population kinetics of the given tissue. Injury will not become apparent until the number of functional cells falls below a critical level. The time and onset of damage is less dependent on dose and more dependent on the cell kinetics of a given tissue. For this reason, in rapidly dividing tissues, such as skin, intestinal mucosa and testis, cell death and cell depletion will occur earlier than in slowly dividing tissue, such as the kidney and lung. In the kidney and lung, the damage may be delayed for months, although the onset of damage when it occurs may be quite acute. These are often referred to as “delayed acute reactions” [1, 10].

The mechanisms leading to radiation induced lung damage are not well understood, but are thought to be made up of many continuous processes. One of the

proposed events leading from radiation exposure to pulmonary pneumonitis and fibrosis is damage to the lung cells directly [11]. The alveolar epithelium consists of Type I and Type II epithelial cells in a near balanced proportion. The type I pneumocytes cover about 90% of the alveolar epithelial surface and are fixed in a post-mitotic state. These cells facilitate gas exchange in the lung. These cells will not enter mitosis after injury. This is in contrast to the type II (or granular) pneumocyte that has been shown to have a turnover time of about 28 to 35 days in mice and humans [1, 11]. The type II pneumocyte is responsible for the secretion of pulmonary surfactant lining the inside of the lung, whose role is to reduce the surface tension on the inside of the lung to enhance pulmonary compliance and prevent atelectasis. Type II pneumocytes act as a cellular reservoir for type I cells, dividing at a rate sufficient to maintain the normal cell population in the epithelium but also capable of rapid division and differentiation into type I pneumocytes upon response to injury [1].

The fact that the lung is the highest oxygenated of all the tissues contributes to radiation damage. After toxic insult or radiation exposure the alveolar surface is denuded because of the high vulnerability of the flat type I epithelial cells, due to its inability to proliferate as an end stage cell. Proliferation of the type II pneumocyte can be accelerated ten-fold under such conditions, with a number of cytokines, growth factors, and cyclins regulating this response [11, 12]. Over the course of several days the type II pneumocyte proliferation results in alveolar re-epithelialization. The immediate proliferative reaction of the type II pneumocyte after injury may be the key event in the sufficient restoration of the alveolar epithelial surface. For this reason it is speculated

that radiation via its role in damaging both type I and type II cells induces the irreversible reaction leading to pulmonary fibrosis. In addition, type II pneumocytes are specialized cells that produce enzymes involved in tissue remodeling after an injury. Of particular interest are the metalloproteases that are responsible for the proteolysis of the extracellular matrix, tissue degradation and remodeling after lung injury during the early and late stages of pulmonary fibrosis. Interestingly, these same enzymes are also secreted by the alveolar macrophage [11, 12].

The alveolar macrophage has been implicated as a key factor in the damage following irradiation of the lung. Under normal circumstances alveolar macrophages have a short turnover time of about one week. This is likely due to their migration from the general circulation to the alveoli rather than their reproduction inside the lung itself. It is a widely known fact that whole body irradiation suppresses both the cellular and humoral immune systems. This is true in the lung as well. Studies have shown that the alveolar macrophage population in the bronchoalveolar lavage (BAL) decreases following radiation exposure, but increases a few weeks later [7, 13]. This depletion was dose related and sensitive to fractionation in that a second thoracic irradiation three days after the first resulted in a greater depletion than a double dose on the first day [13]. It is hypothesized that the macrophages/monocytes that infiltrate the lung following irradiation play an important role in the response of the lung [1, 7, 15]. The exact response of macrophages to radiation, however is unclear, as some authors have reported suppression of activity [16], while others have reported little loss of function [17]. The alveolar macrophage plays an important role in host defense mechanisms against foreign

invaders such as bacteria in the lung. Hahn *et al.* [14] found that the ability of irradiated mice to remove radio-labeled bacteria from the lung was normal; however, the pulmonary bactericidal function was decreased from Day 12 to Day 21. In summary, the relationship between the alveolar macrophage population and pulmonary damage following radiation exposure is not clear.

Radiation has also been shown to cause significant damage to the capillary endothelium [1, 3, 5, 7, 18]. The vascular damage caused by radiation results in a sloughing off of dead and dying endothelial cells. This in turn causes capillary leakage both into the tissue interstitium and the alveolar surface [1]. Phillips and Margolis [22], reported that the earliest damage to rat lung following a 20 Gy exposure was primarily to the microvasculature. The damage to the endothelium was prominent after 24 hours, but there were no alterations in the alveolar epithelium or the basement membrane [23].

In the model suggested by Casserett (see Figure 6), the vascular mechanism is responsible for many of the late radiation effects. This mechanism originates from the damage caused to the microvasculature initiating a powerful feedback loop, thereby promoting fibrosis and vascular insufficiency in the specific organ, principally the smaller blood vessels and capillary network. The damage to the tissue can be a direct effect via the interaction of radiation with the cells making up the capillary endothelium and causing cellular death. The damage can also be an indirect effect by either an obstruction of the endothelial lumen by radiation induced cellular proliferation, thrombi formed from the initial injury, or a reduced functional integrity resulting from cell loss [3, 6]. This model proposes a series of steps starting with increased permeability of the

microvasculature. This is followed by a classical inflammatory response in the damaged area where progressive permanent change occurs in the endothelial tissue. These changes include fibrosis and vessel wall thickening that results in a decrease in the perfusion to the damaged tissue, increased barriers to trans-vessel wall diffusion, and an osmotic imbalance. Once these processes begin, the degeneration of the vascular system becomes worse. Fibrosis and lack of perfusion lead to further fibrosis, functional loss, and ultimately, severe impairment.

The histopathological changes to the lung have been documented in numerous review articles [1, 4, 5, 7] and are summarized in Table 1. Much of the work done in the study of radiation induced lung damage has been done in animals, especially in regard to histopathological changes. Most of the work done in humans is a late response effect, stemming from the course of radiation treatment used for certain cancer types in the thorax. It is generally accepted that the histopathological outcome following exposure to ionizing radiation is similar between animals and humans. However, the dose-effect relationship has been observed to be greater (~ 40%) in the mouse than in humans [1]. As stated earlier, the literature divides the pathological response into three phases: early, intermediate and late effects, each with its own clinically significant features. The acute phase consists of edema and inflammation of the interstitium and air spaces. Excess numbers of mononuclear inflammatory cells including foamy alveolar macrophages infiltrate the area. The air spaces usually contain excess protein and fibrin-rich exudates [1]. In a number of cases there was very little gross damage observed by light microscopy. The intermediate phase has been characterized most extensively in humans.

Histologic studies in humans 4-12 weeks post radiation treatment found that vascular lesions were evident as judged by the engorgement and thrombosis of capillaries and arterioles, resulting in edema and intimal proliferation. Medial changes in the lung tissue were noted together with subintimal accumulations of lipid-laden macrophages. Atypia, hyperplasia, and desquamation of alveolar epithelial cells (possibly type 2 pneumocytes) were also noted in almost all reports. Investigators have also reported the presence of fibrin-rich deposits in the alveolar spaces, hyaline membranes, and the early thickening of the alveolar septa due to edema, mononuclear cell infiltrates, and lying down of connective tissue [1, 38]. Inflammatory cells are notably absent. In the later stages of pulmonary injury, which occur at 6 months or more, the histology consists mainly of dense fibrosis and thickening of the alveolar wall. In addition, a reduction in the fine vasculature and gross architectural abnormalities are apparent [5]. The clinical syndrome of pulmonary pneumonitis occurs at about 2-6 months post radiation exposure (after sufficient exposure to cause the disease). This buildup of fibrotic material and thickening of the alveolar wall reduces gas exchange in the lung. A loss in lung compliance and lung volume is observed along with altered mechanics of surfactant production. This can lead to mild to moderate arterial hypoxemia with normal CO₂ tension [5]. This can be measured physiologically, as a small increase is seen in the residual volume over total lung capacity (RV/TLC), which is indicative of an “air trapping” effect. A change in the minute ventilation can also be used as a pulmonary function diagnostic tool. In experiments done in animals, it has been shown that lung function measurements such as respiratory rate, tidal volume, lung resistance, time of inspiration, time of expiration, and

dynamic compliance have proven to be useful physiologic measurements for the assessment of lung fibrosis and damage [21]. The first clinical sign of radiation pneumonitis in humans is a cough that is irritative and unproductive. In the most severe cases radiation pneumonitis is fatal. The cause of death is usually due to respiratory failure. In some patients however, the pneumonitis is transient and does not persist indefinitely [5].

