Mechanisms Regulating Pulmonary Sensitivity to Radiation

by

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Department of Pathology
Duke University

Date: ______________________
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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology in the Graduate School of Duke University

2012
ABSTRACT

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Abstract

At the present time, there is no approved medical countermeasure (MCM) for mitigating or treating pneumonitis/fibrosis following acute radiation exposure. Since it is neither ethical nor feasible to evaluate potential MCMs against radiation injury in the clinical setting, the FDA permits MCM licensure under an alternative drug development pathway (“Animal Efficacy Rule”) that relies on the predictive validity of animal models. The purpose of the current project was to design a research platform that addresses many of the critical knowledge gaps associated with successful adherence to the FDA Animal Rule.

In these studies, we evaluated the dose-response relationship for survival and functional injury among CBA/J, C57L/J, and C57BL/6J mouse strains. These strains were previously identified to represent the full spectrum of pulmonary pathology associated with acute radiation exposure to the thorax. We next evaluated ultrastructural pathology to identify differences in tissue response among strains as early as twenty-four hours after radiation. Global differential gene expression analysis was utilized to identify the major signaling pathways and genes associated with the development of radiation pneumonitis and/or fibrosis by exploiting the phenotypic differences in radiation-injury among strains. Genes with significant differences were validated by quantitative real-time polymerase chain reaction and their protein products validated by western blot.
Finally, we performed longitudinal analysis of hypoxia-associated gene expression to elucidate the natural history of disease progression in “fibrosis prone” C57BL/6J mice.

In these studies, we identified significant differences in the dose-response, temporal onset, disease progression, and pathologic manifestations of radiation lung injury among murine strains. The severity of ultrastructural damage at twenty-four hours also differed among strains indicating the early tissue response to the radiation insult was dissimilar. A significant difference was found in gene expression among strains. The most interesting differences were associated with the acute-phase response, iron homeostasis, cell cycle/proliferation, and cell death. Lastly, hypoxia-associated gene expression, including hypoxia-inducible factor-1 alpha (HIF-1α) and HIF-2α mRNA and protein stabilization, was dynamically altered during the temporal course of radiation pathogenesis in the “fibrosis-prone” C57BL/6J mice. As the C57BL/6J strain is more “resistant” to radiation-induced lung injury, a better understanding of the pathways involved in tissue response to radiation in this strain might elucidate the mechanisms that make the lungs of this strain significantly more radiotolerant than their counterparts.

The research platform developed in this project provides essential information to interpret and define the complex interrelationships in clinically relevant models of the human response to potentially lethal irradiation and treatment. The overall goal is to provide a rigorous scientific platform for MCM development under the Animal Efficacy
Rule with reasonable expectation that MCMs acquired for the Strategic National Stockpile will effectively prevent, treat, or mitigate radiation-induced lung injury and improve survival among the exposed population.
Dedication

To my Father, who taught me the value of hard work;

To my Mother, who taught me to believe in myself;

To my Sister, for my being my closest friend;

To Zeljko, for always providing support and encouragement
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It is quite ironic to me that I am working towards a PhD in the field of radiation biology. When I entered the University of Georgia in the fall of 2000, I intended to pursue an undergraduate degree in political science and journalism. I had just completed Perestroika by Mikhail Gorbachev, the former President of the Soviet Union, and I was fascinated by the philosophy of “mutually assured destruction” and nuclear counterproliferation. Within two years, however, I transferred to NC State and it was there that I was first introduced to laboratory research. It was the book, A commotion in the blood: life, death, and the immune system, by Stephen S. Hall that ignited the first sparks of passion for medical science and influenced the direction I would eventually take.

Dr. Mark W. Dewhirst was the first person I met at Duke University. He took a chance on an inexperienced undergraduate student with a mediocre grade point average and invited me to his laboratory at Duke University Medical Center for an interview as a summer student. I had only been taking sciences courses at the time for a few months and was still unsure of the role science would eventually play in my future career. I remember being extraordinarily excited about the interview and discussing it with my
mentor, Dr. Hosni Hassan, at NC State University who ensured that I was well prepared. To my initial disappointment, Dr. Dewhirst had already hired another summer student, but he took a huge leap of faith and introduced me to his colleague, Dr. Zeljko Vujaskovic. It was that introduction that would change the course of my career. Both Dr. Hassan and Dr. Dewhirst would continue to have important roles in my career development and I hope they will continue to serve as mentors as I continue on in the field of science.

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Dr. Tom MacVittie at the University of Maryland-Baltimore also played a substantial role in the direction my PhD career would eventually take. Dr. Tom MacVittie introduced me to the field of medical countermeasure development for acute radiation sickness. Dr. MacVittie is an internationally recognized expert in the acute radiation syndrome serving as a member of the NATO Radiation Study Group and CDC Strategic National Stockpile working group, and as an advisor to WHO Collaborating Centers in Radiation Emergency Medical Preparedness and Assistance, among others. I learned a great deal from Dr. Tom MacVittie regarding ARS medical management, organ-specific sequelae associated with acute radiation sickness, and the dire need for a reproducible and consistent model for screening radiation countermeasures within the confines of the animal rule. It was Dr. Tom MacVittie who first envisioned the MCART Product Development and Support Services program to develop a research platform “linking” the various pathologic sequelae between rodents, NHPs, and humans that provided the overall theme for my PhD dissertation. Apart from science, however, Dr. MacVittie also taught me the importance of humility.

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completed during my PhD career. For three decades, Dr. Down has stressed the importance of proper animal models in radiation biology research and the inferiority of the C57BL/6J strain as a model for radiation-induced lung injury. It was his persistence that drove us to re-evaluate animal models in radiation-induced lung injury and provided the basis for this work. Dr. Down taught me a great deal about radiation physics, dosimetry, and the history of the field of radiation biology.

I’d also like to thank Dr. Mike McCreery for the hours he spent teaching me about FDA policy, Emergency Use Authorizations, and the Strategic National Stockpile. It was great fun to learn about federal regulations, requirements, and government needs from Dr. McCreery. He always made the topic really interesting and piqued my curiosity to learn more.

I also must thank our contracting officer at NIAID/NIH, Dr. David Cassatt, for ensuring that I remained funded throughout my PhD career (and for which he continues to remind me). I’d also like to remind him that Duke basketball will always be superior to Kentucky no matter how many articles he sends me to the contrary.

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program in Pathology and I hope that my scientific career will always make Dr. Hale proud.

I also need to thank Dr. Pizzo who took a chance and offered me a spot in the PhD program. Dr. Pizzo has a love of politics that may outmatch mine, which is fairly hard to do. I’d like to thank Dr. Sunday for providing her expertise in pulmonary pathology and for the long-term, fruitful collaboration that we have established.

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Abbreviations

ANOVA/ANCOVA, Analysis of variance/covariance

AP-PA, Anterior-posterior/posterior-anterior

ARS, Acute radiation syndrome

BARDA, Biomedical Advanced Research and Development Authority

BEIR, Biological effects of ionizing radiation

BPM, Breaths per minute

CDC, Centers for Disease Control and Prevention

CT, Computed tomography

DEARE, Delayed effects of acute radiation exposure

ED, Effective Dose

EUA, Emergency Use Authorization

f, respiratory frequency

FDA, U.S. Food and Drug Administration

GI, Gastrointestinal

GI-ARS, Gastrointestinal acute radiation syndrome

Gy, Gray, equivalent to 1 J kg⁻¹

H-ARS, Hematopoietic acute radiation syndrome

HIF-1α, Hypoxia-inducible factor –1 alpha

IACUC, Institutional Animal Care and Use Committee
IAEA, International Atomic Energy Agency

IPA, Ingenuity Pathway Analysis

IPKB, Ingenuity Pathway Knowledge Base

IR, Ionizing radiation

LD, Lethal dose

LET, Linear energy transfer

LINAC, Linear accelerator

MCM, Medical countermeasure

MIT, Massachusetts Institute of Technology, Cambridge, MA

MRI, Magnetic resonance imaging

MV, Megavolts

NHP, Non-human primate

PAM, Prediction Analysis for Microarray

PENH, Enhanced Pause

PBI/BM5, Partial body irradiation/5% bone marrow shielding

PBS, Phosphate buffered saline

MV, Minute Ventilation

NRDC, Natural Resources Defense Council

OCTEC, Office of Counter-Terrorism and Emergency Coordination

ORISE, Oak Ridge Institute for Science and Education
RAD, Radiation absorbed dose
REM, Roentgen equivalent man
RNS, Reactive nitrogen species
ROS, Reactive oxygen species
RT, Radiation therapy
SD, Standard deviation
SEM, Standard error mean
SNS, Strategic National Stockpile
SV, Sievert
TBI, Total body irradiation
TEM, Transmission electron microscopy
TGF-β1, Transforming growth factor-beta 1
TV, Tidal Volume
TLV, Total lung volume
Tr, Relaxation time
UHBI, Upper Half Body Irradiation
UMCG, University Medical Center Groningen, Groningen, The Netherlands
WMD, Weapons of Mass Destruction
WTI, Whole thorax irradiation
1. Background

1.1 Ionizing Radiation

1.1.1 From Discovery to Application

The discovery of radioactivity in the late 19th century heralded the dawn of a new scientific era. At the forefront of the field were notable scientists such as Roentgen, Becquerel, the Curies, Rutherford, and others who laid the foundation for the medical and technological advances that would occur in the 20th century.

In 1895, Wilhem Conrad Roentgen, a prominent German physicist, discovered a new kind of electromagnetic wave called “x-rays” (the x being a reference to their unknown origin) that were capable of penetrating human tissue. The first public radiograph was taken that year and by the following year physicians around the globe were routinely using x-rays as a modality in medical diagnostics and therapeutic treatments for various pathologic conditions.

Prior to Roentgen’s discovery, it had been known for centuries that certain minerals glowed phosphorescent when left in light. However, it wasn’t until 1896 that Antoine-Henri Becquerel discovered the emission of invisible penetrating rays from the element uranium did not require a stimulating agent. The phenomenon of spontaneous emissions was later coined “radioactivity” by Marie Curie, who along with her husband Pierre, discovered the elements radium and polonium while researching the phenomena...
of radioactive emissions from natural elements. Several years later, Ernest Rutherford, the “father of nuclear physics”, differentiated the various types of radiation as alpha, beta, and gamma rays according to their penetrance and determined the nature of radioactive decay. The observations made by Roentgen, Becquerel, the Curies, and Rutherford provided the essential foundation for the myriad of technological and scientific advancements that would occur during the 20th century.

Today, radiation plays an active role in our everyday lives. Nuclear scientists have learned to harness the heat energy produced from nuclear fission to provide power to millions of individuals around the world and the medical treatment of three out of every four patients in the industrialized world will be impacted by nuclear medicine, diagnostic imaging, and radiotherapy.

The discovery of radiation and its uses, however, did not come without a price. The new knowledge led to the creation and detonation of the atom bomb over Hiroshima and Nagasaki, Japan in 1945 by the United States and a nuclear arms race between the United States of America and the former Soviet Union under the doctrine of “mutually assured destruction” during much of the 20th century.

Today, the number of countries interested in developing peaceful nuclear technology is rising. As this trend continues, there is growing risk for a nuclear accident or weapons-grade materials being diverted for non-peaceful purposes. Since 9/11, there has been increasing concern among the U.S. government agencies that weapons-grade
materials will end up in the hands of a terrorist organization or rogue state\(^4\). In 2008, Senator Joseph Lieberman, speaking before the Committee on Homeland Security and Government Affairs in Washington, D.C., stated,

“Between 1993 and 2006, there were 1080 confirmed incidents of illicit trafficking in nuclear materials. Eighteen of those cases involved weapons-grade materials, and another 124 involved material capable of making a so-called “dirty bomb” that would use conventional explosives to spread nuclear materials”\(^4\).

In 2011, more than 10,000 drums of yellowcake uranium were found in the Libyan desert following the overthrow of Muammar el-Gaddafi’s regime\(^5\). It is unclear how much, if any, of the yellowcake may have been smuggled from Libya into Egypt and sold on the black market. Thus, the question for many has become not if, but when an attack will occur.

In addition to the growing threat of a nuclear/radiological incident, other threats for radiation overexposure exist, including nuclear power plant accidents, overdose in clinical radiotherapy for cancer treatment, or during the transport or decommissioning of nuclear material. At the current time, there is no FDA-approved treatment for the various syndromes associated with acute radiation exposure (discussed in Section 1.2.3). As a result, the United State’s government has allocated more than six billion U.S. dollars towards the development of medical interventions to mitigate or treat radiation injury (Project Bioshield, 2003), with a specific focus on the acute gastrointestinal and hematopoietic syndromes and delayed-lung injury.
Based on this, the purpose of the current project was to develop a pre-clinical research model of radiation-induced lung injury that could be utilized for high-throughput screening of medical countermeasures and assessment of the biological effects of radiation on healthy lung tissue. The conceptualization of the project proceeded with respect to current knowledge gaps in the field of radiation normal tissue biology that are of immediate concern to the U.S. government (discussed in Section 1.4). The experiments were designed to build a research platform that would meet the objectives of the Food and Drug Administration (FDA) Animal Efficacy Rule (21 C.F.R. § 314.610, drugs; § 601.91, biologics) for pivotal efficacy testing and approval of radiation countermeasures.

1.1.2 Principles of Radiation

1.1.2.1 Radiation Units

Although the term “radiation” evokes fear in the majority of individuals, radiation plays an important role in our everyday lives. For more than 4500 million years, the earth has been radioactive. Each year, humans are exposed to approximately 6.2 mSV of ionizing radiation, half of which comes from natural sources. We are exposed to radiation through the air we breathe, the foods we consume, the ground on which we walk, and the buildings where we live, work, eat, and sleep. The other half of annual radiation exposure comes from medical procedures, for example diagnostic imaging (ex. computed tomography) and nuclear medicine.
The amount of radiation an individual is exposed to is described by the *medically relevant dose*, or the amount of energy absorbed by tissue. The SI unit of absorbed dose is the Gray (Gy), where one Gy is equivalent to one joule of energy per kilogram of tissue. Another unit often used is the Sievert (Sv), sometimes called the *equivalent dose*, which takes into account the *quality* of the radiation in tissue and is therefore considered to be a measurement for the risk of biological harm. The quality factor is selected based on the relative biologic effectiveness (RBE) of the type and energy of the radiation in tissue\(^1\). For example, neutrons (neutrally charged particles with large mass) are more biologically damaging than x-rays or gamma rays and thus have a higher quality factor. For x-rays and gamma rays, the RBE is identical and the quality factor is 1. Thus, 1 Sv is equal to 1 Gy when comparing the equivalent and the absorbed doses for x-rays and gamma rays. It is also worth mentioning that the old unit of equivalent dose is the rem, or roentgen equivalent man, as this term is often still used. One rem is equal to 0.01mSv (Table 1).