Another confounding factor of this disease is that the clinical syndrome depends on a variety of factors. The first is the volume of the lung irradiated. In many animal studies, the whole lung is irradiated for experimental purposes. In the clinic however, this is not the case. Only the volume necessary to eradicate the malignant growth is irradiated, thus leaving a volume of lung out of the radiation exposure field. In addition, physicians use dose fractionation, where the dose is given in increments to minimize the damaging effects of radiation, in part, to give time for tissue repair processes. In mice, for example, the $LD_{50/180}$ (dose required to kill 50% of the animals in 180 days) has been shown to be about 10-14 Gy using X-rays (non-fractionated). Conversely, in humans the same effects occur at about 8 Gy (~ 40% less) when delivered to the whole lung meaning that humans are more susceptible to the effects of radiation. Clinical symptoms of cough, fever and respiratory distress occur in about 10% of people in the clinic that receive a dose of 40 Gy, given to one-third to one-half of the lung, fractionated over the course of 4-6 weeks [1, 4]. The second factor associated with radiation induced lung damage is the threshold effect. This means that a certain “threshold” dose must be reached before a response is evoked. In terms of radiation doses to the lung, the shape of the dose

response curve is steep and has been shown to have similar sigmoid shapes whether or not the radiation is fractionated. When the radiation dose is fractionated, however; the curve is shifted to the right due to repair processes within the lung, thus it can tolerate a higher total radiation dose if it is fractionated over time.

One of the manifestations of radiation induced lung damage that occurs in the clinic is sometimes called hypersensitivity pneumonitis. This is when pneumonitis occurs in the volume of lung that was not irradiated. Figure 7 is a diagram that illustrates the events that are thought to be involved in this process. In this model there is a substantial immune component that modulates the effects of the radiation on the lung. It is proposed that the lung damage caused in the radiation field elicits an immune response that spreads to the area of the lung not affected by the radiation. Studies have shown that in these cases there is a lymphocytic alveolitis and an increase of T-lymphocytes present in the bronchoalveolar lavage [4, 19]. It has also been shown that the chronic form of hypersensitivity has been associated with an increase in T-cell numbers from both the CD4⁺ and the CD8⁺ subsets, although more so through the CD8⁺ subset [4, 20]. The clinical symptoms for this hypersensitivity pneumonitis are similar to those found in radiation induced pneumonitis.

As stated earlier, the exact mechanism leading from radiation exposure to the clinical disease of radiation pneumonitis is not known. It is theorized that the acute inflammatory response following radiation exposure is a major contributing factor in the development of fibrosis. Recent studies have attempted to correlate how biological and molecular mediators such as cytokines and growth factors may orchestrate the

immunological and cellular responses to tissue damage caused by radiation. It has been a goal of those in the field of radiation biology research to have a molecular indicator for pulmonary fibrosis.

Fibronectin is an extracellular matrix molecule involved in cellular migration during wound healing and development and is found in the interstitial matrix. Laminin is a major component of the basement membrane and has numerous biological activities including promotion of cell adhesion, migration, growth, and differentiation. Rosenkrans and Penney [23], found alterations in both pulmonary fibronectin and laminin in LAF/1 mice following 5, 9 and 13 Gy x-ray exposures to the lung. There was a dose related increase in the amount of fibronectin starting at 1 week with a peak of seven times that of the control at 28 weeks, and then a decrease toward 63 weeks. Laminin was found to be near baseline until 13 weeks, where it increased five-fold at 41 weeks, and was at baseline by 63 weeks, again in a dose dependant fashion.

ICAM-1, known as Intracellular Adhesion Molecule-1, is an immunoglobulin-related molecule expressed on vascular endothelial cells. It has been shown to mediate the adhesion of circulating leukocytes to the endothelium and modulate their subsequent extravasation into the interstitial space in response to lung injury [24]. It has also been shown to have a role in radiation-induced lung damage by virtue of modulating the immune response. Hallahan [25] reported that ICAM-1 null mice had significantly fewer LCA-positive cells (leukocyte common antigen) and a higher mean dynamic pulmonary compliance. In addition, they found that incidence of respiratory insufficiency at 18 months after irradiation was lower in the ICAM null mice vs. the wild-type control. This

group also found that the histology scores for fibrosis and thickening of the alveolar septum also contained less hydroxyproline (measurement for collagen content) than that of the controls.

The CD40-CD40 ligand interaction has also been proven to play a role in the pathology of radiation-induced lung damage. CD40 is an extracellular marker displayed by antigen presenting cells such as macrophages, dendritic cells, and B-lymphocytes. Its engagement with CD40 ligand bearing cells, such as T-lymphocytes, mast cells and eosinophils, allows their activation [26]. These activated dendritic cells and macrophages then promote the inflammatory response by producing cytokines such as IL-1, TNF- α and the chemotactic cytokine IL-8 [27]. An experiment performed by Adawi *et al.* [28] showed that a monoclonal antibody against CD40 ligand was efficacious against death from pulmonary pneumonitis and fibrosis in C57BL/6 mice given 15 Gy of radiation to the upper thorax. There was also a dramatic improvement in the lung pathology, as the influx of inflammatory cells was decreased along with decreases in collagen deposition and septal thickening. Also noted was a blunted expression of COX-2, a pro-inflammatory enzyme that is responsible for prostaglandin synthesis.

The role of cytokines in the process leading from radiation exposure to lung damage has been an area of much investigation. One theory holds that a main mechanism involved in the pathogenesis is the dysregulation of cell proliferation and inflammatory cytokines that may eventually lead to pneumonitis and eventual lung damage. One of the most studied cytokines in this process is TGF- β (Transforming Growth Factor- β). TGF- β is a cytokine with 3 isoforms: β_1 , β_2 , and β_3 . These isoforms

are involved in: 1) modulation of cellular proliferation, 2) enhancement of the deposition of extracellular matrix molecules, and 3) immunosuppression. TGF- β modulates these activities by binding to the target cell receptor and activating a signal transduction pathway, which in-turn activates or suppresses certain genes in the DNA sequence involved in the above activities. Johnston *et al.*, [29] found that mRNA from TGF- β_1 and β_3 isoforms was upregulated at 8 weeks following 5 and 12 Gy radiation exposure in C57BL6 mice, but not in C3H/HeJ mice. Additionally, there was no difference in mRNA levels at 16 and 24 weeks following exposure. In the same mouse strain, Rube *et al.* [30] found that there were elevations in mRNA transcribing for TGF- β as soon as 1 hour after exposure to 12 Gy of radiation. It has been confirmed in humans [31, 32] that TGF- β is increased in the bronchoalveolar lavage fluid (BAL) following radiation exposure to the lung. To further implicate the role of TGF- β , Rabbani [33] demonstrated that when rats were transfected with an adenoviral vector for the TGF- β_2 receptor, there was a significant reduction in the amount of lung fibrosis caused by the radiation injury.

Another cytokine, TNF- α , has been demonstrated to play an important role in the pathogenesis of radiation-induced lung damage. TNF- α (Tumor Necrosis Factor- α), when bound to its receptor, is responsible for transducing growth regulatory signals into the cell. The signal transduction pathway can then lead to cell death via the programmed cell death pathway (PCD), which is mediated by caspases resulting in DNA fragmentation. Alternatively, this pathway can mediate cell survival via the transcription factor NF- κ B pathway. The NF- κ B then translocates into the nucleus where it turns on genes involved in proliferation and inflammation. Rube *et al.* [34], found that both TNF-

α mRNA and protein were upregulated in mice (C57BL6) during the pneumatic phase of radiation-induced lung damage. Other authors have demonstrated that TNF- α is elevated following irradiation of the lung [35, 36, 37]. Other important potent inflammatory cytokines have been shown to be upregulated following radiation exposure to the lung. These include: IL-1 α [35, 36, 38], IL-1 β [35, 36] and IL-6 [32, 37, 41].

NF- κ B has also been shown to be elevated in radiation-induced lung damage. Hasse *et al.* [41] has shown that NF- κ B is elevated out to 6 months post-radiation exposure. NF- κ B is a family of transcription factors that has been shown to bind to promoter/enhancer regions on genes encoding for growth factors, cytokines and adhesion molecules involved in fibrosis and inflammation, including TNF- α , TNF- β , ICAM-1, VCAM-1, ELAM-1, E-selectin and IL-6. NF- κ B also plays a central role in downstream regulation of TNF- α .

Researchers have also demonstrated that numerous inflammatory chemokines (chemotactic peptides) are upregulated following radiation exposure. Johnston *et al.* [39] demonstrated that Lymphotactin (Ltn), RANTES, eotaxin, macrophage inflammatory protein (MIP-1)-1 α , 1 β and -2, interferon-inducible protein 10 (IP-10) and monocyte chemotactic protein-1 (MCP-1) are all involved in the pathogenesis of pneumonitis and fibrosis. In addition, this same group of researchers [40] showed that in addition to the cytokines, many of the above mentioned receptors are up regulated following radiation exposure.

Given the above stated observations, it has been postulated that altering or modifying the inflammatory response by mediating inflammatory cytokine levels might

prove beneficial to the pathogenesis of radiation-induced lung damage. Studies have been done using soluble receptors and agonist/antagonists for certain cytokines involved in this enigmatic process, often with limited success. With this in mind we tested the effect of a novel anti-inflammatory peptide, RDP58 (Sangstat, Inc.), in a mouse model of radiation-induced lung damage.

RDP58 is a 10 amino acid polypeptide that has a molecular weight of 1228 Daltons [49]. It was synthesized by rational design which, in summary, is formulating a drug's structure by using a computer program to model a biological property of a molecule. RDP58 is classified as an immunomodulatory polypeptide based on its biologic activity. There are two main activities of RDP58: 1) inhibition of TNF- α synthesis, and 2) upregulation of hemeoxygenase (HO-1) enzyme activity. These have been demonstrated both *in-vitro* and *in-vivo*. In an *in-vitro* study, RDP58 was shown to inhibit TNF- α secretion from Lipopolysaccharide (LPS)-induced macrophages in a dose dependant fashion. It was also suggested that it acted at a post-transcriptional step, since the amount of mRNA encoding TNF- α was unchanged [49]. RDP58 has also been tested in various *in-vivo* diseases where TNF- α has been implicated in an etiologic role. These include LPS-induced septic shock, concanavalin (Con) A-stimulated cytokine overproduction in mice, scid-hu-mouse graft-versus host disease (GVHD), UVB-induced skin injury, chemical-induced rodent colitis and spontaneous IBD (inflammatory Bowel Disease) in non-human primates. RDP58 has been demonstrated to have a beneficial effect versus that of the control in each of these models [49]. This has also been confirmed by Murthy *et al.* [50] and Boismenu *et al.* [51] who both demonstrated that

RDP58 showed beneficial effects in a model of dextran sodium sulfate induced colitis (DSS) with respect to histology scores and Disease Activity Index (DAI). They also demonstrated that RDP58 was effective at down regulating TNF- α synthesis, which is thought to be one of the causative agents in Inflammatory Bowel Disease.

The goal in this experiment is to test the hypothesis that RDP58, through its ability to act locally to reduce the production of TNF- α , will reduce the severity of radiation induced pneumonitis and fibrosis in the mouse. As discussed, TNF- α has been implicated as a key agent in the pathogenesis of radiation-induced lung damage.

In summary, the pathogenesis of radiation induced pulmonary pneumonitis and fibrosis has remained an enigma after many years of research. The modern generally held theory leans toward pathology stemming from an imbalance of inflammatory cytokines and chemokines that elicit an exaggerated immune response to the lung. This immunological response leads toward a cascade of events that develops into pneumonitis and fibrosis. More research needs to be done however, to fully understand how this process fully manifests itself. This will lead us to therapeutic options that will allow us to reduce the deleterious side effects of radiation therapy in the treatment of cancer.

MATERIALS AND METHODS

Animals

We used 100 adult female BALB/c mice (Harlan, Indianapolis, IN), 6-8 weeks of age at the time of delivery. The animals were housed in micro-isolator “shoebox” type cages in ventilated wall mount units in the Animal Care Facility at San Jose State University. All mice were housed in a specific pathogen free environment and were acclimated for at least 7 days prior to the start of the experiment. The mice were housed four to five animals per cage. All mice had access to food and water *ad libitum* for the duration of the experiment. The bedding consisted of wood-pulp fiber, Carefresh® (International Absorbents Inc., Ferndale, WA) and was changed in accordance to San Jose State University’s Standard Operating Procedures. The fluorescent lights were automatically controlled to offer a 12-hour light and 12-hour dark cycle. The diet consisted of a standard rodent diet, Harlan-Teklad 2014 (Harlan-Teklad, Madison, WI). All animal procedures were reviewed and approved by the San Jose State University Institutional Animal Care and Use Committee (IACUC), under protocol number 870.

Experimental Design

The animals in this experiment were arranged into four (4) cohorts with four (4) groups of animals corresponding to survival time after radiation exposure (see Table 2). Cohorts A, B, and C were euthanized on days 4, 7, and 14 post-radiation exposure, respectively. These animals would be subject to bronchoalveolar lavage (BAL)

evaluation for total cells present, cell differential counts, and total protein concentration. Also the cytokines IL-12 and TNF- α were measured in both BAL and homogenized lung tissue. Cohort D would consist of four groups of nine mice each, held for 21 days post radiation exposure. The endpoint for this experiment was histopathology of the lung. The groups within each cohort were arranged as follows: Group 1 would receive both RDP58 and radiation, Group 2 would receive RDP58 only (Control), Group 3 would receive sterile water (vehicle control) and radiation, and Group 4 would be the untreated controls. Based on data supplied by the drug sponsor (Sangstat, Inc), the recommended dose of RDP58 was 50 μ L of a 0.1 mg/mL solution delivered intra-nasally once per week. At the scheduled sacrifice time, the animals were anesthetized with a rodent cocktail of ketamine and xylazine, given intra-peritoneally (IP) at a dose of 80 mg/kg and 10 mg/kg, respectively. The mice were subsequently sacrificed either by inhaled CO₂ gas for histopathologic evaluation or by anesthetized cervical dislocation for *in vitro* diagnostic procedures. The animals in Cohorts A, B, and C were used for in-vitro diagnostic procedures (cytokine levels, protein levels and cell differentials). Animals in Cohort D were subject to gross necropsy and histopathological evaluation.

Treatment

All mice in groups 1, 2, and 3 received either 50 μ L of sterile water (vehicle) or a 0.1 mg/mL solution of RDP58, intra-nasally, 5 minutes prior to radiation exposure and weekly thereafter (Cohorts C and D only). The mice were dosed by first anesthetizing them as previously described. The animals were then restrained in a vertical position and

either drug or vehicle was administered intra-nasally, using a P-100 micro-pipettor (Gilson Pipet-man®). The pipette tip was placed on the tip of the nose and upon inspiration a small amount was injected into the animal. This was repeated until all 50 uL were instilled. The mice were held vertically for approximately two minutes after dosing to facilitate full penetration into the deep lung areas. The animals were then placed in their respective cages and allowed to recover.

Irradiation

All mice in Groups 1 and 3 from Cohorts A through D were irradiated using a J.L. Shepard & Associates, Mark I, Model 25, ^{137}Cs γ - ray source operating at a dose of about 3 Gy/min. The irradiator is located in the Nuclear Science Facility at San Jose State University. Pictures of the ^{137}Cs irradiator can be seen in Figures 1 and 2 in Appendix I. The dose of radiation was confirmed prior to the experiments using the Fricke ferrous sulfate chemical dosimeter system. All mice in the radiation treatment groups were given 12 Gy of γ -radiation and fell within this lab's previous pilot experiments and values stated in the literature [10]. All mice were confined to plastic restraint devices that were placed in lead shielding in such a way as to shield the lower part of the body (everything caudal to the xyphoid process) from radiation exposure. A picture of the lead shielding device is shown in Figure 3 of Appendix I. This device ensured that only the upper part of the body was irradiated. The mice were anesthetized with a ketamine/xylazine cocktail given intra-peritoneally (IP) as stated above, for the duration of this procedure.

The mice were then placed back into their cages and allowed to recover until their scheduled sacrifice time.

BAL (Bronchoalveolar Lavage)

BAL was performed on all of the animals in the 4, 7 and 14 day recovery periods. Once the mice were cervically dislocated a midline incision was made at the level of the abdomen and both the abdominal aorta and the vena cava were cut to fully exsanguinate the animal. Next, a midline incision was made over the trachea. The trachea was then carefully dissected free. A small incision was then made across the trachea and a blunted 20 Ga., 1" needle (BD Biosciences, San Jose, CA) was passed into the lumen just cranial to the carina (first bifurcation of the trachea). The needle was then secured into place using 3.0 silk suture. The lungs were then infused with 600 uL of Ca^{+2} and Mg^{+2} free Dulbecco's Phosphate Buffered Saline (Sigma Chemicals, St. Louis, MO). for one minute while gently massaging the chest. The fluid was then withdrawn and placed into a 1.5 mL Eppendorf® style centrifuge tube (VWR International, West Chester, PA). This lavage step was then repeated once more and the recovered contents were pooled. The pooled lavage fluid was then spun at 400 x g in a microcentrifuge to pellet the cells. The supernatant was then aliquotted off and snap frozen on dry ice and stored at -80°C for later cytokine analysis. The cell pellet fraction was re-suspended with 1 mL of Dulbecco's Phosphate Buffered Saline with Ca^{+2} and Mg^{+2} (Sigma Chemicals, St. Louis, MO). This fraction was then placed on a hemocytometer and the total number of cells per mouse lavage was counted. The counts were adjusted taking into account the amount

of fluid withdrawn. In addition, slides for differential cell cytology were made using a Shandon Cytospin® (Thermo Electron Corp, Franklin, MA). In summary, 100 uL and 150 uL of the resuspended BAL cells were placed into the two-chamber Cytofunnel® that was attached to the double cytoslide® (Thermo Electron Corp, Franklin, MA). The slides were then placed into the Cytocentrifuge® (Thermo Electron Corp, Franklin, MA) and spun at 800 x g for 10 minutes. The slides were then allowed to air dry. Once the slides were dry, they were stained with Wright's stain (Diff-Quik, Dade Behring, Newark, DE.). The procedure consisted of dipping each slide in the "Fix" solution 5 times, allowing the slide to remain in the solution for about 1 second on each dip. The excess solution was allowed to drain off. Each slide was then dipped in the next solution labeled "Solution 1" 5 times, allowing the slide to remain in the solution for about 1 second on each dip. The excess was then allowed to drain off. Last, each slide was dipped in the solution labeled "Solution 2" allowing the slide to remain in the fluid for about 1 second on each dip. The slide was then rinsed with deionized water and allowed to air dry on a microscope slide rack. Differential counts were then made with respect to alveolar macrophages, polymorphonuclear leukocytes (PMN's) and lymphocytes. A minimum of 150 cells were counted to get a representative and accurate count of the respective cell populations in the BAL. The individual count was then divided by the total and expressed as a percentage of the whole.