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<th>Definition</th>
<th>Unit</th>
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<td>Absorbed Dose</td>
<td>Amount of energy absorbed per kg/tissue.</td>
<td>Gray (Gy), 1 J kg(^{-1})</td>
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<tr>
<td></td>
<td></td>
<td>Rad, 0.01 J kg(^{-1}) (old unit)</td>
</tr>
<tr>
<td>Equivalent Dose</td>
<td>Absorbed dose x radiation quality</td>
<td>Sievert (Sv)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rem (old unit), 100 rem = 1 Sv</td>
</tr>
</tbody>
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Table 1: Units of Radiation
1.1.2.2 Radiation Types

Radiation is the transfer of energy through the passing of electromagnetic waves or particles in matter. Radiation can be either non-ionizing or ionizing. Non-ionizing radiation occurs when the energy of an electromagnetic wave or particle excites an electron to a higher energy state, but does not strip the electron from the atom. Examples include radio waves and microwaves. In comparison, ionizing radiation occurs when there is enough energy to knock an electron from an atom or molecule, a term called “ionization”. Because ionizing radiation, in contrast to non-ionizing radiation, has enough energy to break chemical bonds, exposure can result in biological damage that may or may not have health consequences. Examples of ionizing radiation are electrons, photons, alpha and beta particles, neutrons, and heavy ions.

The primary source of natural background radiation exposure is alpha (α)-particles. As α-particles are found in the earth’s crust and soil, they may also sometimes be present in construction materials. These highly charged particles are emitted during the radioactive decay of elements such as polonium and radium. Radon, an inert radioactive gas produced from the decay of radium, is considered to be the second leading cause of lung cancer in the United States next to tobacco smoke (polonium 210 was found in tobacco in 1959). Due to their large mass and minimal penetrance, α-particles are not considered to be biologically dangerous when exposure is limited to external contamination. However, when α-particles are ingested, inhaled, or internalized
(ex. wound contamination), they are approximately twenty times more destructive to DNA and other cellular macromolecules than either x-rays or gamma-rays due to their strong ionization properties.

Approximately five percent of natural background radiation exposure each year comes from cosmic (or space) radiation. Although the hazards of space radiation are minimal for the majority of individuals, it is of particular concern for astronauts traveling on long space flights. The association between the duration of space flight and biological damage was first identified in the late 1970s when a larger number of chromosomal aberrations were seen in astronauts from the moon missions compared to the Gemini missions that did not go to the moon. As a result, the unacceptable risk for health effects due to radiation exposure is a major impediment to space exploration.

The primary types of cosmic radiation are high energy protons, charged particles with high mass (>2000x greater than electrons) and to a lesser extent, neutrons and heavy ions. In 1946, Robert Wilson first proposed the use of protons in cancer therapy. The biological rationale for use of protons lay in their sharp Bragg peak and minimal side scatter thereby allowing more precise delivery of radiation to tumor while sparing normal tissue. Today, protons and heavy charged particles are both used in clinical radiotherapy, albeit sparingly when taking into account the large number of individuals receiving radiotherapy each year. This is in large part due to the cost associated with construction and maintenance of proton facilities (>125 million). Over recent years
there has been a growth in specialized proton facilities across the U.S., however, heavy ion facilities for cancer therapy no longer exist in the U.S. and are primarily localized to Germany and Japan\(^9\).

Neutrons, neutrally charged particles similar in mass to protons, are also used in cancer radiotherapy primarily to treat inoperable tumors resistant to conventional radiotherapy\(^10\). Since neutrons are a form of high linear energy transfer (LET) radiation, they deposit a greater amount of energy in tissue than photons (low LET radiation). This results in a greater number of double stranded DNA breaks and a higher rate of cell kill when compared to low LET radiation and forms the biologic basis for neutron therapy to treat cancers resistant to conventional radiotherapy\(^10\). The majority of clinical trials in the first half to mid-20\(^{th}\) century demonstrated no overall survival advantage with neutron therapy when compared to electron or photon therapy, but serious, adverse side effects (i.e. severe normal tissue damage) were noted\(^9,11\). Many of the early clinical trials were flawed, however, due to limitations in basic radiobiological knowledge and use of equipment with poor dose-distribution characteristics. As a result, the use of neutrons in clinical radiotherapy was later revisited and shown to be more effective than photon or electron therapy in treating some cancer types including locally advanced, inoperable salivary gland tumors\(^9\). Currently there are only three facilities in the United States specializing in neutron radiotherapy. These facilities are located at the University of
Washington (Seattle, WA), Karmanos Cancer Center/Wayne State University (Detroit, Michigan), and FermiLab/Northern Illinois University (Chicago, Illinois).

Understanding the risk of biological harm to healthy tissue from high LET radiation is of particular importance for individuals exposed to acute doses of radiation in a nuclear meltdown or attack with a “dirty bomb” since neutrons are emitted as a by-product of nuclear fission. Since neutrons cause far greater toxicity than low LET radiation, knowledge of the radiobiological effects of high LET radiation on normal tissue cannot be extrapolated from pre-clinical studies with low LET radiation.

Due to the broad use of photon therapy in clinical cancer treatment, the majority of pre-clinical radiobiological research is based on data derived from radiation experiments using gamma or x-rays. Gamma rays and x-rays differ only in the way they are produced, meaning they have similar physical properties (both are photon emitters). Gamma rays are produced by radioactive decay of unstable nuclei whereas x-rays are produced when electrons are accelerated within a device and then abruptly stopped in tungsten or gold leading to the emission of energy in the form of electromagnetic waves. Gamma rays and x-rays are both highly penetrating and are used in diagnostic radiology, nuclear medicine, as well in cancer radiotherapy. This project will deal with these types of radiation.
1.2 Medical Aspects of Ionizing Radiation Exposure

1.2.1 Radiation Threats

Over the past century, the number of individuals exposed to radiation overdose resulting in acute radiation sickness or death has been relatively minimal (Appendix A). On a day-to-day basis, exposure to acutely injurious levels of radiation is primarily a concern for individuals who work with or around nuclear or radioactive materials in which accidents can and do occur. Radiation damage to healthy tissue is also a concern for those undergoing medical procedures such as cancer radiotherapy in which normal tissue surrounding the tumor is inevitably exposed to radiation during treatment. Of the majority of documented radiation incidents involving occupational exposure or medical-related overdose, most occurred as a result of worker negligence, equipment error, or a combination of both. In addition, there is also the threat of prolonged exposure to low dose radiation (cumulative dose <10cGy) due to routine occupational exposure or environmental contaminants (ex. unexpectedly high levels of radionuclides have recently been found in artic sea ice\(^{12}\) and Jordanian sandstone aquifers\(^{13}\) and an unknown amount of radioactive material leaked from Fukushima after the reactor meltdown).

In “Lessons from Major Radiation Accidents” written by Ortiz, Oresegun, and Wheatley for the International Atomic Energy Agency, a retrospective analysis of radiation accidents suggests the following:
“a) accidents involving industrial radiography are the most frequent cause of severe or fatal overexposure to workers and the public; b) accidents involving gamma irradiators normally result in fatalities of workers, whereas accidents with electron beam accelerators often result in amputation of limbs, c) accidents in radiotherapy can affect large numbers of patients, resulting in their death (directly or indirectly) or severe degradation in quality of life; and d) the loss of control of sources (“orphan” sources) has resulted in death and severe deterministic effects to members of the public and has caused widespread contamination of the environment”14.

Aside from the aforementioned events, which are generally rare and involve only a limited number of individuals exposed at any one time, the immediate concern of the U.S. government is related to the likelihood for a mass-casualty scenario in which a large number of individuals are exposed at any one time and for which the U.S. remains unprepared. Specifically, radiation threats where a large number of individuals might be exposed include 1) an attack with a nuclear bomb (e.g. Hiroshima/Nagasaki), 2) detonation of an explosive radiological dispersal device (“dirty bomb”), 3) use of a radiological dispersal device hidden in a high-traffic public area or 4) a nuclear meltdown (e.g. Chernobyl)15.

In today’s political environment where borders are fluid and communication is instantaneous, the ability of a rogue state or terrorist group to obtain and use radioactive isotopes or weapons grade materials for a terrorist attack is a key threat to our national security. The use of radioactive sources for criminal purposes is well documented. There were 827 confirmed incidents among International Atomic Energy Agency (IAEA) member states between 1993 and 2005 involving illegal acquisition, possession, transfer, or disposal of nuclear or other radioactive material, including highly enriched uranium
and plutonium. Table 2 summarizes the radiation incidents that have been reported since 1950. Since the fall of the Soviet Union, the international community has placed increasing emphasis on securing nuclear facilities and radioactive material stockpiles, particularly within the former Soviet block. Since 9/11, the sense of urgency with which the U.S. government and international community have worked to document and secure radioactive materials has only escalated.

Currently, there are more than twenty thousand nuclear weapons in existence around the world. This does not include the numbers held by Israel, India, and Pakistan who do not report their nuclear weapons to the IAEA.

The most often misused radioactive source is cesium, a gamma-emitter. Cesium-137 is deeply penetrating, has a relatively long half-life, and can cause biological harm through external exposure. This is in contrast to alpha-emitters (ex. plutonium) which require ingestion or inhalation to cause biological damage thereby limiting their ability to cause mass casualties. Cesium and other gamma emitters are the primary radioisotopes used in industrial radiography and industrial irradiators and the majority of incidents of radiation misuse have been carried out by individuals with direct access to these sources.

The lack of efficient post-exposure treatments for victims experiencing acute radiation toxicity presents a serious problem should an attack with a radiological device or nuclear bomb occur. As a result, a large number of organizations and industry
partners have been working together to develop a plan for emergency preparedness, response, and treatment. These organizations include the following with a primary focus on drug development: Office of Counterterrorism and Emergency Coordination (OCTEC) within the Federal Drug Administration (FDA), the radiation/nuclear group at the National Institute of Allergy and Infectious Disease (NIAID), a division of the National Institutes of Health (NIH), and the Biomedical Advanced Research and Development Authority (BARDA). Participants focusing specifically on emergency response and triage include the Radiation Injury Treatment Network and the Radiation Emergency Assistance Center/Training Site (REAC/TS) managed by the Oak Ridge Institute for Science and Education. REAC/TS is a member of the international Radiation Emergency Medical Preparedness Assistance Network (REMPAN) and the IAEA’s Response Assistance Network (RANET). Federal Emergency Management Agency (FEMA) is also a participant with a wide focus on development of computer models mimicking the various types of attacks that could occur and the scenarios associated with each. It is a truly collaborative effort among these groups that identifies the current logistical, medical, and scientific challenges that we, as researchers, must appropriately address when developing radiation countermeasures for use in emergency situations (see Section 1.4).
Table 2: Criminal incidents involving radioactive sources (1950-2005)

<table>
<thead>
<tr>
<th>Date and Location</th>
<th>Radiation Source</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>July 1969, USA</td>
<td>Enriched uranium</td>
<td>University professor</td>
</tr>
<tr>
<td>April 1974, Austria</td>
<td>Iodine-131</td>
<td>Transportation system</td>
</tr>
<tr>
<td>January 1979, USA (NC)</td>
<td>Uranium-dioxide</td>
<td>Employer/Extortion</td>
</tr>
<tr>
<td>May 1979, France</td>
<td>Radioactive graphite</td>
<td>Employer</td>
</tr>
<tr>
<td>October 1979, USA</td>
<td>Tritium</td>
<td>Business</td>
</tr>
<tr>
<td>July 1981, USA</td>
<td>Iridium-192</td>
<td>Suicide</td>
</tr>
<tr>
<td>April 1985, USA</td>
<td>Plutonium</td>
<td>Extortion</td>
</tr>
<tr>
<td>August 1992, Lithuania</td>
<td>Low enriched uranium</td>
<td>Unknown target/theft/sale</td>
</tr>
<tr>
<td>October 1992, Bulgaria</td>
<td>Plutonium</td>
<td>Business</td>
</tr>
<tr>
<td>November 1992, Ukraine</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>January 1993, France</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>January 1993, Lithuania</td>
<td>Cesium, Beryllium,</td>
<td>Unknown/smuggling/sale</td>
</tr>
<tr>
<td></td>
<td>Uranium</td>
<td></td>
</tr>
<tr>
<td>November 1993, Russia</td>
<td>Nuclear warheads</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>1994, Estonia</td>
<td>Cesium</td>
<td>Unknown/theft</td>
</tr>
<tr>
<td>February 1994, Bulgaria</td>
<td>Radium</td>
<td>Unknown</td>
</tr>
<tr>
<td>May 1994, Russia</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>July 1994, Bulgaria</td>
<td>Uranium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>October 1994-February 1996, China</td>
<td>Phosphorus-32</td>
<td>Individual/student</td>
</tr>
<tr>
<td>December 1994, Russia</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>November 1995, Russia</td>
<td>Cesium</td>
<td>Unknown/acquired by Chechen rebels</td>
</tr>
<tr>
<td>March 1996, Romania</td>
<td>Low enriched uranium</td>
<td>Unknown/sale</td>
</tr>
<tr>
<td>March 1996, Tanzania</td>
<td>Cesium</td>
<td>Unknown/sale</td>
</tr>
<tr>
<td>November 1996, Georgia</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>April 1997, Russia</td>
<td>Cesium</td>
<td>Unknown</td>
</tr>
<tr>
<td>March 1998, USA (NC)</td>
<td>Cesium</td>
<td>Unknown</td>
</tr>
<tr>
<td>May 1998, Russia</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>June 1998, Azerbaijan</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>March 1999, Lebanon</td>
<td>Uranium</td>
<td>Illegal sale to Iran/Syria</td>
</tr>
<tr>
<td>August 1999, USA</td>
<td>Phosphorous-32</td>
<td>Co-worker/lab technician</td>
</tr>
<tr>
<td>August 1999, Romania</td>
<td>Nuclear components</td>
<td>Illegal sell to states and terrorist organizations</td>
</tr>
<tr>
<td>September 1999, Chechnya</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>September 1999, Ukraine</td>
<td>Strontium-90</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>Date</td>
<td>Location</td>
<td>Material</td>
</tr>
<tr>
<td>--------------------</td>
<td>------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>December 1999, S. Korea</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>March 2000, Kazakhstan</td>
<td>Strontium-90</td>
<td>Likely delivery to Al Qaeda</td>
</tr>
<tr>
<td>May 2000, Cambodia</td>
<td>Uranium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>July 2000, Ukraine</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>October 2000, Russia</td>
<td>Cesium</td>
<td>Accidental; Thieves trying to sell scrap metal</td>
</tr>
<tr>
<td>December 2000, Japan</td>
<td>Iodine-125</td>
<td>Infrastructure</td>
</tr>
<tr>
<td>May 2001, Moldova</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>June 2001, Russia</td>
<td>Strontium</td>
<td>Accidental; thieves trying to extract lead</td>
</tr>
<tr>
<td>June 2001, Russia</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>September 2001, Russia</td>
<td>Cesium</td>
<td>Unknown/sale</td>
</tr>
<tr>
<td>December 2001, Georgia</td>
<td>Strontium</td>
<td>Unknown</td>
</tr>
<tr>
<td>May 2002, Russia</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>May 2002, China</td>
<td>Iridium-192</td>
<td>Business</td>
</tr>
<tr>
<td>May 2002</td>
<td>Cesium-133, non radioactive isotope</td>
<td>Unknown/theft/sale (thieves thought they had Ce-137)</td>
</tr>
<tr>
<td>July 2002, UK</td>
<td>Plutonium</td>
<td>Plot by Real IRA to steal plutonium for “dirty bomb”</td>
</tr>
<tr>
<td>July 2002, Russia</td>
<td>Plutonium, Cesium, Strontium, Uranium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>September 2002, Kazakhstan</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>June 2003, Thailand</td>
<td>Cesium</td>
<td>Presumed sell to militant Islamic organization for RDD</td>
</tr>
<tr>
<td>July 2003, Russia</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>September 2003, Poland</td>
<td>Cesium</td>
<td>Sting operation/sale of 60 g of Ce-137 to polish police</td>
</tr>
<tr>
<td>May 2004, Ukraine</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
<tr>
<td>January 2005, Ukraine</td>
<td>Cesium</td>
<td>Unknown/theft/sale</td>
</tr>
</tbody>
</table>