Lung Tissue Homogenate

At the time of sacrifice, the right lobes of the lung were taken from each animal in Cohorts A, B and C. The lung lobes were placed in a sterile 5 mL plastic round bottom conical vial filled with 1 mL of Dulbecco's Phosphate Buffered Saline (Sigma Chemicals, St. Louis, MO). The tissue was then homogenized for one minute using a hand-held polytron type tissue homogenizer (Handishear AC Mechanical Homogenizer made by Virtis Company Inc., Gardiner, NY) set on "medium" speed. The resulting homogenate was then centrifuged at 1000xG for 5 minutes. The supernatant was then poured into 1.7 mL plastic cryo-vials, snap-frozen on dry ice, and subsequently stored at -80° C until analysis for cytokine levels.

Protein Analysis

Analysis of protein content of the recovered BAL supernatant was performed using a micro BCA Protein Assay Kit #23235 (Pierce, Rockford, IL.). The linear range of this kit, according to the supplied literature, is 1-20µg/mL when used in the microtiter plate method. The reagents were diluted according to the product literature. In control BAL samples it was found that a twenty (20)-fold dilution was necessary to bring the BAL protein levels into the linear range of the assay. The microtiter plates were read at 562 nm on a SpectraMax 340PC microplate reader (Molecular Devices, Sunnyvale, CA). The sample concentration was compared to a bovine serum albumin (BSA) standard curve. This was performed automatically by using Softmax® software package for

Windows® (Molecular Devices, Sunnyvale, CA). The results of this assay were then expressed in $\mu\text{g/mL}$ for each animal.

Cytokine Analysis

Analysis of the cytokine concentrations of mouse IL-12 +p40 and mouse TNF- α were performed on both the recovered BAL supernatant and homogenized lung tissue using a sandwich type ELISA (Biosource, Camarillo, CA). The samples were removed from the -80°C freezer and allowed to come to room temperature. At this time, the lung tissue homogenate was filtered to remove the remaining particulate matter using a $0.2\mu\text{m}$ filter (Pall Corporation, East Hills, NY) attached to a sterile syringe. The product supplied protocols were strictly followed and the results were read @ 450 nm on a SpectraMax 340PC microplate reader (Molecular Devices, Sunnyvale, CA). Cytokine levels were quantified by comparing each sample versus a standard curve. This was performed automatically by using Softmax® software package for Windows® (Molecular Devices, Sunnyvale, CA). The results of this assay were then expressed in pg/mL for each animal.

Lung Tissue Histopathology

Lung samples for histopathology and immunohistochemical staining were taken from all of the animals on Day 21. The animals were first humanely euthanized by inhaled CO_2 administered via bell jar exposure chamber. The lungs were then excised and inflated with 1 mL of 0.9% saline. The lungs were then fixed in 10% neutral

buffered formalin (NBF) and shipped to QualTek Molecular Laboratories (Santa Barbara, CA) where they were imbedded in paraffin and sectioned at 3-5 μm . They were then stained with hematoxylin and eosin (H&E). The slides were evaluated for inflammation, edema, congestion, collagen deposition and fibrosis by a board-certified veterinary pathologist at Idexx Laboratories (West Sacramento, CA).

Immunohistochemistry (IHC) Staining

At the time of H&E staining, the sections were also stained by immunohistochemistry. The tissues were de-paraffinized and stained with rat anti-mouse F4/80 to stain the macrophages present in the tissue. At the same time, additional sections were also stained with CD45^{RB} to stain the lymphocytes present. All staining procedures were performed by QualTek Molecular Laboratories (Santa Barbara, CA) according to their standard operating procedures. Each slide was then examined under a microscope at 200x using a grid pattern with an area of 625 μm and the lymphocytes counted. Six different random representative areas of each lung were then chosen and the number of positive staining cells was counted. These counts were averaged and the groups compared. The number of macrophages and lymphocytes were subjected to statistical analysis.

Statistical Analysis

Statistical analysis was performed by first subjecting the data to a one-way analysis of variance (ANOVA). If a significant difference was found between the

groups, ($P < 0.05$), then a Tukey's *post-hoc* Multiple Comparison Test was performed.

All statistical parameters and Kaplan-Meier survival curves were calculated using

GraphPad's Prism® software (San Diego, CA).

RESULTS

Survival

The survival curves for the animals in the 21 day survival Cohort (D) are shown in Figure 8. The only group to experience mortality was the radiation exposed vehicle treated group (Group 3). There was one mouse that died on Day 10 and two additional mice that died on day 11. The animals in both irradiated groups (Groups 1 and 3) had clinical symptoms of radiation exposure such as hair loss around the irradiated area (upper thorax), weight loss and anorexia. None of the animals in the RDP58 treated group died. When the animal's from Group 1 and Group 3 were compared to one another, the survival was found to be significantly different ($P = 0.02$). Not included in this figure are the animals from day 4, day 7 and day 14 Cohorts. One animal from each of the irradiated (drug and vehicle treated) groups died on day 10 [data not shown]. No other mortality was seen.

Total Cell Count

The total cell counts from the bronchoalveolar lavage fluid (BAL) for animals recovering to days 4, 7 and 14 can be seen in Figure 9. On day 4, there was a greater number of cells present (62% increase) in the irradiated vehicle control (group 3) vs. the irradiated RDP58 treated group (group1), but this difference did not reach statistical significance. The other groups were not significantly different from each other. By day 7, there were about 5×10^5 more cells present in the BAL fluid of Group 3 versus that of

Group 1, though it also did not reach statistical significance. It was noted that, at the time of doing the cell counts, there was a great deal of cellular debris or mucous present in the irradiated vehicle group on two animals (Group 3). These two animals were also found to have a decreased cell count in the BAL fluid compared to the other animals in that group. In general, the total cell counts were less on day 7 than those recovered on day 4. On day 14, the only significant difference was found to be with respect to the RDP58 control (Group 2). Out of the four animals in Group 2, two were found to be clinically “high” (average of 1.65×10^7) and one was found to be clinically “low” (about 1.4×10^5). This is about a 100 fold difference and is evident by the wide error bars and skewed cell data (day 14, Group 2). This made Group 2 significantly different than the untreated control group ($P < 0.01$). Overall, the total BAL recovery averaged about 83% of the infused volume for all animals.

Differential Cell Count

The differential cell counts from the bronchoalveolar lavage fluid for animals recovering to day 4, day 7 and day 14 can be seen in Figures 10-12. The cells were differentiated into macrophages, lymphocytes and polymorphonuclear leukocytes (PMN's) groups. With respect to macrophages (figure 10), on day 4, it was found that there was a significant decrease (89.6% vs. 93.7%) in the number of macrophages in the irradiated, RDP58 treated (group 1) versus the irradiated vehicle control group (group 3) ($P < 0.01$). There was a significant increase (86.0% vs. 80.7%) seen in the RDP58 control (group 3) versus the control group (group 4) ($P < 0.05$). On day 7 there was no significant

difference with respect to macrophages between any treated and control groups. Also noted, there was a difference in cell counts between irradiated and non-irradiated animals at this time ($P < 0.05$), regardless of RDP58 treatment. On day 14, there was a significant decrease (61.3% vs. 80.4%) in the number of macrophages in the irradiated RDP58 treated (group 1) animals versus the vehicle control (group 3) ($P < 0.001$). In addition, there were a decreased number of macrophages in the irradiated animals versus the non-irradiated animals at the 14-day timepoint. In general, it appears that radiation increased the number of macrophages present until 14 days, where there is a decrease below the control.

Figure 11 shows the percentage of lymphocytes present in the BAL fluid. On day 4, there was no significant difference between the treated groups versus the control groups. There was a significant decrease in the number of lymphocytes between the irradiated and non-irradiated groups ($P < 0.01$). An interesting finding was the high percentage of lymphocytes (17.9%) present in the control group on day 4. On days 7 and 14, the non-irradiated controls appeared to be more in line with a “typical” (about 5-7%) percentage of lymphocytes normally found in the lung [45]. On day 7, there was no significant difference between the irradiated RDP58 group and the irradiated vehicle control. There was a difference between both of these groups and the RDP58 control (non-irradiated) group ($P < 0.01$). On Day 14 there was no statistical difference between any of the groups. Overall, though not significant, it appears that there is a decrease in the number of lymphocytes present in the irradiated RDP58 group versus the irradiated control (vehicle treated) group for all the days measured.

Figure 12 shows the percentage of the polymorphonuclear leukocytes made up of the neutrophils, eosinophils and basophils. On Day 4, there was a significant increase in the PMN's present in the BAL of Group 1 compared to those in Group 3 ($P < 0.05$). Group 3 had about a 45 % reduction compared to that of Group 1 ($P < 0.05$). There also was a significant increase in the control animals versus the RDP58 treated controls (7.2% vs. 4.5%). This represents a 62 % increase ($P < 0.001$). Ironically, the control group (group 4) had the highest number of PMN's present in the BAL on day 4. On day 7, there was about a 35% decrease in the number of PMN's in Group 3 versus that in Group 1. This difference, however, did not meet statistical significance. Interestingly, the untreated controls had the highest value at this time point again, as the PMN's made up 9% of the cell population. At the 14-day time-point however, the results changed. Group 1 had a high number of PMN's present in the BAL (26.9%) versus Group 3 (11.6%). This represents an increase of about 43%, but it was not statistically significant. The lowest percentage was seen at 14 days and was in the RDP58 control group (Group 2), which had 2.5% of the cells being PMN's. At 14 days there was no statistical significance between any of the groups. All the data for cell counts and cell differentials are summarized in Table 3.

Protein Concentrations of the BAL

Figure 13, shows the protein content of the BAL. On Day 4, there appeared to be no appreciable difference between any of the groups. The values in the irradiated groups were slightly higher (about 18%) as they averaged 297 and 291 $\mu\text{g/mL}$ for Groups 1 and

3, respectively. The non-irradiated controls averaged 257 and 240 ug/mL for Groups 2 and 4, respectively. On day 7, the overall protein content was lower compared to the animals on day 4. In contrast, the protein content was highest in the non-irradiated animals. The protein content as a whole was about 45% lower in the animals that received radiation treatment. In comparing the irradiated groups, there was only a 2 ug/mL average difference (Group 1 = 150 ug/mL, Group 2 = 152 ug/mL). Again, none of the values were statistically significant. On day 14, the protein concentration was increased for all groups. The highest value was in Group 1, (469 ug/mL). This is compared to Group 3, (410 ug/mL). This represents a 12% decrease. The lowest protein measured was in the untreated group, at 292 ug/mL).

Cytokine Concentrations

The TNF- α concentration in the bronchoalveolar lavage fluid (BAL) for days 4, 7 and 14 can be seen in Figure 14. For both BAL and lung tissue homogenate assays, we found the lower limit of detection to be about 6.0 pg/mL, which was stated to be the performance of this ELISA assay according to the supplied literature. On Day 4, the animals in Group 1 had a 65% reduction in the TNF- α level versus the control group ($P < 0.05$). As seen by the error bars in the above figure 10, there was a significant amount of variability for Group 1 at Day 4. The TNF- α level for three of the five animals were below the limit of detection. These three animals were assigned the lowest limit of detection of 1.0 pg/mL. The RDP58 Control (Group 2) animals showed a similar result as they had a 36% reduction in the TNF- α level in the BAL. The irradiated vehicle control

(Group 3) had the highest level of TNF- α present (21 pg/mL) on Day 4. On Day 7, this trend was not seen. The concentrations were similar between the groups with the highest level being in the non-irradiated control (23 pg/mL) and a low of 17 pg/mL measured in the RDP58 control group. There were no significant differences between groups at 7 days post radiation treatment. On day 14, the differences between the groups were similar to the 7 day animals. Again, the highest TNF- α concentrations were found to be in the untreated control group (Group 4). The levels in Group 1 were slightly elevated (25%) versus the irradiated vehicle control group, but this value was not significant. At 14 days, the trend between the groups appeared to be identical to the levels seen on day 7, but overall the values were about 50% lower.

The TNF - α concentration in the BAL fluid measured by ELISA in the lung tissue homogenate are shown in Figure 15. On day 4, there was no significant difference between the groups. The highest concentration was in Group 2. In contrast, the lowest concentration of TNF- α was measured in Group 3. Overall, on day 4, the concentrations of TNF- α were lower in the animals exposed to radiation, whether or not they were treated with RDP58 or vehicle. This trend was continued on day 7 (except Group 2), and the measured levels were slightly elevated. The average TNF- α concentration was identical (174 pg/mL) for both Groups 1 and 4. Group 2 had the lowest value at 138 pg/mL and Group 3 was 160 pg/mL. The data for day 14 showed that the level of TNF- α present in the lung tissue was about the same for groups 1-3. Group 4 was decreased compared to the rest by about 63% on day 14. This number was not found to be significantly different. It was also noted that the amount of TNF- α expression in the lung

tissue was found to be about 10 times higher than that of the BAL fluid. Of course it is also true that BAL fluid is inherently more diluted than that of the lung tissue homogenate.

The amount of IL-12 p40 in both the BAL and the lung tissue can be seen in the both Figures 16 and 17. For both assays, the lower limit of detection was about 2.0 pg/mL. This value was exactly what was stated to be the performance of this ELISA assay according to the supplied literature. In Figure 16, it can be seen that on Day 4, there was a decrease in the amount of IL-12 p40 between the irradiated animals and the non-irradiated animals. These data showed statistical significance comparing Groups 1 and 4 ($P<0.05$) and Groups 3 and Group 4 ($P<0.01$). There was very little difference between Groups 1 and 3. There was a fair amount of inter-animal variability associated with group 4 (non-treated) on day 4, as can be seen by the large error bars. On day 7, there was no difference in treatment regimens as Groups 1 and 3 were nearly identical; however, there was a difference between irradiated and non-irradiated animals as Groups 1 and 3 were statistically significant when compared against Group 2 ($P<0.05$). The non-irradiated animals showed a slightly higher amount of IL-12 present in the BAL. By day 14, there were no significant differences between groups. It was noted that Group 1 had an elevated amount of IL-12 (50% higher) than Group 3 and 42% higher than Group 4.

In the lung tissue homogenate, it was found that there was only a difference with regard to radiation treatment, independent of drug treatment. These results are shown in Figure 17. It should be noted that, on Day 4, the only differences were between the irradiated and non-irradiated animals. The difference is statistically significant

($P < 0.001$). The mean values for the irradiated RDP58 treated animals were nearly identical to the vehicle control group on day 4. Also noted was the fact that the RDP58 controls were slightly elevated versus the untreated controls. On day 7, the result was nearly identical. The mean values were higher across the board, but the differences remained about the same as those seen on day 4, but no statistical significance was found between radiation and/or drug treatment. On day 14, we again witnessed similar results compared to day 7, but the values were slightly lower. Again, the irradiated animals showed lower levels of IL-12 versus the controls, but the values were not statistically significant.

Immunohistochemistry (IHC)

The immunohistochemical staining results are graphed in Figure 18. With respect to lymphocytes, it can be seen that the number of positive staining cells is about 50% greater in the non-irradiated animals when compared to the irradiated groups 21 days post-irradiation. This value was statistically significant ($P < 0.001$). There was no difference between the irradiated RDP58 treated animals and their respective control group. Looking at macrophages, it can be seen that there was a difference between Groups 1 and 3. Group 1 had a significant reduction (46%) in the number of macrophages compared to Group 3 ($P < 0.001$). Again, there was a significant difference between the irradiated and the non-treated control animals ($P < 0.05$), but in this case there was a decrease in the number of cells.

Lung Tissue Histopathology

Lung tissue histopathology was performed on all of the animals in the 21-day recovery cohort (Cohort D). There were no significant changes observed between any of the groups with regard to histology scores for inflammation, edema, collagen deposition, or thrombosis (No data shown). The observed changes were characterized by focal or multifocal hemorrhages in the lung with a varying degree of congestion that was consistent with the method of euthanasia (CO₂ gas inhalation). For this reason histopathology did not prove to be a useful determination of lung damage in this study.