*Table modified from Mohtadi and Murshid, 2006*\(^7\). Here, the target is designated as “unknown/theft/sale” when individuals were apprehended in possession of radioactive sources with presumed intent to sell. Although some of the radioactive material was recovered in these incidents, in many cases some material remains missing and untold amount of material has ended up on the black market from documented and undocumented cases of nuclear trafficking.
1.2.2 Medical Syndromes Associated with Radiation Exposure

1.2.2.1 Adverse health effects of low dose radiation exposure

It was not long after Roentgen’s discovery of x-rays that the biological hazards associated with radiation exposure were first recognized. Within several years, a number of investigators, including Becquerel and Pierre Curie, noticed the development of erythema (reddening of the skin) and ulcerations following exposure to radioactive sources and a number of adverse events from medical imaging, including skin necrosis leading to limb amputations, were reported\(^2\). The health effects of radiation overexposure are dependent on the total absorbed dose, dose rate, route of exposure, the quality of radiation, the area/volume of the body exposed, and the presence or absence of secondary injuries (blast injuries, thermal burns) or co-morbidities\(^{15}\). Depending on the radiation dose and duration of exposure, tissue damage may range from increased risk for fetal abnormalities, sterility, cataracts, and carcinogenesis to rapid death due to acute radiation sickness\(^{21}\).

The biological harm associated with radiation exposure can be classified as either stochastic or deterministic. The *stochastic* effects of radiation exposure suggest the *probability* of an event occurring (i.e. gene mutation, lens opacification) increases with respect to radiation dose. This is based on the linear-no-threshold (LNT) model that states biological damage caused by ionizing radiation is directly proportional to the dose received. As a result, any exposure to radiation would be considered harmful and it is the accumulation thereof that ultimately determines the relative risk for developing
cancer or other adverse health effects over a lifetime. However, several studies, including long-term follow-up of survivors from Chernobyl and Hiroshima/Nagasaki, have challenged the LNT model. Aside from the significant increase in thyroid cancers among children and adolescents exposed to the radioactive fallout at Chernobyl, radioepidemiological studies among survivors who received less than 0.05 Sv have found no definitive increase in carcinogenesis above that expected to occur naturally in an aging population. However, the reliability of current estimates is not at all comprehensive and further follow-up of survivors is necessary before long-term risk assessment can be established. A recent study published in PNAS by Dr. Neumaier and colleagues draws into question the reliability of the LNT model from a scientific point of view based on new evidence for “DNA repair centers.” Using time lapse imaging and mathematical kinetic modeling of radiation-induced foci (RIF) formation in mammary epithelial cells, the investigators found the number of RIF do not increase proportionally with the yield of double stranded DNA breaks (DSB). These data coupled with alterations in RIF formation/disappearance suggest DSB may migrate over distances of up to 2 µm to cluster in centralized regions for more efficient recognition and repair. As a result, previous studies demonstrating a linear association between radiation dose and DNA damage based on the relationship between “radiation-induced foci” and radiation dose should be viewed with skepticism. Thus, it is now being acknowledged that the
LNT model may not accurately predict individual risk of injury although it is still unclear whether the LNT model under or overestimates relative risk\textsuperscript{24}. Since the relative risk of biological harm is unclear at low radiation doses, the international community maintains a conservative limit for individual members of the public (<1 mSV/year excluding natural background radiation) and occupational workers (<50 mSv/year). In 2006, the BEIR VII report based on follow-up of more than 400,000 persons exposed to radiation (average dose 19.4 mSV), stated, “it is unlikely that there is a threshold below which cancers are not induced”\textsuperscript{25}. Since the threshold dose for which radiation becomes harmful is not known, the doses listed above are maximal limits set by the U.S. Government, expecting that individuals and occupational workers will maintain appropriate safeguards against radiation exposure, a concept defined by ALARA or “as low as reasonably achievable”. In contrast, to stochastic effects, deterministic effects suggest the severity of tissue damage/injury rises with increasing dose. The threshold dose before deterministic effects are observed is considered to be approximately 50cGy and the following chapter discusses the acute and delayed radiation syndromes associated with doses >50cGy in more detail.

1.2.2 Acute radiation syndrome/delayed effects of acute radiation exposure

The organ-specific sequelae that arise following exposure to high radiation doses over a short period of time can be categorized as either the acute radiation syndrome (ARS)
or the delayed -effects of acute radiation exposure (DEARE). Knowledge regarding the
pathogenesis of ARS/DEARE has been empirically derived through past experience with
radiation accident victims and atomic bomb survivors from Hiroshima and Nagasaki.
The syndromes associated with total body or partial body irradiation (TBI/PBI) are
defined by the mode of death. The organ-specific sequelae that fall under ARS are
cerebrovascular (>20 Gy), gastrointestinal (>5 Gy), and hematopoietic syndromes (>0.5
Gy). Without moderate to intensive supportive care, the lethal radiation dose for acute
exposure for 50% of the population within the first 60 days (LD50/60) is between 3.4 to
4.0 Gy. However, with excellent intensive supportive care survival can be prolonged for
several months improving the LD50/60 to greater than 5.4 Gy. If survival is prolonged
due to intensive supportive therapy, an individual may experience more than one of
these syndromes and death ultimately results from multiple organ failure, including
respiratory distress due to radiation pneumonitis/fibrosis. As a result, ARS is more
accurately described as multiorgan dysfunction syndrome or MODS.

Immediately after acute TBI/PBI at high doses, there is a prodromal period in
which early symptoms appear. These include nausea, vomiting, and diarrhea. At
supralethal doses, victims may also experience a transient loss of consciousness,
neurologic dysfunction, fever, and hypotension. The timing, scope, and severity of
clinical manifestations in the prodromal period are reliable indicators of approximate
radiation dose (i.e. sublethal, lethal, or supralethal; see Section 1.2.2).
After the initial symptoms have abated, there is a so-called “latent period” in which the individual seemingly appears to have recovered. The latent period may last for several hours (cerebrovascular syndrome) to days (GI) or weeks (hematopoietic/respiratory syndromes) before the initial symptoms return with greater severity (manifest illness phase). During the manifest illness phase, rapid decline in health status is observed and is followed by either recovery or death. The absence of a notable latent period is indicative of supralethal exposure and imminent mortality from CNS syndrome.

The timing of the latent period and manifest illness phase is inversely proportional to the total dose received (Figure 1). Thus, individuals experiencing the cerebrovascular syndrome may succumb within the first 48 hours of exposure whereas individuals with hematopoietic syndrome may survive for several weeks. If individuals survive through the initial, acute syndromes, then there is a strong likelihood for later development of the delayed GI syndrome, lung injury (pneumonitis/fibrosis), and/or kidney failure as well as injury to other organs (Table 3)\textsuperscript{27,28}. 
Figure 1: Approximate time course of clinical manifestations of acute radiation syndrome.

Shown are the approximate times for hematopoietic, gastrointestinal (GI), and central nervous system (CNS) symptoms at different dose ranges of total body irradiation. Almost all individuals exposed to doses greater than 6 Gy will experience vomiting within the first 8 hours of exposure. If symptoms appear within the first 5-15 minutes, the individual has likely experienced a lethal dose and will succumb to CNS syndrome within twenty-four to forty-eight hours following exposure. The signs and symptoms of CNS syndrome include headache, impaired cognition, disorientation, seizures, and hypotension. GI symptoms occur at doses greater than 5 Gy and include nausea, vomiting, or diarrhea. Hematopoietic changes (>0.5 Gy) include development of lymphopenia, granulocytopenia, or thrombocytopenia. If vomiting does not occur within the first 8 hours, the individual has likely received a sublethal dose (<2 Gy). Persistent vomiting during the first six to eight hours after exposure is useful for distinguishing between psychosocial symptoms (fear; anxiety) associated with a radiation event and exposure to significant doses of total body irradiation. In the former, vomiting episodes will be limited to only one or two whereas in the latter, vomiting will persist for several hours. Note that the clinical manifestations of injury to different organ systems overlap. Reprinted with permission from Waselenko et al21.
Table 3: Phases of Radiation Injury*

<table>
<thead>
<tr>
<th>Dose Range, Gy</th>
<th>Prodrome</th>
<th>Manifestation of Illness</th>
<th>Prognosis (without therapy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5-1.0</td>
<td>Mild</td>
<td>Slight decrease in blood cell counts</td>
<td>Almost certain survival</td>
</tr>
<tr>
<td>1.0-2.0</td>
<td>Mild to moderate</td>
<td>Early signs of bone marrow damage</td>
<td>Highly probable survival (&gt;90%)</td>
</tr>
<tr>
<td>2.0-3.5</td>
<td>Moderate</td>
<td>Moderate to severe bone marrow damage</td>
<td>Probably survival</td>
</tr>
<tr>
<td>3.5-5.5</td>
<td>Severe</td>
<td>Severe bone marrow damage, slight GI damage</td>
<td>Death within 3.5-6 weeks (50% of victims)</td>
</tr>
<tr>
<td>5.5-7.5</td>
<td>Severe</td>
<td>Pancytopenia and moderate GI damage</td>
<td>Death probable within 2-3 weeks</td>
</tr>
<tr>
<td>7.5-10.0</td>
<td>Severe</td>
<td>Marked GI and bone marrow damage, hypotension</td>
<td>Death probable within 1-2.5 weeks</td>
</tr>
<tr>
<td>10.0-20.0</td>
<td>Severe</td>
<td>Severe GI damage, pneumonitis, altered mental status, cognitive dysfunction</td>
<td>Death certain within 5-12 days</td>
</tr>
<tr>
<td>20.0-30.0</td>
<td>Severe</td>
<td>Cerebrovascular collapse, fever, shock</td>
<td>Death certain within 2-5 days</td>
</tr>
</tbody>
</table>

* Table reprinted with permission from Waselenko et al.21
Cerebrovascular Syndrome. Total body exposure to radiation doses in excess of 20 Gy almost invariably results in death within the first 48 hours due to cerebrovascular/central nervous system syndrome (CNS). Immediately after the radiation incident, victims may experience a brief loss of consciousness, disorientation, hypotension, and fever. Such signs are indicative of supralethal radiation exposure for which there is no treatment. The latent period is brief, generally lasting only a few hours. During the subsequent manifest illness phase, the victim may experience disorientation, cognitive impairment, cerebral edema and increased intracranial pressure, seizures, fever, and hypotension. While the pathogenesis of gastrointestinal and hematopoietic syndromes leading to death is relatively clear, the cause of death in cerebrovascular syndrome is less well known. However, it is thought that death is a result of increased intracranial pressure due to endothelial cell damage and vascular leak. As doses in excess of 20 Gy are considered supralethal, radiation countermeasure efforts have focused on the potentially survivable gastrointestinal (GI) and hematopoietic syndromes, and radiation-induced lung injury.

Gastrointestinal Syndrome. The gastrointestinal syndrome occurs following total or partial body radiation doses between 8 and 12 Gy, although minor signs and symptoms may be recognized at doses as low as 5 Gy. Following radiation exposure, there is a short prodromal period where the individual may experience abdominal pain, nausea, diarrhea, and vomiting, after which the symptoms will dissipate for several days.
However, the individual's health rapidly declines with reappearance of vomiting and diarrhea followed by malnutrition/ malabsorption, dehydration and electrolyte imbalance, sepsis, and multiorgan failure\textsuperscript{21}. Without supportive care, death occurs within 1-3 weeks.