DISCUSSION

The goals of this study were two-fold. The first goal was to evaluate the effect of a local acting anti-inflammatory compound, RDP58, in a murine model of radiation-induced lung inflammation. The second goal was to test the hypothesis that by inhibiting both IL-12 and TNF- α , a therapeutic benefit may result. In the case of IL-12, there has been no literature found to date implicating it in the pathogenesis of radiation-induced lung damage, although there have been reports implicating this cytokine with bleomycin-induced lung damage in mice [42, 43]. However, this has been contradicted by Keane *et al.* [44], who showed that IL-12 given as a treatment regimen, decreased hydroxyproline scores (marker for collagen deposition). This reduction was proposed to act via the ability of IL-12 to modulate IFN- γ production. The pathology of bleomycin-induced pulmonary fibrosis is thought to involve the release of chemotactic factors (chemokines) from alveolar macrophages that attract lymphocytes and polymorphonuclear leukocytes [18]. It is the opinion of some investigators that a similar process contributes to the effects of radiation-induced lung damage. Most recently, Williams *et al.* [45] stated that “with the refinement of molecular assays and techniques, together with other investigators, they have come to recognize an immediate response in normal tissues after radiation injury in the form of cytokine upregulation.” Based on these findings they suggest: “as an alternative to the target cell theory, it is the immediate and more importantly persistent dysregulation of cytokines and growth factors that is the perennial underlying mechanism leading to the induction of late effects.” This was the reasoning in

designing this experiment; to test the hypothesis that RDP58 can reduce certain cytokines and in turn reduce the amount of radiation damage to the lung.

It has been a goal for many researchers to find a predictive assay that could forecast the late effects of radiation damage to the lung. Rubin *et al.* [46] states that “Ideally a biochemical marker that could be detected early, at the time of completion of radiation and could forecast late effects. The predictive assay needs to be simple, reproducible and sensitive enough for clinician’s to terminate radiation treatment to avoid severe and life threatening toxicity.” It would also have to correlate with the pathophysiology of the injurious event. This type of an assay would also be of particular use to the researcher as well as the clinician. Methods used to assess lung damage used biomarkers of effect, such as protein content of the bronchoalveolar lavage fluid (BAL) for detection of breakdown of microcirculation [8], surfactant analysis secreted from type II pneumocytes [46], hydroxyproline content of the tissue indicative of collagen content [21], and cell differential of the BAL fluid [45]. More recent methods use techniques such as ELISA and RNase protection to detect TNF- α and IL-1 α [35] and detect cytokines of specific interest, and northern blotting for genes such as collagens I-III [29]. This is in addition to the tool of tissue histopathology. In this experiment, we used a select set of these tools to test our hypothesis.

Analyzing the results in the experiment proved to be both difficult and surprising in light of past literature. When examining the survival curves, there appeared to be an advantage for the RDP58 treated group, as there were no deaths associated with this group at 21 days post radiation exposure. The survival curves proved to be significantly

different ($P=0.02$). The total cell counts of the BAL fluid proved to be of little value, as there were no significant differences between groups. The values obtained fell within what is seen in the literature [45]. The cell differentials indicated that there were differences with respect to drug treatment and radiation exposure. In general, it appeared that the radiation exposure affected macrophages the most. There was an increase in the number of macrophages at the early timepoints, both 4 and 7 days; followed by a decrease at 14 days (Fig. 10). This is a paradox because the reverse is true with respect to IL-12p40 (Figs.16-17). IL-12p40, both in the BAL and the lung tissue homogenate, was generally reduced in the radiation exposed groups. A particular interesting observation was the high percentage of polymorphonuclear leukocytes present in the irradiated animals on day 14 (Fig. 12). Both the treated and vehicle control groups showed a significant increase in these cells on day 14.

Another interesting observation was the lack of difference between the groups with regard to protein content of the BAL. As seen in Fig.13, there was little difference between the irradiated animals or the non-irradiated animals with respect to protein content. This does not correlate well with early research using protein as a measure of increased permeability caused by lung damage. Anderson *et al.* [8] found that protein content of the BAL fluid exhibits a biphasic increase following radiation with 5-28 Gy of x-ray exposure. During the first 6 weeks following radiation, they found that mean protein content was increased about 4 times the normal level. The amount of protein then decreases to baseline for the next 6 weeks. A large subsequent increase in protein to about 20-fold normal levels then occurs at about 15 weeks post-irradiation. This then

sharply declines back to baseline at 43 weeks. It was also noted that at the higher doses of radiation the second phase of protein leakage was much higher than that observed at lower doses. There was also no indication of a dose response over this period of time. Comparing these data to previously published data on surfactant levels also suggests that surfactant and protein responses are largely unrelated.

The immunohistochemical staining also showed a significant decrease in the number of lymphocytes residing in the irradiated lung tissue vs. the non-irradiated control. In this study, a correlation between TNF- α and radiation treatment was present (Figs. 14-15), like much of the previous literature has reported [34, 35, 36, 37]. Our data showed that the TNF- α in the lung tissue homogenate was slightly decreased for both the radiation treated animals and the non-irradiated control groups. TNF- α has been shown to be a potent proinflammatory cytokine that triggers inflammatory cell recruitment which is thought to be an important initial step in the host defense against pulmonary infection, irradiation and inhaled toxic substances [39]. RDP58 was shown to be effective in decreasing the amount of TNF- α on Day 4 in the BAL fluid. After Day 4; TNF- α , either in the BAL fluid or lung tissue homogenate, did not appear to correlate with radiation treatment or drug treatment. RDP58 did show to be effective in controlling the number of interstitial macrophages residing in the lung at 21 days post exposure (Fig. 18). The number of macrophages was decreased significantly compared to the controls.

As stated previously, the results observed with respect to TNF- α in this model were found to disagree with that of other authors. The reason for this is not certain. One

possible reason may be the difference in the mouse strain used. Rube *et al.* [34], found that the peak (~4 times the control) mRNA expression for TNF- α occurs at about 8 weeks in lung tissue. There was about a 2 fold-increase starting at about 2 weeks post-irradiation. These results were obtained in the C57BL/6 strain of mice, which are commonly known as a “fibrosis prone” strain. The same author also labeled the TNF- α present in the cells by immunohistochemistry (IHC). It was noted that significant increases were seen in the cells of the lung as soon as 1 hour post-irradiation, and persisting through 24 weeks. The peak activity was at 2 weeks. Supporting this work, Johnston *et al.* [36] demonstrated that in C57BL/6 mice, the levels of TNF- α mRNA were at about 5-fold of the control level at 7 days post-irradiation and about 4 times the control level at 14 days. These levels then subsided to the control level until day 182, where they rose back to about 3 times that of the control. Contradicting this, Hong *et al.* [35] showed that the levels of mRNA for TNF- α were not significant versus the controls after irradiation; however this was at only a 2 Gy dose. At 20 Gy, the same group saw no upregulation of TNF- α mRNA (C57BL/6 mice). It is noted that a 12 Gy dose was used in this experiment.

In this experiment, there were no significant differences between any groups in the lung histopathology at day 21. It has been noted that, in some cases [1, 5, 7], there is an absence of gross histopathologic lesions at these somewhat early time points. The cause behind the absence of histologic lesions has not been documented. How this lack of histopathologic lesions correlates to the dysregulation in specific cytokine levels has not been well documented. More research could be done in this area to elaborate on the

mechanism behind this. An interesting note in this research was the lack of dysregulation of TNF- α , both in the BAL and the lung tissue homogenate. We did not observe the extensive change in TNF- α level that, upon reviewing the literature, has been documented by other authors [15, 29, 36, 39]. The possibility may exist that this may have been the reason for the absence of histopathologic lesions. More studies would have to be done to prove this point.

One of the difficulties encountered in this experiment was interpreting the data obtained from the cohorts used for in-vitro diagnostic procedures. The reason for this was due to the small group size of 4-5 animals. The reason for this small group size in the in-vitro portions of this experiment was the funding limitations for this project. It became difficult at times to discern changes due to a radiation insult and in the subsequent evaluation of the therapeutic potential in the acute phases of this experiment from that of normal animal variability. It is of note that with respect to some of the endpoints measured such as cell counts of the BAL, cytokine levels, and BAL differentials can vary depending on the state of health of the test animals. The statistical analysis in this experiment attempted to eliminate any such errors when comparing groups, but in some cases, the results seen were not significant due to small group size.

Of interesting note when reviewing the literature is the amount of strain variability in mice and the susceptibility to radiation induced lung fibrosis. Numerous authors have demonstrated that there is a significant variation with respect to mouse strain and the response of the lung to radiation exposure [29, 30, 36, 39, 47] and bleomycin treatment[47]. These authors have reported that mice that are considered

sensitive express more pro-inflammatory cytokines than those that are considered resistant. The typical sensitive mouse is considered to be the C57BL/6 strain. It has been shown to have a significantly higher cytokine (IL-1, TNF- α , and TGF- β) and extracellular matrix (tissue markers of collagen I-III mRNA) in response to radiation of the lung than that of a typical resistant mouse strain like the C3H/HeJ [29, 36, 39]. A study performed by Sharplin [47], showed that C57Bl/6 mice developed significantly more fibrotic areas of the lung compared to the BALB/c which showed only small foci of contracted fibrosis at 28 and 52 weeks post-irradiation. The C3H mouse showed no fibrotic scarring throughout the study. It was also noted that the histological data for the BALB/c mice did not appear to be significant enough to account for the respiratory distress and deaths witnessed in that study. The reason for this was thought to be a lack of pulmonary blood perfusion, as 50% of the lung acini were found to be non-functional.