The pathogenesis of gastrointestinal syndrome follows a well-defined course. The initial target cells for radiation injury appear to be the crypt cells of the microvilli. Under physiologic conditions, the intestinal crypt cells migrate up the microvilli to replenish the epithelial cell layer that is sloughed off every few days. Clonogenic death of the stem cell population due to radiation damage leads to shortening of the microvilli resulting in disruption of the mucosal barrier, bacterial translocation, inflammation and sepsis that may be exacerbated by lymphocyte depletion\textsuperscript{30,31}.

*Hematopoietic Syndrome.* The onset of hematopoietic syndrome occurs after TBI with a dose of 0.5 Gy or greater. At doses in excess of 2.5 Gy, immune suppression resulting in hemorrhage and infection becomes particularly life threatening and without supportive care, death due to the hematopoietic syndrome will occur between several weeks to 2 months after exposure. With superior medical care, such as cytokine therapy, antibiotics, and bone marrow transplant, the hematopoietic syndrome can be a potentially survivable condition at doses as high as 6-7 Gy\textsuperscript{29}.

The primary focus for medical countermeasure development has been on the gastrointestinal, hematopoietic, and pulmonary syndromes. There has also been some
focus on development of topical MCMs for a fourth ARS subsyndrome, radiation-induced skin toxicity (cutaneous syndrome), when funding allows (personal communication with NIAID and BARDA). Skin toxicity is characterized by erythema, edema, dry and/or moist desquamation, ulceration, hair loss (alopecia), and fibrosis.

Understanding the sequential progression of organ-specific sequelae following acute TBI/PBI are important not only to medical management of acute and delayed radiation sickness (Section 1.2.3) but also to the development of pre-clinical research models for screening of therapeutic interventions to prevent, mitigate, or treat radiation disease (Section 1.4). As this project focuses primarily on DEARE-lung, the following chapter discusses radiation-induced lung injury in more detail (Section 1.3).

1.2.3 Medical Management of Acute and Delayed Radiation Sickness

The medical management of acute and delayed radiation sickness is challenging. In a mass casualty setting, there will be a high volume of victims, limited resources, loss of infrastructure, and presence of combined injuries such as thermal burns, wounds/crush injuries, and infection. The majority of our knowledge concerning the medical management of ARS/DEARE has been derived from experience with nuclear accident victims and well-controlled, pre-clinical animal studies of TBI/PBI.

In 1990, a computerized database was established by researchers in Moscow, Russia and Ulm, Germany to address the limitations associated with the database registries operated by the IAEA and Oak Ridge Institute of Science and Education. The registries
operated by the IAEA and Oak Ridge describes the conditions that led to individual radiation accidents and the medical consequences of radiation exposure, respectively. Neither registry, however, addresses the hour-by-hour clinical manifestations of disease, therapeutic regimens used to treat the illness, or clinical outcomes\textsuperscript{33}. Therefore, the International Computer Database for Radiation Exposure Case Histories (ICDREC) was established to document the clinical day-to-day management of acute radiation sickness\textsuperscript{33}. When data was unavailable in the public realm, the treating physicians and institutes were contacted such that thorough documentation could be completed\textsuperscript{33}.

There are three routes for radiation exposure. These are external or internal contamination with radionuclides and external irradiation. Since there is no rapid method for estimating the total body radiation dose at the time of presentation, the physician must treat based on the predicted clinical course. The temporal onset and duration of prodromal symptoms provide a good estimate of the total dose and are generally well correlated with physical dose estimates\textsuperscript{34}. For example, development of diarrhea, fever, and hypotension within the first hour suggests the individual has received a fatal TBI/PBI dose ($>10$ Gy). If these initial signs and symptoms are present within 5-15 minutes of exposure, death is certain within one to two days. In contrast, if there is a delayed onset of mild prodromal symptoms for 6-12 hours with dissipation by 24 hours, the dose is likely to be less than 2 Gy and the likelihood for survival is favorable\textsuperscript{28}. 
Until two decades ago, the prognosis for individuals experiencing gastrointestinal or hematopoietic syndromes was dire. Without supportive care, the lethal dose for 50% of individuals before 60 days is 3.2-3.6 Gy due to bone marrow failure. However, today, bone marrow failure may no longer be the limiting factor for survival as intensive supportive care with IV fluids, antibiotics, cytokine therapy and bone marrow transplant has been shown to extend survival\textsuperscript{27, 34}. With excellent supportive care, the LD50/60 can be improved to >5.4 Gy.

There is ample data to support the use of cytokine therapy in ARS-heme. Cytokine therapy was first used to treat ARS-heme victims at Chernobyl. Since then, it has been shown to significantly improve survival in radiation accident victims in Israel\textsuperscript{35}, Belarus\textsuperscript{27}, and Japan\textsuperscript{34} as well as in pre-clinical studies with canine and NHP models of ARS. In the majority of cases, cytokine therapy with human recombinant granulocyte-colony stimulation factor (G-CSF; Neupogen\textsuperscript{®}) or pegylated G-CSF (Neulasta\textsuperscript{®}) and granulocyte-macrophage colony stimulating factor (GM-CSF; Leukine\textsuperscript{TM}) are given concurrently. In victims treated with cytokine therapy, neutrophil recovery occurred 3-6 days earlier than non-treated individuals\textsuperscript{21}. In addition to cytokine therapy, blood transfusions and bone marrow transplant (BMT) may also be used for individuals experiencing complete myelosuppression. However, due to the risk for graft versus host disease, BMT is reserved for individuals with the highest radiation doses (>6 Gy).

The ability for BMT to enhance recovery and improve survival is not known. A
retrospective analysis of 29 individuals receiving lethal doses of total body irradiation in which BMTs were a part of the treatment regimen was performed by Densow et al.\textsuperscript{33}. Only three individuals ultimately survived out of twenty-nine\textsuperscript{33}. Although the number of individuals included in the analysis was too small to derive statistically meaningful results, there is some question whether BMT improved survival outcome or whether the three individuals would have survived anyway\textsuperscript{33}.

Since there is no precisely defined treatment for ARS, physicians must use their best judgment to treat the clinical signs and symptoms\textsuperscript{21}. These include antibiotics specific to gram-negative bacteria, antiemetics, and antifungals, topical burn creams, and the like\textsuperscript{21}.

As survival can now be extended through the hematopoietic syndrome, death due to multiorgan dysfunction syndrome (MODS) has become the predominant concern. Specifically, failure of the gastrointestinal tract, skin, lungs, and sometimes kidneys, appear to be the primary causes of death\textsuperscript{28}. In a careful review of ICDREC data of 49 radiation-related fatalities, Densow et al.\textsuperscript{33} determined deaths were attributed to radiation burns in 32 cases, gastrointestinal syndrome in 24 cases, infection in 9 cases, respiratory syndrome in 9 cases, graft versus host disease in 7 cases, and 7 died due to other causes\textsuperscript{28}. At the current time, there is no FDA-approved treatment for the syndromes associated with MODS after radiation.
For more information concerning the daily medical management of radiation accident victims, there are a number of thorough reviews. In particular, Waselenko et al. provides an excellent overview of the recommendations from the Strategic National Stockpile Radiation Working Group.21

1.3 Radiation-Induced Lung Injury

1.3.1 Radiation-induced lung injury: relevance and clinical pathology

Radiation induced lung injury is characterized by either an acute, inflammatory reaction (pneumonitis/alveolitis) or delayed, chronic fibrosis. The dose-response for lethal radiation pneumonitis is derived from individuals receiving upper half body or total body irradiation (Figure 2). The threshold for lethal pneumonitis is approximately 7.5 Gy after which the incidence rises steeply with a lethal dose for 50% of individuals by day 180 (LD50/180) being roughly 9.3 Gy and an LD100/180 of ~11 Gy36,37.

Radiation pneumonitis has an acute onset between 6 to 16 weeks after radiation exposure and becomes particularly life threatening when the entire lung tissue is exposed38. In contrast, chronic fibrosis occurs months to years later and is characterized by diffuse interstitial fibrosis, focal scarring, alveolar collapse, and impaired ventilation38.
Figure 2: Actuarial incidence of radiation pneumonitis among patients receiving upper half body or total body irradiation.

Shown is a best fit sigmoidal complication curve\(^1\) using simplified probit regression analysis for patients who were treated between 1960-1976 with a single large dose of radiation to the whole lung as palliative care for advanced lung disease or total body irradiation as adjuvant therapy for Ewing’s sarcoma or prior to bone marrow transplant\(^2\). Patients with significant additional irradiation, previous lung diseases, or metastatic disease were excluded from the analysis. The threshold for clinical pneumonitis was 7.5 Gy (actuarial onset ~ 8 Gy) after which the incidence increased rapidly. Probit analysis of data provided in Van Dyk et al.\(^3\) by our group identified the lethal dose for 50% of individuals to be 10.60 Gy (95% confidence interval: 9.97-12.05 Gy). Reprinted with permission from Van Dyk et al.\(^3\)

\(^{1}\text{Image is crooked in original manuscript.}\)
Approximately 8-12 weeks following radiation exposure, changes in pulmonary function can be seen. These include a decrease in lung volume and pulmonary compliance, impaired gas exchange, decreased tidal volume, and increased respiratory frequency. At that time, mild symptoms may begin to appear such as dyspnea, non-productive cough, and mild fever. If unresolved, the initial symptoms may rapidly worsen. Within two weeks, the individuals will present with tachypnea, cyanosis, and fulminant organ failure leading to death due to respiratory insufficiency. Although clinically symptomatic injury has a delayed onset, radiographic changes may be observed earlier. These changes are characterized by a diffuse haze that will progress to focal opacity within and outside the irradiated field \(^{39}\). Within a few hours following the initial exposure, a transient drop in tissue perfusion may be observed. There is a brief renormalization period prior to a second, progressive decrease in perfusion beginning 1-2 months after exposure\(^{40}\).

Upon gross morphologic exam, lungs from individuals succumbing to radiation-induced lung injury are described as “heavy, rubbery, dark red and poorly aerated”\(^{36}\). Histologically, the pneumonitis reaction is characterized by edema, atypia and desquamation of alveolar epithelial cells, intimal proliferation and medial thickening, mononuclear cell infiltrates, giant cell formation, and inflammatory exudates with an absence of polymorphonuclear leukocytes\(^{36, 39}\). Hyaline membranes are also seen\(^{36, 39}\). Progressive interstitial fibrosis with fibroobliteration of capillaries, thickened alveolar
walls, and lipid-laden foamy macrophages is seen during the late phase. For a detailed review of radiation pathology in the lung with excellent illustrative images from the now-defunct Armed Forces Institute of Pathology, please refer to Roswit and White.

Pleural effusions are observed in approximately 5% of individuals with radiation pneumonitis/fibrosis. However, these rarely impede lung function and are generally asymptomatic. In non-human primates, pleural effusions are present in at least 30% of subjects experiencing acute radiation pneumonitis/fibrosis; however, the effusions resolve upon treatment with dexamethasone (M. Garofalo, personal communication).

Pneumothorax has been observed in a small number of radiotherapy patients with Hodgkins Disease or breast cancer and in radiation accident victims receiving greater than 100 Gy. As a result, pneumothorax is considered to be an exceedingly rare condition and is unlikely to be directly related to radiation injury to the lung.

The current standard of care for pneumonitis is corticosteroid treatment. Prednisone (60-100 mg/day) may be given at the onset of symptomatic injury and gradually tapered as symptoms resolve. However, there is a narrow window for which corticosteroid treatment is useful. Prophylactic use of steroids has shown no benefit in preventing radiation pneumonitis, nor do they reduce symptomatic injury and improve survival if there is a delay in treatment after the initial onset of symptoms. Ventilation may also be required. In many patients, corticosteroid treatment has no effect on the pneumonitis phase and in those where it is effective at reducing pneumonitis, once it is
removed, a more severe pneumonitis often returns leading to respiratory distress and eventual death. While corticosteroids may significantly reduce the pneumonitis reaction, they typically have no effect on development or treatment of fibrosis. There are a number of explanations for this disparity, including the hypothesis that radiation pneumonitis is a delayed hypersensitivity reaction. This theory is supported by the long latency period and the ability of anti-inflammatories to reduce the severity of the reaction as long as they are being administered.

At this point, it is unclear whether pneumonitis gradually progresses to fibrosis or whether these are unconnected pathologies resulting from injury to different target cell populations. Evidence for two separate pathologies comes from the ability of therapeutic agents to pry apart the pneumonitis reaction from fibrosis (or vice versa) as well as the development of fibrosis in patients without prior evidence of pneumonitis.

For more detailed information, there are two excellent reviews one by Nicholas Gross and the other by John Coggle and colleagues, concerning the clinical symptoms and physiologic changes associated with radiation lung injury in individuals exposed to high dose radiation.

### 1.3.2 Physiologic effects and molecular mechanisms of radiation-induced lung injury

As ionizing radiation transverses the cell, the deposition of energy in tissue results in the production of free radicals and breakage of chemical bonds. This leads to locally multiply damaged sites in DNA including base damage, single strand and double
stranded breaks, and crosslinking\textsuperscript{45}. Single stranded DNA breaks are rapidly repaired with a half-life of less than 4 minutes\textsuperscript{45}. DNA double stranded breaks, on the other hand, have a much longer half-life and therefore the initial DNA damage may be amplified by subsequent production of hydroxyl radical after irradiation. In the presence of oxygen, DNA damage may be “fixed” in an irreversible form resulting in anaphase death or loss of reproductive capacity\textsuperscript{42}.

While the initial chemical reaction lasts for only a few milliseconds, the ensuing tissue response to cell injury sets off a chain of events leading to chronic oxidative stress, vascular dysfunction, tissue hypoxia, and inflammation and fibroproliferation\textsuperscript{46}. The clinical manifestation of tissue injury may take hours, days, weeks or even months to appear after the initial exposure. Elucidating the physical, chemical, and biologic basis for tissue injury that culminates in debilitating disease or organ failure has formed the basis of decades of research since the declassification of the Manhattan Project.

It is well established that one of the primary mechanisms by which radiation kills cells is through DNA damage. Depending on the extent of DNA and macromolecular damage, cells may undergo several cell divisions before entering the pathway that leads them towards cell death. This observation formed the basis of the classical “target cell theory” that suggested the timing of symptomatic normal tissue injury was directly related to the proliferation kinetics of the tissue\textsuperscript{47}. Thus, tissues with large stem cell populations, i.e. the gastrointestinal tract or hematopoietic system, would exhibit earlier
overt tissue damage than tissues with more slowly proliferating cell populations (i.e. lung).

However, the target-cell hypothesis has come under increasing scrutiny due to the observation of bystander effects up to 1 mm away and the absence of a clear link between in vitro cellular radiosensitivity and clinical outcome. Therefore, while loss of reproductive capacity among the functional cell population may serve as a trigger for the molecular and physiological events that drive radiation-damage, it is the coalescing of microvascular dysfunction, dynamic cytokine surges, chronic oxidative stress, and tissue hypoxia that ultimately facilitate the pathogenic outcome.

One of the primary targets of radiation is the microvascular endothelium. Within hours to days, endothelial cell swelling and detachment from the basement membrane is observed. Radiation damage to the endothelial membrane activates acid sphingomyelinase leading to high intracellular levels of ceramide. Ceramide, a sphingolipid, acts as a second messenger to mediate radiation- and oxidative stress-induced apoptosis of endothelial cells. Activation of the sphingomyelin pathway has been implicated in acute respiratory distress syndrome. Ceramide-mediated apoptosis of endothelial and Type I epithelial cells in the alveolar septae leads to vascular leak and pulmonary edema.

Radiation damage to the endothelium leads to vascular dysfunction and upregulation of vascular adhesion molecules important for leukocyte trafficking into the
damaged tissue\textsuperscript{54}. Within the first week following radiation, there is an initial, but transient, decrease in macrophages, most likely due to mitotic cell death\textsuperscript{55}. Inflammatory cells are recruited to the damaged tissue in waves during the course of disease progression. The recurrent patterns are likely associated with tissue remodeling processes and reproductive death of cell populations in the lung with variable mitotic rates\textsuperscript{55}. Inflammatory cells found in lung tissue after radiation includes monocytes, macrophages, neutrophils, and lymphocytes. McBride et al. showed the importance of lymphocytes in facilitating the pneumonitis reaction in a murine model of total body irradiation with bone marrow transplant\textsuperscript{56}. When a thymectomy was performed prior to irradiation, C3H mice displayed a significant reduction in pneumonitis compared to mice with intact thymus\textsuperscript{56}.

A perpetual cascade of cytokines occurs during the so-called clinically latent period\textsuperscript{57}. The temporal expression of cytokines during the progression of radiation-injury in pre-clinical rodent models has been characterized both in bronchoalveolar lavage fluid as well as in whole tissue\textsuperscript{57}. Chiang et al.\textsuperscript{58} found BAL did not correlate to the inflammatory cell types in whole tissue as these reflect inflammatory cell infiltrates in different compartments of lung tissue.

After radiation, there is an early increase in IL-1\(\beta\), IL-6, keratinocyte chemoattractant (IL-8 in humans), TNF-\(\alpha\), IFN-\(\gamma\), and TGF-\(\beta\)1. However, this appears to subside until 6-8 weeks after radiation, where again they appear to be elevated. In
Rubin’s study, IL-1α mRNA was elevated at 2 weeks post-radiation and briefly renormalized before increasing approximately 8 weeks after radiation and remaining elevated through 26 weeks⁵⁹. Chiang et al. also found a bi-phasic response in inflammation in C57BL/6 mice after 12 Gy⁵⁸. In that study, TNF-α, IL-1α/β, and IFN-γ were expressed during the clinically latent period⁵⁸. However, during the fibrotic phase, TNF-α was absent, whereas IL-1α/β and IFN-γ remained elevated⁵⁸.

Johnston et al. found in the late, fibrosis phase of C57BL/6J mice, chemokines and chemokine receptors families predominate⁶⁰. In those studies, the authors found an increase in mRNA encoding for the chemokines MCP-1, MCP-3, RANTES and the chemokine receptors, Ccr1, Ccr2, Ccr5, and Ccr 6 in lung tissue during the late phase of injury, which was not observed in fibrosis-resistant C3H/HeJ mice following 12.5 Gy whole thorax irradiation. The authors concluded the differential expression of chemokines and their receptors during the late phase of injury between the two strains suggests macrophages and lymphocytes are significant contributors to radiation-induced fibrosis.

Transforming growth factor-beta 1 (TGF-β1), a pro-inflammatory and pro-fibrogenic cytokine, plays an important role in the development of fibrosis after radiation. TGF-β1 is sequestered in its inactive form in the extracellular matrix. Upon exposure to ionizing radiation, TGF-β1 is activated by hydroxyl-mediated cleavage of its latency-associated peptide (LAP) where it then can participate in autocrine and
paracrine signaling. Binding of TGF-β1 to its cell-surface receptor activates a Smad-mediated second messenger signaling pathway that transduces the signal from the cell surface to the nucleus. This, in turn, leads to activation of genes involved in number of host responses including apoptosis, cellular proliferation, extracellular matrix remodeling, and chronic inflammation.

The pathogenic process of radiation damage has been described as a “wound that does not heal” (Figure 3). The histopathologic features of radiation fibrosis are characterized by a replacement of mesenchymal cells and excessive collagen deposition.
Figure 3: Phases of normal wound healing and radiation-induced fibrosis over time

The normal wound healing process pictured above the timeline is a precisely orchestrated response to tissue injury. It begins with the initial platelet response immediately after the trauma and continues to the final tissue remodeling and scar tissue formation more than a year later. Radiation activates the wound healing machinery, but in addition to these processes the unique nature of radiation damage initiates a series of processes (below the timeline) that are distinct from those involved in normal wound healing. These processes span the whole timescale of normal wound healing and it is this continued interference with the normal control of wound healing that leads to the excessive deposition of extracellular matrix and collagen that are the hallmarks of radiation fibrosis. ROS, reactive oxygen species; RNS, reactive nitrogen species, TGF-beta, transforming growth factor-beta. Figure republished with permission from Bentzen Nature Reviews 2006 46.
TGF-β1 latency-associated peptide (LAP) is differentially expressed between the lungs of pneumonitis-prone and fibrosis-prone mice after irradiation\textsuperscript{64}. In studies by Franko et al., focal areas of acute inflammation and fibrotic lesions in C57L/J mice expressed disproportionately lower levels of TGF-β LAP than uninvolved healthy tissue suggesting higher levels of active TGF-β1 within damaged areas\textsuperscript{64}. In comparison, the non-fibrosis prone C3HeB/FeJ strain had significantly higher levels of latent TGF-β\textsuperscript{64}. Based on these results, Franko and colleagues identified Type II pneumocytes and macrophages as the predominant producers of TGF-β1 following radiation in fibrosis-prone mice.

In addition to its role in fibrosis, TGF-β1 is also known to participate in pro-inflammatory tissue responses. Intratracheal administration of manganese-superoxide dismutase (SOD) plasmid/liposome complex has been shown to lower levels of TGF-β1, IL-1, and TNF-α mRNA levels and improve median survival time after acute radiation exposure in mice\textsuperscript{65}. However, at the time of death, TGF-β1 levels were not significantly different among irradiated control and treatment groups\textsuperscript{65}. Targeting TGF-β1 or components of its signaling pathway has been shown to significantly reduce collagen deposition and improve lung function\textsuperscript{62, 66-69}.

The decline in pulmonary perfusion during the weeks following radiation leads to the development of tissue hypoxia\textsuperscript{70}. Ward and colleagues evaluated pulmonary perfusion and ultrastructural damage in rat lungs exposed to 25 Gy hemithoracic
irradiation using a $^{60}$Co source$^{71}$. Endothelial damage was observed within 24 hours following irradiation and persisted for the first 30 days with evidence of endothelial blebbing and degeneration$^{71}$. Angiotensin converting enzyme, a molecule that modulates blood pressure by converting angiotensinogen to angiotensin II, decreased in the irradiated right lung with a concomitant reduction in pulmonary perfusion$^{71}$. Angiotensin II (Ang II) was not evaluated in Ward’s study, but it would be expected that a reduction in ACE activity would result in lower Ang II levels and vasodilation. This would be a classical tissue response to compensate for the decline in perfusion$^{71}$.

Vujaskovic et al. were the first to recognize the influence of tissue hypoxia in radiation pathogenesis$^{72}$. The development of hypoxia can induce profound changes in the tissue microenvironment$^{73}$. Hypoxia develops due to a number of factors including vascular dysfunction, increased metabolic demands of cells, atelectasis, and tissue scarring leading to obliteration of the alveoli$^{73}$. Tissue hypoxia stimulates production of pro-inflammatory and pro-fibrotic cytokines both in vitro and in vivo$^{74,76}$. The induction of NF-κB by tissue hypoxia enhances leukocyte recruitment to further amplify the inflammatory response$^{73}$. Neutrophils, macrophages, mast cells, and dendritic cells are recruited to the hypoxic areas where they are activated to undergo the respiratory burst and secrete pro-inflammatory and pro-fibrotic cytokines$^{73}$. Hypoxia-induced stabilization of the alpha subunit of the hypoxia-inducible factors facilitates myeloid cell adaption to environments of low oxygenation$^{73}$.  

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Hypoxia participates in tissue remodeling and wound healing; however, a number of hypoxia-associated genes are sensitive to the redox status of the cellular environment. Dewhirst and colleagues proposed that the cytokine surge during wound healing processes is initiated by mechanisms other than hypoxia\textsuperscript{77}. One potential mechanism underlying the initiation of the cytokine surge and its persistence after radiation is the development of chronic oxidative stress. Oxidative stress develops at the time of irradiation and is sustained throughout the course of disease development through induction of oxidant-generating enzymes, such as the families of NADPH oxidase and nitric oxide synthase, and mitochondrial leakage. A number of transcription factors (HIF-1α, NF-κB) and pro-inflammatory/pro-fibrogenic cytokines (TGF-β1, TNF-α) are stabilized by free radicals. It has been proposed that reactive oxygen/nitrogen species are stronger stabilizers of the alpha subunit of hypoxia-inducible factors than hypoxia\textsuperscript{78}.

Oxidative damage to DNA has been observed to increase continuously in the irradiated lungs of mice\textsuperscript{29} and in rats\textsuperscript{70} throughout the in-life follow-up period. Furthermore, mice over expressing extracellular superoxide dismutase are less prone to developing radiation fibrosis than their wild-type counterparts\textsuperscript{80,81}. As a result, a number of potent antioxidants have been evaluated for their ability to prevent, mitigate, and/or treat radiation-induced lung injury in rodent\textsuperscript{82-85} and non-human primate models (personal communication with Dr. MacVittie, UMB and Dr. M. Cline, WFU).
In summary, after pulmonary irradiation, a series of events are initiated that culminate in promoting tissue regeneration and healing\textsuperscript{46}. When the process is dysfunctional, acute pneumonitis and/or fibrosis may develop. The severity of these reactions is dependent on the total volume of lung irradiated, the total accumulated dose, and the dose rate. The initial ionizing event causes damage to genetic and non-genetic cellular macromolecules\textsuperscript{45}. DNA damage in the form of crosslinks, single and double-stranded breaks, and base damage can be lethal\textsuperscript{45}. Damage to genetic and non-genetic macromolecules initiates cellular repair processes that participate in tissue healing and remodeling\textsuperscript{55}. How the “danger” signal is relayed to neighboring cells influences the overall orchestration of the tissue response\textsuperscript{86}. Although the period between the time of irradiation and manifestation of pneumonitis/fibrosis may be clinically latent, it is by no means molecularly silent. Waves of cell death occur due to the differences in mitotic rates of target cells. This stimulates recurrent inflammation and cytokine storms that further exacerbate the original damage and impair the ability of tissue to properly regenerate. The underlying environment of oxidative stress further leads to DNA and macromolecular damage and chronic activation/inactivation of redox sensitive signaling pathways involved in modulation of endothelial function, inflammation, and fibroproliferation. Endothelial damage and oxidative stress lead to early changes in tissue perfusion that declines over time due to microvascular loss and vascular dysfunction. As a result, the tissue becomes increasingly hypoxic. This further
stimulates leukocyte recruitment/activation and oxidative damage. The injurious process becomes a self-sustaining event that negatively impacts the healing process. Finally, lung function is overcome through massive inflammation, scarring, and atrophy for which it can no longer compensate. If the process is not stopped either on its own or through pharmacologic intervention, then the result will be severe respiratory insufficiency leading to debilitating disease or death.

1.3.3 Pre-clinical murine models of radiation-induced lung injury

A number of species have been used to model clinical radiation pathogenesis in the laboratory. These include rodents, mini-pigs, dog, hamsters, rabbits and others. The genetic and physiologic differences among species and strains used as pre-clinical models impede the ability to appropriately compare and interpret the outcomes of those studies (Figure 4). There is currently no consensus among the radiobiology community as to which species and strain is most appropriate for studying the radiation effects on healthy lung tissue.87, 88

The most common experimental model used in radiation research is the mouse. As such, it one of the most well characterized species in respect to the pathogenesis of pulmonary injury following thoracic or total body irradiation. There is considerable variability in histopathologic sequelae and severity of lung injury among mouse strains exposed to wide field, thoracic irradiation.89, 90 These differences were first recognized in 1983 by Julian Down and Gordon Steel in a comparison between CBA and C57BL
strains\textsuperscript{91}. Franke et al. extended this to evaluate nine murine strains and characterized them as being either “fibrosis” or “pneumonitis” prone\textsuperscript{92, 93}. It was these elegant studies that led to the subsequent adoption of the C57BL/6 strain as the standard model for evaluating the molecular mechanisms associated with radiation-induced lung injury and efficacy screening of candidate MCMs over the past two decades.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{pneumonitis.png}
\caption{Incidence of lethal pneumonitis among different species at 2-6 months post-radiation.}
\end{figure}

Shown are species-dependent differences in pulmonary radiotolerance. The variations in slopes illustrate the importance of species and strain choice in designing pre-clinical research models of radiation-induced lung injury. Dose response curves for different species are based on data from Van Dyk et al., 1981 (Human, UHBl)\textsuperscript{37}; Hopewell et al., 2000 (Pig)\textsuperscript{94}; Poulson et al., 2000 (Dog, fractionated IR)\textsuperscript{95}; Rubin et al., 1986 (NZ Rabbit); Jackson et al, 2010 (C57L & CBA/J mice)\textsuperscript{99}. 