Recently, other investigators have linked other inflammatory mediators in the pathology of radiation-induced lung damage. Hasse *et al.* [41] found that NF- κ B binding activity was elevated in rats following 20 Gy of hemithorax radiation. This was accompanied by an increased level of mRNA for IL-6 that suggests transcriptional activation of NF- κ B dependant genes. Nitric oxide, a gaseous chemical messenger, has been implicated as an inflammatory mediator in radiation lung damage. In a unilateral lung irradiation study in rats performed by Nozaki *et al.* [52], it was found that radiation caused an increase in nitric oxide (NO) production from macrophages from both the irradiated and non-irradiated sides of the lung starting 7 days after radiation exposure. This effect continued until 56 days after exposure in both sides of the lung. This

correlated with the increase in procollagen- α_1 mRNA. This effect was attenuated using the NOS inhibitor L-NAME. As stated in the literature review, reactive oxygen species (ROS) such as $\cdot\text{O}_2$ and $\text{OH}\cdot$, are commonly generated in tissues exposed to radiation. Recently, Kang *et al.* [53] showed that mice that were transgenically modified to over express superoxide dismutase showed significant improvement in terms of survival, breathing parameters, macrophage count and activated TGF- β levels. Measurements for collagen and fibrosis deposition were markedly improved for the transgenic mice versus the wild type.

Many researchers feel that the pathogenesis of radiation-induced lung damage is a result of the dysregulation of the cytokines associated with many of the cell types in the lung [4, 29, 40]. This dysregulation then leads to a process resulting in the eventual pneumonitis and fibrotic scarring of the lung. In this study, we only found the dysregulation in the cytokine TNF- α following irradiation at 4 days post-radiation exposure, and only in the BAL fluid. By day 7 and thereafter, there was no statistical difference between the groups. There were no histopathological differences between the groups at the 21 day time point. We were able to find a difference between the RDP58 treated group and the vehicle control in the number of interstitial macrophages present. The RDP58 treated group showed a significant decrease in the number of macrophages present in the lung tissue compared to that of the control group ($P < 0.05$). We did notice a significant reduction in IL-12p40 in both BAL and lung tissue in the irradiated groups versus the non-irradiated groups on day 4 post-exposure ($P < 0.05$). The same was true on Day 7, but the result was not statistically significant. The alveolar protein level showed

little variability in the experiment, as the levels were not statistically significant with respect to drug or radiation treatment. The data for the total cells present in the BAL showed that, on Day 4, the RDP58 treated group had a decreased number of cells present versus the control group; however this value did not reach statistical significance. With respect to cell differentials, the RDP58 treated groups showed a statistically significant decrease in the number of macrophages on days 4 and 14. On day 14, the RDP58 treated animals had only 61.3% of the BAL cells being macrophages. This led to a concomitant increase in the number of polymorphonuclear leukocytes. This number would be considered low, as different authors have shown that the “average” nonirradiated percentage for macrophages is typically about 90-95 %.

In conclusion, we were able to demonstrate that RDP58 delivered intra-nasally into the lung, increases the survival of mice out to 21 days post-radiation exposure. We were able to demonstrate that RDP58 is effective in decreasing the number of macrophages present in both the BAL and lung tissue following radiation exposure. It was also shown to suppress the amount of TNF- α present in the BAL fluid at 4 days post-radiation exposure. There appears, however, to be some conflicting data when comparing the values of TNF- α and protein levels in the BAL, and cell differentials with that of the literature. In some cases we failed to see a significant difference versus the untreated control group. The primary reason for the results is postulated to arise from the strain of mouse used in the current study. From the literature [47], the BALB/c mouse could be classified as a traditional “fibrosis resistant” mouse. Mice usually used for these types of studies are the “fibrosis prone” C57BL/6, based on the cytokine profile in

correlation to the histopathology. The absence of damage seen in this mouse assessed by the cytokine levels and lack of histopathological changes may be due to the resistance of the BALB/c mouse to radiation-induced lung damage at the radiation doses used in the current study.

We failed to measure any differences in IL-12 p40 in either the BAL fluid or the lung tissue and only saw improvement in TNF- α on day 4. Since the earliest time-point measured in this experiment was on day 4, a possibility exists that a significant increase in the cytokines levels measured went unnoticed because we were passed the relevant time at which they are critically expressed following lung tissue damage. With this in mind, more work need to be done to demonstrate the mechanism behind why RDP58 improves the survival of BALB/c mice 21 days post-radiation exposure to the lung. More acute endpoints can be measured, such as 6, 12, and 24 hours post-radiation exposure in order to ascertain the cytokine expression at these times. Additionally, more long term studies need to be run in order to correlate this with the longer term fibrosis and vascular insufficiency often seen in the clinic. Including physiological measurements such as breathing rate and ventilation rates by data acquisition systems would also prove useful, along with the long term pathology to correlate how these act together.

TABLES AND FIGURES

Table 1. Early, intermediate and late histopathological changes in the lung following radiation exposure to the lung. Reprinted from Gross [5] with permission.

Site	Early (0-2 months)	Intermediate (2-9 months)	Late (9 months +)
Capillaries	2 hr: Endothelial changes leading to increased permeability. 2 days: Endothelial separation from basement membrane. Sloughing of cells giving obstruction of capillary lumen.	Abnormalities. Widespread obstruction due to platelets, collagen and fibrin. Reduced capillary permeability	Loss of many capillaries. Regeneration of new capillaries. Reduced permeability.
Type I pneumocyte	Degenerative changes and sloughing decreased numbers or Normal.	Further decrease in numbers.	
Type II pneumocyte	Early degenerative changes becoming more marked with time or Normal	Large increase in size and number. Abnormal appearance.	Return to normal size and number.
Basement membrane	Swollen, becoming irregular	Folded and thickened	Folded and thickened.
Interstitial space	Edema and debris. Increased number of inflammatory cells. Slight increase in coarse connective tissue.	Infiltration on mononuclear, mast and inflammatory cells. Increase in connective tissue	Few inflammatory cells. Large increase in collagen.
Alveolar space	Hemorrhagic. Filled with fibrin and cell debris. Increase in number of alveolar macrophages.	Alveolar space becomes smaller.	Alveolar space small or absent. Architecture destroyed.
Bronchial Epithelium	Early transient inflammatory reaction, ciliary paralysis, increase in goblet cells or normal	Epithelial proliferation	

Table 2. Experimental design.

Cohort	Group	n	Animal #'s	Treatment	Radiation	Duration
A	1	5	101-105	RDP58	Yes (12 Gy)	4 Days
	2	5	201-205	RDP58	No	
	3	5	301-305	Vehicle	Yes (12 Gy)	
	4	5	401-405	Untreated	No	
B	1	5	101-105	RDP58	Yes (12 Gy)	7 Days
	2	5	201-205	RDP58	No	
	3	5	301-305	Vehicle	Yes (12 Gy)	
	4	5	401-405	Untreated	No	
C	1	4	101-104	RDP58	Yes (12 Gy)	14 Days
	2	4	201-204	RDP58	No	
	3	4	301-304	Vehicle	Yes (12 Gy)	
	4	4	401-404	Untreated	No	
D	1	9	101-109	RDP58	Yes (12 Gy)	21 Days
	2	9	201-209	RDP58	No	
	3	9	301-309	Vehicle	Yes (12 Gy)	
	4	9	401-409	Untreated	No	

Note: Cohorts were used to denote recovery timepoints. Each cohort within the study was run separately to facilitate ease of dosing and animal holding space.

Table 3. Cellular changes in the Bronchoalveolar Lavage Fluid at 4, 7, and 14 days post-radiation exposure. All numbers are group averages $\pm$ Standard Deviation. Minimum of 4 animals per group. * indicates significant difference ($P < 0.05$). ** indicates significant difference ($P < 0.001$).