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It is understandable that the C57BL/6J strain has become the “gold-standard” in radiation biology research. In large part this was due not only to the studies performed by Franko and Sharplin, but also the broader use of the C57BL/6J strain in biomedical research. As such, it has become one of the best-characterized models of radiation-induced lung injury and is used ubiquitously across laboratories today.

A number of studies have drawn into question the use of the C57BL/6J strain as the gold standard for pre-clinical research due to the long latency period prior to the development of focal fibrosis (6-9 months), mild pneumonitis, and, more importantly, the confounding effects of pleural effusions on survival (Figure 5)\textsuperscript{96-99}. Notably, pleural effusions are rarely seen in radiation accident victims, and when they are, their contribution to morbidity/mortality appears to be minimal. Travis and Tucker highlighted the problem of pleural effusions in their excellent synopsis of radiation lung research at the Twelfth L.H. Gray Conference on “Assays of Normal-Tissue Injury, and Their Cellular Interpretation”\textsuperscript{96}.

During the past few years, there has been a growing interest in re-evaluating many of the common research models used in biomedical research, particularly in radiation-induced tissue injury. The FDA requires efficacy screening of MCMs in a well-characterized animal model that reflects the pathogenesis of radiation injury in human lung to optimize the predictive validity of the model.
Figure 5: Radiographic changes illustrate the complication of pleural fluid accumulation on lung expansion in C57BL/6J mice following 15 Gy WTLI

Shown are micro-CT images with three-dimensional reconstructions together with coronal and axial T2-weighted MRI of moribund C57BL/6J mice at 28–30 weeks after whole-thorax irradiation (WTLI) with a single dose of 15 Gy. Images show increased lung density and decreased lung volume associated with the presence of pleural effusions. The circle (*) shows a polypropylene tube containing pleural fluid isolated from another irradiated C57BL/6 mouse. Survival studies utilizing the C57BL/6J strain are complicated by the presence of pleural effusions that contribute to respiratory insufficiency and mortality in this strain. Pleural effusions are not a pathology frequently observed in humans following WTLI and present a confounding factor when screening radiation countermeasures using the C57BL/6J strain. Reprinted from Jackson et al. 89 with permission.
The mouse is the best species for modeling radiation-induced lung injury due to its close DNA sequence similarity to humans and the ability to utilize gene-targeted mutagenesis to study the influence of specific genes or pathways on radiation pneumonitis/fibrosis\textsuperscript{100}. However, there are a number of physiological and immunological dissimilarities between mice and humans. Mice have a shorter life span, are skewed towards a Th1 immunological response, and are less diverse due to singular use of inbred strains\textsuperscript{100}. The presence of bronchus-associated lymphoid tissue at sites of bronchial tree bifurcation in mice may also play a role in the immunological responses of lung tissue to pathogenic insults that may not adequately reflect the pathology in humans.

These often-overlooked differences are one of the primary reasons there is a widespread failure of therapeutic compounds to successfully achieve the desired benefit in humans. The broad availability of genetically modified models on C57BL/6J background often makes this strain appealing due to the elegance of experimental design. However, in a number of disease-types, the C57BL/6J strain is an inferior model as these mice less adequately reflect the pathogenesis of the disease in humans when compared to other strains\textsuperscript{101}.

A broader approach should be taken for the study of human disease in preclinical models. To improve the extrapolation of results from bench to bedside, models should reflect the currently known pathogenesis of the disease in humans. This
may require backcrossing of strains to improve the “humanness” of the model or use of more than one strain in research experiments. While this is more costly, it will improve the predictive validity of research outcomes and be more cost-effective in the end in both money spent and in lives saved.

1.4 Drug Discovery, Development, and Acquisition for the Strategic National Stockpile: Meeting the FDA Animal Efficacy Rule Criteria

1.4.1 Drug licensure through the FDA “Animal Efficacy Rule”

To address the growing concern over the possibility for a terrorist attack on U.S. soil with a radiological or nuclear device, in 2003, the U.S. Government authorized more than six billion dollars towards the development of radiation countermeasures to treat acute radiation sickness (ARS) and the delayed-effects of acute radiation exposure (DEARE). MCMs approved by the FDA for treatment of ARS/DEARE will be acquired by the U.S. Government for the Strategic National Stockpile (SNS) for use in a radiation emergency. Up until now, this chapter has addressed the medical syndromes associated with ARS/DEARE and their medical management. However, this section will address the medical and logistical challenges associated with medical management in mass casualty scenarios and the work by the U.S. government to address the current gaps in our knowledge.

In the rare instances when it is neither ethical nor feasible to evaluate the therapeutic efficacy of drugs in humans, the FDA may approve MCMs through the
“Animal Efficacy Rule”. The FDA Animal Rule has four criteria. These are “1) a reasonably well-understood pathophysiological mechanism of radiation and its prevention by the MCM, 2) the effect must be demonstrated in more than one animal species expected to react with a response predictive for humans, 3) the animal study endpoint must be clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity, and 4) sufficient data on the pharmacokinetics and pharmacodynamics of the product in animals and humans to allow for the selection of an effective dose in humans”\textsuperscript{102}. Although efficacy testing is not required to be conducted in humans, safety trials in healthy, human volunteers are still required\textsuperscript{102}.

In 2004, the National Institutes of Health was authorized to support the basic research and drug discovery efforts for radiation countermeasures in three primary areas-gastrointestinal-ARS, hematopoietic-ARS, and DEARE-lung\textsuperscript{103}. To meet this objective, the Centers for Medical Countermeasures against Radiation (CMCR) were established and a contract was awarded to the University of Maryland School of Medicine to establish product support services (MCART program). The MCART program is designed to conduct pre-clinical studies under Good Laboratory Practice guidelines. Currently, drugs are being developed to treat injuries associated with external radiation exposure and internal radiation exposure, although pre-clinical models of the latter are more difficult to establish.
Due to the rarity of radiation accidents, the government learned that large pharmaceutical companies were not interested in pursuing developing of MCMs to treat radiation toxicity due to the lack of financial incentive. Primarily the companies who were interested were small, start-up companies that lacked the funding to proceed with drug development. Thus, in 2006 the Biomedical Advanced Research Development and Authority (BARDA) was created within the U.S. Department of Health and Human Services (U.S. HHS) Office of the Assistant Secretary for Preparedness and Response (ASPR) to guide drug development through FDA licensure.

1.4.2 Importance of logistical, medical, and scientific challenges to drug development for DEARE-lung

A number of logistical, medical, and scientific challenges must be addressed to develop therapeutic countermeasures that can be successfully used to improve survival and reduce morbidity in a radiation emergency. One of the challenges to MCM development for ARS-CNS and ARS-GI is the short window of opportunity for treatment. The timing for treatment initiation for ARS-heme and DEARE-lung are less critical, but still important and little is known regarding the optimal window for treatment.

There is likely to be considerable delay between the time of exposure and arrival of emergency personnel in a radiation event. Prior to triage, the first step will be to determine the type of radiation incident that occurred (radiological dispersal device, weapon of mass destruction, “dirty bomb”; neutrons vs. γ-rays). The next priority will be
to treat life-threatening injuries such as blast wounds and limb amputations. Individuals closest to the hypocenter will receive the most dire physical injuries (blast wounds, crush injuries, thermal burns) and highest radiation doses. As combined injury is considered fatal, these individuals are likely to receive only palliative care.

The U.S. government has estimated a minimum of twenty-four hours before treatment of ARS/DEARE will be initiated. Therefore, at this time, the FDA is prioritizing the development of MCM effective when started twenty-four hours or later after exposure.

The U.S. government estimates up to 1 million persons may be exposed to radiation if a WMD or “dirty bomb” were detonated in a large U.S. city, such as New York City or Washington, DC. Conditions are also likely to rapidly deteriorate. Therefore, orally available MCMs are considered to be superior to therapeutics that must be administered by intravenous or subcutaneous injection.

At this time, the radiation dose estimate is based on the temporal onset and duration of the prodromal period, lymphocyte depletion kinetics, and the dicentric chromosome assay. Each of these has limitations for use in a mass casualty scenario.

First, there will be significant difficulty in discerning between the prodromal and psychosocial symptoms that manifest themselves in similar ways (nausea, vomiting diarrhea). Second, lymphocyte depletion kinetics requires absolute lymphocyte counts (ALC) to be performed two to three times per day during the first week. Lack of
resources and personnel in a mass casualty scenario diminishes the feasibility of performing multiple blood draws and laboratory measurements of ALC. Third, the dicentric chromosome test requires specialized laboratories and a minimum of 48-72 hours for results. Again, with limited resources and high patient volume, there is likely to be a delay in test results diminishing the window of time for treatment intervention.

Clearly, there is a need for improved biodosimetry that can be assessed at the point-of-care. During the past decade the majority of radiation biodosimetry studies have focused on identifying a method to determine the physical radiation exposure dose. Knowledge of the total body dose can facilitate rapid assessment and triage of individuals acutely exposed to radiation. However, knowledge of the total body dose has limited predictive value regarding organ-specific damage or individual risk for development of severe organ injury. This is because the total body dose does not take into account intrinsic or environmental variability in radiation response among a large population as well as the likelihood for heterogeneous radiation exposure.

Radiation damage to the lung is the leading cause of death after accidental total body exposure when GI and hematopoietic syndromes are successfully treated\(^\text{27}\). The threshold dose for lethal lung injury after upper half body irradiation (UHBI) is estimated to be around 7.5 Gy\(^\text{104}\). However, the threshold dose for lung injury is likely to be much lower in the event of a radiation accident or attack, as a number of confounding factors will influence pulmonary sensitivity. These include radiation damage to other
organs, opportunistic infections, and combined injuries such as thermal burns and crush injuries. Therefore, knowledge of organ-specific biomarkers of radiation toxicity and probability for delayed lung injury is a critical component of risk assessment and medical decision-making in a mass casualty radiation event.

At this time, our ability to assess risk for lung injury is impeded by the lack of a known robust and sensitive biomarker(s) detectable prior to the onset of symptoms. Identification of a biomarker or biomarker panel would improve point-of-care (POC) diagnostics to facilitate medical decision-making and increase overall survival by allowing early therapeutic intervention.

Ideally, biomarkers will be assayable using minimally invasive or non-invasive diagnostic tools such as plasma or urine analysis. A biomarker-based diagnostic test will need to be sensitive to early radiation damage and relatively specific to the lung rather than total body dosimetry to minimize individual variability, heterogeneity of dose distribution, and confounding factors such as influence of other syndromes and co-morbidities.

Additionally, development of a robust and reliable biomarker for organ-specific injury will assist in defining the “trigger to treat”. Due to the current lack of a point-of-care diagnostic dosimeter, it is at the physician’s discretion to treat the clinical signs and symptoms with currently available therapeutics. For example, steroids are given at the
onset of dyspnea to treat radiation pneumonitis. However, starting treatment prior to the onset of symptoms is likely to provide a survival advantage.

In a mass casualty setting, it is unknown whether individuals will receive supportive care. Therefore, it is unclear whether pre-clinical research programs should or should not use the clinical standard of care in their models. The majority of pre-clinical radiation research models do not employ supportive care in the form of antibiotics, IV fluids, or BMT. The use of supportive care in the laboratory is often dictated by Institutional Animal Care and Use Committee (IACUC) requirements. For example, to comply with IACUC standards of care, NHPs are treated with IV fluids, antibiotics, and steroid treatment, as symptoms require. Since supportive care may be used clinically it will be important to determine any potential interaction of MCMs with the current standards of care (Filgrastim, etc).

In the current project, supportive care (i.e. steroids) was not required by Duke University’s IACUC and therefore was not given. This allowed us to evaluate the natural progression of organ-specific injury and identify molecular mechanisms associated with radiation pathogenesis without confounding factors. For our pre-clinical model, supportive care will be reserved for the advanced stages of drug development to determine their influence on tissue injury and survival in combination with candidate MCMs. Candidate MCMs will be defined by their ability to improve survival (primary parameter) and/or significantly reduce morbidity (secondary parameter). Drugs with a
dose modification factor greater than 1.2 are considered potential candidates for further development.

1.5 Research Objectives

The overall goal of this project is to address many of the critical knowledge gaps associated with successful adherence to the FDA Animal Efficacy Rule criteria required for licensure of radiation countermeasures. To meet this goal, studies were performed to first establish a well-characterized animal model that sufficiently represents the pathophysiology associated with radiation-induced lung injury in humans. Once established, the models were then utilized to improve current knowledge of the molecular mechanisms of lung toxicity as related to human radiation-induced pulmonary pathogenesis.

The results obtained herein will provide a rigorous scientific platform for MCM development under the FDA Animal Efficacy Rule with reasonable expectation that MCMs acquired for the strategic national stockpile and deployed during a radiation emergency will effectively prevent, treat, or mitigate radiation-induced lung injury and improve survival among the exposed population.

In order to meet these goals, the research objectives were as follows:

Objective 1. Establish pathophysiological outcome (survival, functional injury, histopathology, and natural history of disease) of radiation-induced lung injury in three murine strains, C57BL/6J, CBA/J, and C57L/J. Establish dose response curves for
development of lethal pneumonitis. Determine the lethal dose for 30-70 percent of subjects 180 days after thoracic irradiation. Determine the temporal onset of functional injury and assess the type and severity of histopathologic damage.

**Objective 2:** Identify patterns of differential gene expression in lung tissue twenty-four hours after thoracic irradiation among three genetically different but related murine strains with variable phenotypic expression of lung injury. Compare gene expression changes at twenty-four hours with expected phenotypic expression of injury among each individual strain to determine those genes and/or pathways likely to be associated with the initiation of radiation pneumonitis and/or fibrosis.

**Objective 3.** Validate changes in gene expression and their protein products in non-irradiated and irradiated lung tissue from three murine strains. Determine ultrastructural changes in lung tissue twenty-four hours after thoracic irradiation.

**Objective 4.** Establish the natural history of lung injury progression. Determine temporal changes in hypoxia-associated gene expression in the pathogenesis of radiation-induced lung damage.