	Total cells ($\times 10^6$)	Percent alveolar macrophages	Percent lymphocytes	Percent Polymorphnuclear leukocytes (PMN's)
4 days post irradiation	n=5	n=5	n=5	n=5
Untreated Control	2.74 ± 0.70 *	80.7 ± 2.5	17.9 ± 2.4	7.2 ± 0.9
RDP58 + radiation	4.33 ± 1.61	87.9 ± 1.9 *	7.1 ± 2.1	5.0 ± 1.9 *
RDP58 only	4.13 ± 1.54	86.0 ± 4.4 **	9.5 ± 2.6 *	4.5 ± 1.9
Vehicle + radiation	6.99 ± 3.12 *	93.7 ± 1.0 **	5.3 ± 1.2 *	2.3 ± 0.9 *
7 days post irradiation	n=5	n=5	n=5	n=5
Untreated Control	1.17 ± 0.82	85.2 ± 5.5 *	5.8 ± 1.5 *	9.0 ± 4.6
RDP58 + radiation	1.65 ± 1.07	87.6 ± 6.3	7.6 ± 4.1 *	4.8 ± 2.7
RDP58 only	1.63 ± 1.34	77.6 ± 7.6 *	14.8 ± 3.2 **	7.6 ± 5.7
Vehicle + radiation	2.01 ± 1.39	95.7 ± 3.1 *	2.6 ± 1.6 **	1.7 ± 1.6
14 days post-irradiation	n=4	n=4	n=4	n=4
Untreated Control	2.74 ± 2.39	88.8 ± 4.7	7.2 ± 3.8	4.0 ± 1.3
RDP58 + radiation	2.51 ± 1.07 *	61.3 ± 6.9 **	11.9 ± 3.0	26.9 ± 9.0 *
RDP58 only	10.0 ± 8.46 *	87.8 ± 3.1 **	9.8 ± 1.7	2.5 ± 1.6
Vehicle + radiation	2.01 ± 1.07 *	80.4 ± 4.0 **	8.0 ± 0.4	11.6 ± 3.9 *

Notes: Animals in the 4 day recovery cohort were dosed with RDP58 or vehicle on day 0.
Animals in the 7 day recovery cohort were dosed with RDP58 or vehicle on day 0
Animals in the 14 day recovery cohort were dosed with RDP58 or vehicle on days 0 and 6.

Figure 1. A diagram summarizing the events in the photoelectric scattering process. This figure shows an incoming photon interacting with the electron of the K orbital. The incoming photon disappears and a photoelectron is ejected, with energy, $E = h\nu - B.E.$ $h\nu$ = Planck's constant x frequency of photon, B.E. = binding energy. This vacated orbital is then filled by an electron from an outer orbital, in this case the M orbital. This shift in orbital results in the emission of fluorescent radiation with energy, $E = \Delta B.E.$ Also shown is the possibility that an electron can move from the L orbital to fill the vacancy. Reprinted with permission from Radiation Biophysics by Edward L. Alpen (pp. 59) by Prentice Hall (1998).

Figure 2. A diagram summarizing the events in the Compton Scattering Process. The incoming photon interacts with the electron (dark circle). This results in a photon that is scattered at angle θ , with a specific energy ($h\nu$) and momentum ($h\nu/c$). The recoiled electron is also scattered at angle ϕ with velocity $=V$, momentum $=p$ and energy $=E$. h = Planks constant, ν = frequency of photon, c = speed of light (3.0×10^8 m/s). Reprinted with permission from Radiation Biophysics by Edward L. Alpen (pp. 63) by Prentice Hall (1998).

Figure 3. A diagram summarizing the events in the pair production scattering process. The incoming photon, with energy $> 1.02 \text{ MeV}$ interacts with the nucleus of the atom. This creates an electron and a positron *de novo*. The electron is scattered and the positron goes through an annihilation event producing 2 photons, each with an energy = 0.511 MeV . Reprinted with permission from Radiation Biophysics by Edward L. Alpen (pp. 71) by Prentice Hall (1998).

Figure 5. Summary of the processes from radiation energy transfer to biological damage, including the time course of events. Reprinted with permission from Radiation Biophysics by Edward L. Alpen (pp. 52) by Prentice Hall (1998).

Figure 6. The model proposed by Casarett generalizing the role of radiation damage to the microvasculature as the underlying cause of late tissue damage following radiation exposure. Reprinted with permission from Radiation Biophysics by Edward L. Alpen (pp. 231) by Prentice Hall.

Figure 7. The proposed mechanism of the interaction of irradiation, immunology and genetic factors in the pathogenesis of radiation induced sporadic pneumonitis.

Figure 8. Kaplan-Meier survival curve for Cohort D (21 Day survival). Radiation exposed mice received 12 Gy of γ -radiation to the upper thorax. All mice in the control, RDP58 + radiation, and RDP58 control survived to Day 21. Some mortality was seen with the mice in the radiation control group. Group differences were significant at $P = 0.02$. Each group contained 9 animals.

Figure 9. Graph of the total number of cells per milliliter present in the bronchoalveolar lavage fluid (BAL) per group. Each bar is the mean $\pm$ SD of the percentage of total cells counted. A minimum of four animals were in each group. * indicates a significant difference vs. non-treated control ($P < 0.05$).

Figure 10. The mean percentage of macrophages present in the BAL fluid for all groups on Days 4, 7, and 14. Each bar is the mean $\pm$ SD of the percentage of total cells counted. A minimum of four animals were in each group. * indicates a significant difference vs. respective control ($P < 0.05$). ** indicates a significant difference vs. respective control ($P < 0.01$)

Figure 11. The mean percentage of the lymphocytes present in the BAL fluid for all groups on Days 4, 7, and 14. Each bar is the mean $\pm$ SD of the percentage of total cells counted. A minimum of four animals were in each group.

Figure 12. The mean percentage of polymorphonuclear leukocytes (PMN's) present in the BAL fluid per group on Days 4, 7, and 14. Each bar is the mean $\pm$ SD of the percentage of total cells counted. A minimum of four animals were in each group. * indicates a significant difference vs. the respective control group ($P < 0.05$). ** indicates a significant difference vs. the respective control group ($P < 0.01$).

Figure 13. The amount of total protein present in the BAL fluid per group on Days 4, 7, and 14. Each bar is the mean $\pm$ SD. All protein concentrations determined by BCA method as described in Materials & Methods. A minimum of four animals were used in each group.

Figure 14. The mean concentration of TNF- α present in the BAL per group on Days 4, 7, and 14. Each bar is the mean $\pm$ SD. All samples were quantified by using by sandwich-style ELISA as described in the Material & Methods. A minimum for four animals were used per group. * indicates a significant difference vs. respective control group ($P < 0.05$).

Figure 15. The mean concentration of TNF- α present in the lung tissue homogenate per group for Days 4, 7, and 14. Each bar is the mean $\pm$ SD. All samples were quantified by using by sandwich-style ELISA as described in the Material & Methods. A minimum for four animals were used per group.

Figure 16. The mean group concentration of IL-12 p40 present in the BAL per group on Days 4, 7, and 14. Each bar is the mean $\pm$ SD. All samples were quantified by using by sandwich-style ELISA as described in the Material & Methods. A minimum for four animals were used per group.

Figure 17. The mean group concentration of IL-12 p40 present in the lung tissue homogenate per group on Days 4, 7, and 14. Each bar is the mean $\pm$ SD. All samples were quantified by using by sandwich-style ELISA as described in the Material & Methods. A minimum for four animals were used per group. ** indicates a significant difference vs. respective control group ($P < 0.001$)

Figure 18. The average number of positive staining CD45^{RB} and F4/80 cells per group at 21 days post-radiation exposure. Each bar represents the mean $\pm$ SD. Lymphocytes were stained with CD45^{RB} and Macrophages were stained with F4/80. Cell counts were performed as described in Materials & Methods. A minimum of seven animals used per group. ** denotes a statistical difference vs. control group ($P < 0.001$)

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Appendix I

Pictures of the radiator and dosing apparatus

Figure 19. Picture of the ¹³⁷CS irradiator located in the Nuclear Science Facility at San Jose State University

Figure 20. Picture of the inside of the ^{137}Cs irradiator located in the Nuclear Science Facility at San Jose State University

Figure 21. A picture of the lead shielding used when exposing the lungs of the mice in the γ irradiator. Each mouse was anesthetized and inserted into the top of the plastic lumen and secured with tape. Each mouse was inserted to the level of the xyphoid process, meaning all body parts cranial to this were irradiated.

Appendix II

Pictures of Histopathology

Figure 22. Lung section at 21 days after 12 Gy. radiation exposure to the thorax. Slide stained with H & E, viewed at 400X. Animal was treated with RDP58 once per week. No significant difference was found between the RDP58 treated animals and the vehicle control group. All slides were read by a board-certified veterinary pathologist.

Figure 23. Lung section at 21 days after 12 Gy. radiation exposure to the thorax. Slide stained with H & E, viewed at 400X. Animal was treated with RDP58 once per week. No significant difference was found between the RDP58 treated animals and the vehicle control group. All slides were read by a board-certified veterinary pathologist.

Figure 24. Lung section at 21 days after 12 Gy. radiation exposure to the thorax. Slide stained with H & E, viewed at 400X. Animal was treated with vehicle once per week. No significant difference was found between the RDP58 treated animals and the vehicle control group. All slides were read by a board-certified veterinary pathologist.

Figure 25. Lung section at 21 days after 12 Gy. radiation exposure to the thorax. Slide stained with H & E, viewed at 200X. Animal was treated with vehicle once per week. No significant difference was found between the RDP58 treated animals and the vehicle control group. All slides were read by a board-certified veterinary pathologist.