### 1.6 Summary

Radiation-induced lung injury is the leading cause of death following TBI/PBI when gastrointestinal and hematopoietic syndromes are successfully treated. However, there is a concern over the lack of available radiation countermeasures (MCM) to treat radiation pneumonitis/fibrosis following acute radiation exposure.
There are two primary limitations to the successful identification, development, and approval of MCMs for radiation pneumonitis and/or fibrosis. The first is an incomplete understanding of the mechanisms involved in the development of radiation-induced lung injury. The second is the lack of a well-characterized animal model that is consistently used across research groups and institutions for pre-clinical radiobiological research.

It is neither ethical nor feasible to evaluate potential MCMs against lethal normal tissue toxicity in a clinical setting. Therefore, the potential efficacy of MCMs in humans is likely to be inferred from animal studies. As a result, choosing the appropriate model for MCM screening is a critical step towards achieving successful translation from pre-clinical research models to human patients.

The studies in this project were designed to establish a well-characterized animal model of lung injury that can then be utilized to elucidate the mechanisms underlying radiation-effects on healthy lung tissue. In order to optimize the predictive validity of the data, we utilized three murine strains that represent the full spectrum of phenotypic expression of lung injury observed in humans following pulmonary irradiation.

More broadly, the development of a validated model of radiation-induced lung injury using consistent and proper dosimetric parameters, as conducted in this study, will improve data interpretation and understanding of the complex mechanisms involved in lung injury. The overall goal is to establish a research platform for screening
new therapeutic countermeasures to prevent, mitigate, and/or treat lung injury following acute radiation exposure within the framework of the FDA Animal Efficacy Rule.
2. Experimental Procedures

2.1 Methods for development of a pre-clinical animal research model for radiation-induced lung injury

2.1.1 Animals

Female C57L/J, CBA/J, and C57BL/6J mice were purchased 8-10 weeks of age (~20g) from Jackson Labs. Animals were housed 5 per cage at the Duke Genome Science Research Building (GSRBII), a barrier facility, and provided food and water ad libitum. All experiments were performed with prior approval from the Duke University Institutional Animal Care and Use Committee (IACUC).

2.1.2 Radiation

Radiation dosimetry and quality assurance were performed prior to initiation of the study as previously described. Animals were allowed to acclimate for one to two weeks prior to radiation exposure. For irradiation, mice were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg) and simultaneously irradiated in the prone position with 7.5-17.5 Gy of 320-kVp X rays (Precision X-ray Inc., North Branford, CT, HVL = 2.00 mm Al, dose rate = 67 cGy min⁻¹) in 2.5 Gy increments. Radiation was delivered to the thorax through adjustable apertures with 8 mm lead shielding of the head and abdomen. Sham-irradiated animals were treated in the same way, except that the radiation source was not turned on. To validate experimental set-up, port films are taken for each radiation procedure. After radiation, animals are kept on heating blankets.
to help maintain normal body temperature until fully awake and mobile, at which time they are placed back in the housing facility.

### 2.1.3 Survival Analysis

The primary parameter for studying radiation-induced lung injury is radiation pneumonitis, which is expected to be the predominant and lethal response between 3 and 6 months after whole thorax irradiation. Therefore, the primary clinically relevant measurable parameter for radiation pneumonitis was mortality. All animals were closely monitored for respiratory distress over the first 6 months post-radiation exposure. Moribund mice were euthanized by sodium pentobarbital overdose (>250 mg/kg) per IACUC recommendations. Surviving mice were euthanized at a pre-determined final time point of 182 days. A cohort of mice in each group was euthanized at a pre-determined time point of 10, 14, or 26 weeks (n = 5/time point). Body weights were recorded every 2 weeks during the in-life phase until the first sign of weight loss, after which body weights are recorded daily and animals observed for behavioral changes twice per day. Criteria for euthanasia included a combination of animal observation (lethargy, hunchback, ruffled fur) and/or body weight loss >15%. At the time of euthanasia, a final body weight was recorded and final pulmonary function test (whole body plethysmography) was performed.
2.1.4 Analysis of Respiratory Function

Pulmonary function was assessed prior to radiation exposure and every two weeks thereafter during the in-life portion of the study using an unrestrained whole body plethysmograph (Buxco Electronics, Wilmington, NC).

2.1.5 Gross Morphology and Histopathology

At the time of euthanasia, a bilateral thoracotomy was performed. Pleural fluid was absorbed onto pre-weighed tissue paper and weighed again. The whole lung and heart were excised, rinsed in cold phosphate-buffered saline, and wet lung weights recorded. The left lobe was inflated through the bronchus with 10% neutral buffered formalin, paraffin embedded, and sectioned in 5-mm sections.

Hematoxylin and Eosin (H&E) and Masson’s trichrome staining was performed as previously described. H&E stained sections were evaluated for inflammatory cells, alveolar capillary distension or congestion, hyaline membranes, and alveolar wall thickness. Scoring of perivascular and alveolar inflammation was performed as previously described. Perivascular inflammation was scored on a scale of 0-4 where 0 was no cell layers and four was four or more layers of inflammatory cells around the vessel. Alveolar inflammation was scored on a scale of 0-5 with 0 being no alveolar inflammation and 5 being complete consolidation of the tissue. Semi-quantitative assessment of the degree of interstitial fibrosis was determined using a predetermined numerical scale of 0-8 based on the Ashcroft scoring method. Criteria for grading lung
fibrosis were based on histological features such as alveolar wall thickness, fibrotic damage to lung structures, and fibrous lesions.

2.1.6 Statistical Design and Analysis

Statistical analyses were performed and plots generated using SAS version 9.2. Animal identity was included in this analysis to account for inter-animal variations. The proportion of mice that survive for 182 days was computed for each strain and radiation dose. The mean survival times (MST) among decedents were presented by strain (C57BL/6J, C57L/J, CBA/J) and radiation dose (0, 7.5, 10, 12.5, 15, and 17.5 Gy). Kaplan-Meier Survival Curves were used to present survival data by strain and radiation dosage. A time-to-event analysis for overall survival was also performed on the survival data using a Cox proportional hazards regression model to compare the time to death of the strains with radiation dose as a covariate. Secondary parameters (lung weight, breathing rates, etc.) were examined descriptively using descriptive statistics (mean, standard deviation, median, maximum, and minimum). All statistical analyses were performed at alpha = 0.05 (significance) and 0.10 (marginal significance) levels. All tests were conducted as two-sided tests.

2.2 Methods for differential gene expression analysis and validation

2.2.1 Animals and Radiation Exposure

Female C57BL/6J, CBA/J, and C57L/J mice (Jackson Labs, Bar Harbor, ME) were irradiated at 10-12 weeks of age with 12.5 or 15 Gy of 320-kVp X rays (Precision X-ray
Inc., North Branford, CT, HVL = 2.00 mm Al, dose rate = 67 cGy min\(^{-1}\)). Age-matched sham-irradiated mice were included as controls. Unexposed regions were shielded using 8-mm lead shielding. Mice were euthanized twenty-four hours post-exposure by pentobarbital overdose (>250 mg/kg). Lung tissue was excised, embedded in OCT, and frozen over dry ice. Tissue was stored at -80°C until analysis.

### 2.2.2 RNA Isolation

RNA isolation was performed using the Qiagen RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol with slight modifications\(^{108}\). At the time of RNA extraction, the right upper lobe from three to four mice per group was excised from OCT, placed in RNALater for five minutes, and homogenized in 2 mL of lysis buffer (Qiagen, Valencia, CA) with zirconia-silica beads using a BeadBeater (BioSpec, Bartlesville, OK). The samples were not pooled. RNA quality was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) located at the Duke Microarray Facility. Only RNA with an RNA Integrity Number (RIN) above 9.0 was used.

### 2.2.3 Affymetrix mouse gene chip hybridization

Mouse oligonucleotide arrays (1 array per RNA sample for a total of 27 arrays) were printed at the Duke Microarray Core Facility using Affymetrix Mouse Genome 430 2.0 gene chips, which allows 39,000 transcripts to be analyzed in a single array. All GeneChip Mouse Genome Arrays from Affymetrix contain a set of mouse maintenance
genes to facilitate normalization of array experiments prior to performing data comparisons. Amplification, probe preparation and hybridization protocols were performed using the MessageAmp™ Premier RNA Amplification kit (Applied Biosystems, Foster City, CA). 

2.2.4 Data Normalization and Quality Control

To guard against batch effects or other technical factors impacting array data, we took the following procedures. Animals were maintained under identical housing conditions and euthanized on the same day. Mice were irradiated in groups of 10 and alternated by strain along the radiation platform to minimize effects due to non-uniform radiation distribution or internal errors in the radiation procedure. Previous radiation field uniformity tests indicate less than 6% difference across the field. Samples were processed and hybridized in a single batch to protect against batch effects. RNA extraction and preprocessing methods used in this study are well characterized. To ensure reproducibility and minimize error, samples were not pooled, rather run independently. Gene expression values were normalized using Robust Multichip Average (RMA). Unsupervised analysis including principal component analysis (PCA) and hierarchical clustering was performed to understand natural variations among the samples.
2.2.5 Statistics

Statistical analysis was performed at the Center for Computational Biology and Bioinformatics, University of Indiana School of Medicine (Indianapolis, IN). One-way ANOVA was utilized to compare differences in gene expression among strain, radiation, and response (acute pneumonitis vs. delayed injury) separately. Two-way ANOVA was used to compare the strain and radiation dose effect.

2.2.6 Ingenuity Pathway Analysis

Partek® Genomics Suite software (Partek Inc.; St. Louis, MO) was used to calculate the p-value and fold-change for each comparison. Strains were classified as “acute responders” if mortality occurred less than 140 days post-radiation due to acute pneumonitis (e.g. CBA/J, C57L/J). The C57BL/6J strain of mice was classified as “delayed responders” due to the long latency period that extended beyond 140 days. Genes differentially expressed between acute and delayed responders were considered to be genes of interest for functional, biological, and molecular network analysis.

To determine the relationships between the genes of interests, gene identifiers and their expression values were imported into a web-based pathway analysis application, Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com). Each identifier was mapped to its corresponding object in the Ingenuity Pathway Knowledge Base (IPKB). The IPKB is a continuously updated database of known gene functions and their interactions based on published literature. For network generation, a
p-value cutoff of <0.01 and 1.2 fold change in expression was set to identify molecules whose expression was significantly differentially regulated. Molecules were overlaid onto a global molecular network developed from information contained in IPKB. Networks of network eligible molecules were then algorithmically generated based on their connectivity.

Pathways that were most significant to the data set were identified using IPA canonical pathways analysis based on the IPA library of canonical pathways. The significance of the association between the data set and the canonical pathway was measured as described\textsuperscript{111}.

### 2.2.7 Quantitative Real-Time PCR

Gene expression was validated using quantitative real time Reverse Transcriptase PCR (ABI 7900HT, Applied Biosystems) as previously described\textsuperscript{112}. Briefly, the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) was used according to the manufacturer’s protocol to convert RNA to cDNA. Assays-on Demand\textsuperscript{TM} Gene Expression primer sets were purchased from Applied Biosystems. Real-time PCR was performed using \textit{TaqMan} Universal PCR Master Mix according to \textit{TaqMan} Gene Expression Assay protocol (Applied Biosystems). Data was analyzed using one-way analysis of variance (ANOVA) and Tukey’s Multiple Comparisons Test.
2.2.8 Western Blot

The snap frozen right lung lobe (n = 5/group) was placed in a 2mL tube filled with 1mL zirconia/silica beads (BioSpec Products, Bartlesville, OK) and 2mL ice-cold homogenization buffer (1% sodium deoxycholate, 5 mM Tris-HCL (pH 7.4), 2 mM EDTA, 10 mg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin A, 0.1 mg/ml benzamidin with or without phosphatase inhibitors). Tissue was then homogenized using the Mini-Beadbeater (BioSpec Products, Bartlesville, OK). Protein concentration was determined using the Nanodrop Spectrophotometer (ThermoScientific, Wilmington, DE). Western blot was performed as previously described79. The anti-alpha-1 antitrypsin antibody was purchased from Abcam (Cambridge, MA) and for alpha-1 acid glycoprotein was purchased from R&D (Biosystems, Minneapolis, MN). To control for loading efficiency, the blots were stripped and reprobed with GAPDH or α-tubulin antibody (Sigma-Aldrich). Differences between groups was analyzed by Student’s t-test.

2.2.9 Electron Microscopy

C57L/J, CBA/J, and C57BL/6J mice were irradiated to the whole thorax with a single dose of either 0 Gy or 15 Gy using the dosimetric parameters described above. Twenty-four hours later, animals were euthanized by sodium pentobarbital overdose (>250 mg/kg) and a bilateral thoracotomy performed. Lung tissue was extracted, the lobes separated and the right lobes were snap frozen in liquid nitrogen for western blot
analysis and the left lobe fixed with 10% neutral buffered formalin. The left lung was then cut into longitudinal sections for electron microscopy or paraffin embedding. Paraffin embedded tissue was cut into 5 micron thick sections and stained with H&E for evaluation of histopathologic damage as described above.

For transmission electron microscopy, the Duke Electron Microscopy facility, a shared resource, prepared the tissue as previously described\(^{113}\). Briefly, thick sections were cut (0.5 µm) and stained with toluidine blue. Sections were visualized under a light microscope to determine the location for ultrathin sections. Mild inflammation was seen in C57L/J and CBA/J thick sections but not in C57BL/6J sections. Ultrathin sections were stained with uranyl acetate and lead citrate and visualized using a Philips CM12 Transmission Electron Microscope.

### 2.2.10 Multiplex Cytokine Assay

C57L/J, CBA/J, and C57BL/6J (n = 3-4/group) received a single dose of 0 Gy, 12.5 Gy, or 15 Gy to the whole thorax as described previously\(^{114}\). Fourteen weeks after radiation, a bilateral thoracotomy was performed and lung tissue harvested. Whole fresh blood was collected at the same time via cardiac puncture. Blood was placed in BD Vacutainer collection tubes with EDTA (BD, Franklin Lakes NJ), centrifuged, and plasma stored at -80°C for further analysis. The left lung was inflated with 10% neutral buffered formalin, fixed overnight, and paraffin embedded. The right lung was
homogenized in TRIS-HCL buffer with protease and phosphatase inhibitors and stored at -80°C for cytokine analysis.

Hematoxylin and eosin and Masson’s Trichrome stains were performed on 5 micron thick tissue sections as previously described\textsuperscript{114} for evaluation of histopathologic damage and fibrosis.

Lung tissue and plasma samples were analyzed using the Bio-Plex Pro\textsuperscript{TM} Mouse Cytokine 23-plex Assay (cat# M60-009RDLP; Biorad, Hercules, CA). The assay detects the following cytokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, and IL-17A, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP1β, Rantes, and TNF-α. The assay was performed according to the manufacturer’s protocol as previously described\textsuperscript{115}. Statistically significant differences between groups were determined using the Student’s t-test.

2.3 Natural pathophysiology of radiation-induced lung injury in the C57BL/6J mouse

2.3.1 Animals and radiation

Female C57BL/6J mice (8-10 weeks) received a single 15 Gy dose of x-ray irradiation (Therapax 320, Pantak Inc., East Haven, CT) at a dose rate of 67cGy/min using beam energy of 320 kV (75 SSD, HVL= 2.00 mm Al). Radiation was delivered to the whole thorax through adjustable apertures (1.25-1.5 cm) with 8 mm lead shielding of the rest of the body. Sham-irradiated animals were subjected to the same scenario, but the radiation source was not turned on. All mice were anesthetized prior to irradiation.
with an intraperitoneal (i.p.) injection of a ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture. Animals were euthanized and tissue harvested at pre-determined time points of 1 day, 3 days, 1 week, 3 weeks, 6 weeks, and 6 months. At the time of euthanasia (>250mg/kg sodium pentobarbital, i.p.), lungs were snap frozen in liquid nitrogen and stored at -80°C (n = 7/group). All studies were performed and approved by the Institutional Animal Care and Use Committee at Duke University Medical Center.

2.3.2 Visualization of histopathologic damage and tissue hypoxia

To visualize histopathologic damage in sham-irradiated and irradiated tissue at each of the pre-determined time points, hematoxylin and eosin (H&E) staining was carried out as previously described. To assess tissue hypoxia, an endogenous marker, carbonic anhydrase (CAIX), and an exogenous marker (pimonidazole) were used.

Immunostaining for CAIX was performed as previously described by Gauter-Fleckenstein et al. Briefly, 5-micron thick paraffin embedded tissue sections were deparaffinized and rehydrated using xylene and graded alcohol (100-80%) concentrations. Hydrogen peroxide (10%) was used to block endogenous peroxidase activity and antigen retrieval performed with citrate buffer (Biogenex, San Ramon, CA). Slides were incubated at 4°C overnight with a rabbit polyclonal antibody to carbonic anhydrase IX (1:200; Abcam, Cambridge, MA) followed by washing in phosphate buffered saline and incubation for one hour at room temperature with a donkey anti-rabbit secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA). Slides
were counterstained with Harris hematoxylin and visualized under light microscopy using a 40x objective.

Pimonidazole hydrochloride (70 mg/kg i.p, Chemicon International Inc., Temecular, CA) was injected three hours prior to euthanasia and immunostaining performed as previously described\textsuperscript{118}.

\textbf{2.3.3 RT-PCR}

Total RNA isolation was performed as described above. Reverse transcription was performed by incubating 3 µg of pooled total RNA, 2.5 µM of random primer, and 200 units of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), for a total reaction volume of 20 µL, for 10 min at 25\textdegree{}C followed by 60 min at 50\textdegree{}C. PCR amplification was carried out using Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and two pairs of primers in one reaction, one amplifying the target gene fragment and another amplifying a 1 kb GAPDH fragment to serve as an internal control. PCR products were visualized on 1.5\% agarose gel containing 0.5 µg/ml ethidium bromide. The ratio of PCR product of target gene to GAPDH better reflects the RNA level in the samples. Primer sequences and the size of PCR products are listed in Appendix B.

\textbf{2.3.4 Western Blot}

Snap frozen left lungs were prepared for western blot analysis by immersing the lung in homogenization buffer containing protease and phosphatase inhibitors (Roche
Applied Sciences, Indianapolis, IN), and sonicating samples to disrupt cell membranes. Samples were centrifuged to remove undigested tissue. The supernatants were diluted 1:1 in Laemmli Sample Buffer (95% LSB, 5% β-Mercaptoethanol) (BioRad, Berkeley, CA). The prepared samples were then boiled and centrifuged prior to loading (10 µL per well) onto 10% SDS-PAGE gels. Following electrophoresis, protein was transferred onto polyvinylidene fluoride (PVDF) membranes and probed with a specific antibody to HIF-1α (Novus Biologicals, Littleton, CO), HIF-2α (Abcam, Cambridge, MA), or HIF-3α (Novus Biologicals, Littleton, CO) diluted in 5% non-fat milk in TBS+0.1% Tween20. Following secondary antibody incubation (Cell Signaling, Beverly, MA), signals were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Films were scanned using an HP ScanJet 3110. Band intensity was quantified using ImageJ Software (NIH, Bethesda, MD). Measured intensities were normalized using α-tubulin expression and then averaged within groups. Comparisons between irradiated and non-irradiated controls at each time point were made using a student’s t-test.

2.3.5 Oligo array-for hypoxia-associated genes

**RNA Extraction:** Snap frozen whole lung tissue was homogenized in 5 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with the PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA, USA). The total RNA was isolated using TRIzol reagent following the manufacturer’s instruction (Invitrogen, Carlsbad, CA, USA). The RNA
was repeatedly purified until the A260:A280 was greater than 2.0 and A260:A230 was greater than 1.7.

Probe Preparation: cDNA synthesis, cRNA synthesis, and cRNA labeling were performed using the TrueLabeling-AMP 2.0 kit (SA Bioscience, Frederick, MD, USA) according to the manufacturer’s manual. Briefly, 10 µg of pooled total RNA was used for the cDNA synthesis. The RNA pool was produced by mixing equal amount of total RNA from each sample in the same group. The cDNA was then subjected to cRNA synthesis and labeling with biotinylated-UTP overnight at 37°C. After purification with SA Biosciences ArrayGrade cRNA cleanup kit (SA Bioscience, Frederick, MD, USA), 15 µg of biotin-labeled cRNA was used for hybridization.

Hybridization: A mouse hypoxia signaling pathway-specific Oligo GEArray DNA microarray (SA Bioscience, Frederick, MD, USA, Cat#: OMM-032) was used to determine temporal changes in the expression of 113 genes following thoracic irradiation. Prior to hybridization, array membranes were pre-wetted with 5 ml of deionized water for 5 minutes and then pre-hybridized with 2 ml pre-warmed GEAhyb hybridization solution (SA Bioscience, Frederick, MD, USA, Cat#: H-01) for 4 hrs at 60°C. The array membrane was hybridized with 15 µg of purified biotin-labeled cRNA probes overnight at 60°C with continuous agitation. The membranes were then washed with Wash Solution 1 (2x SSC, 1% SDS) for 20 min at 60°C and followed by Wash Solution 2 (0.1x SSC, 0.5% SDS) for 15 min at 60°C with continuous agitation.
Chemiluminescent Detection of Gene Expression and Data Analysis: The signal was visualized using the Chemiluminescent Detection kit (SA Bioscience, Frederick, MD, USA, Cat#: D-01). Briefly, after blocking, the membranes were bound with alkaline phosphatase-conjugated streptavidin. After washing, the membranes were incubated with Chemiluminescent substrate for 5 min and exposed to x-ray film. Gene spots with signal intensity higher than the blank on the same membrane were considered detectable. The densitometry of GAPDH in the radiation group was normalized with that in control group. Densitometry for each gene spot was then normalized with the adjusted GAPDH. For the time point experiment, the average increased densitometry from two repeated experiments was graded in 5 expression degrees. One represents a weak increase and five represents the highest increase. If the gene spot was undetectable in both the control and irradiated mice in same time point, it was designated as 0.

3.1 Introduction

Deliberate or accidental radiation exposure can lead to life threatening or severely debilitating pulmonary disease when the thorax receives an acute dose in excess of 8 Gy\textsuperscript{119}. One of the limitations to extrapolating pre-clinical observations to improvement in clinical outcome is the lack of a well-defined animal model. Ideally, pre-clinical models will replicate the anticipated clinical course of disease in humans. This includes defining the similarity between murine and human lungs in dose-response, natural history, and histopathology of injury following acute thoracic radiation exposure. The purpose of this project was to thoroughly characterize murine strain differences in pulmonary radioresponse to identify the model that most closely reflects the pathogenesis of radiation toxicity in human lung\textsuperscript{89,90}.

One of the requirements of the FDA Animal Rule is for the pre-clinical study endpoints to reflect the desired benefit in humans, generally an improvement in survival or a significant reduction in major morbidity\textsuperscript{102}. Therefore, in this study, survival was chosen as the primary endpoint. Secondary endpoints included those with clinical correlates to maximize translation of animal efficacy data to clinical outcome. In this study, longitudinal measurements of respiratory function were chosen as the secondary endpoints. Retrospective analysis was performed to determine whether any of the
measured endpoints could be useful as early indicators of individual risk for developing lethal lung injury. Finally, lung tissue was harvested to assess histopathologic damage at the time of morbidity/mortality or at the final time point for the in-life portion of the study, depending on which came first.

3.2 Results

3.2.1 Kaplan-Meier survival curves for radiation dose-response among three inbred murine strains

In order to compare the radiation dose-range for temporal onset and survival between murine strains and non-human primates and humans, the first step was to determine the dose response for lethal lung injury among three murine inbred strains (C57L/J, CBA/J, and C57BL/6J). These strains were chosen based on preliminary data\textsuperscript{97,116} suggesting they reacted with a response comparable to humans. For these studies, ten female mice from each strain were irradiated in the prone position with a single dose of 7.5 to 17.5 Gy (320 kVp x-rays, 2.5 Gy increments) delivered to the whole thorax. Non-irradiated age-matched mice were included as controls. Figure 6 shows the resulting survival curves for each strain. In this study, a one Gray (Gy) increase in the radiation dose significantly increased the odds of death (p < 0.0001).

\textsuperscript{2} These results are accepted for publication in Jackson et al. Health Physics 2012.
Figure 6: Kaplan-Meier survival among three murine strains exposed to thoracic irradiation (WTLI)

C57BL/6J (A), CBA/J (B), and C57L/J (C) mice received WTLI with a single dose of 7.5-17.5 Gy X-ray irradiation (69cGy min⁻¹) using beam energy of 320 kV. Animals were followed for survival up to 26 weeks (182 days) after radiation. A significant difference in survival was seen among strains (p = 0.0038). C57L/J mice displayed a significantly higher odds of death compared to C57BL/6J mice (odds ratio = 15.24) or CBA/J mice (odds ratio = 10.26).
3.2.2 Comparison of radiation dose response relationships: From rodents to humans

The overall objective of the study was to assess the natural progression of radiation-induced pulmonary toxicity among three murine strains and identify those strains that tend to react with a response most comparable to humans. In this study, the radiation dose to cause lethality in 30-70% of mice within the first 180 days (LD30-70/180) was determined using probit analysis (Figure 7). The survival data was then compared to data derived from non-human primates at the University of Maryland-Baltimore (personal communication with Dr. Tom MacVittie) and published literature describing the human pulmonary dose-response for lethality.\textsuperscript{104,120} Table 4 describes the median survival times and lethal dose for 30-100% of mice within the first six months post-exposure in comparison to non-human primates and humans. Due to the steep dose response in the C57L/J strain, the LD30-70 could not be accurately assessed. The LD100 is based on the lowest dose in which 100% lethality was observed within the first 180 days post-radiation. Since the dose range was 2.5 Gy increments, the actual LD100 may be significantly lower. This is particularly important for the C57L/J strain in which the LD100 likely falls very close to that observed in the NHPs (11.5 Gy) and humans (11 Gy) (Table 4). Future studies will explore the dose-response relationships using tighter incremental windows to better estimate the LD30-70/100 and LD100/180. However, these results provide a good estimation of the dose-range expected to cause significant morbidity/mortality among the three strains for comparison with non-human primates.
and humans. Establishing a linkage in temporal onset and dose-response between rodent strains, non-human primates, and humans is one step towards designing future studies to screen medical countermeasures with a reasonable expectation that the results from those studies are likely to translate to human lung injury.

Table 4. Mean survival times and dose response relationships among murine strains irradiated to the whole thorax

<table>
<thead>
<tr>
<th>Murine strain</th>
<th>No. of subjects</th>
<th>Radiation dose</th>
<th>Percent lethality</th>
<th>Mean survival time</th>
<th>LD50/180</th>
<th>95% Confidence Interval (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>57</td>
<td>7.5-17.5 Gy</td>
<td>35.1</td>
<td>162 (±14) days</td>
<td>13.43 Gy</td>
<td>12.20-14.69 Gy</td>
</tr>
<tr>
<td>CBA/J</td>
<td>55</td>
<td>7.5-17.5 Gy</td>
<td>36.7</td>
<td>125.5 (±12) days</td>
<td>13.11 Gy</td>
<td>12.08-14.21 Gy</td>
</tr>
<tr>
<td>C57L/J</td>
<td>60</td>
<td>7.5-17.5 Gy</td>
<td>56.4</td>
<td>120 (±17) days</td>
<td>10.24 Gy</td>
<td>0.205-509 Gy</td>
</tr>
<tr>
<td>NHP</td>
<td>46</td>
<td>9-12 Gy</td>
<td>50</td>
<td>144.5 days</td>
<td>10.28 Gy</td>
<td>9.92-10.67 Gy</td>
</tr>
<tr>
<td>Humans</td>
<td>246</td>
<td>2-10 Gy</td>
<td>15</td>
<td>100 days</td>
<td>10.60 Gy</td>
<td>9.97-12.05 Gy</td>
</tr>
</tbody>
</table>

1. Personal communication with Dr. Mike Garofalo and Dr. Tom MacVittie, Univ. of Maryland-Baltimore.
2. Approximate values based on probit analysis of data from Van Dyk 1981104.
3. Lethality for C57L/J mice within the first 180 days post WTLI was 10% at 10 Gy and 100% at 12.5 Gy. The lack of data points between these two values prohibited the ability to establish an accurate LD50/180 value, as evidenced by the 95% CI.

3.2.3 Temporal onset of respiratory dysfunction coincides with morbidity and/or mortality among strains

To assess the dose-response incidence of functional injury, respiratory function was monitored every two weeks throughout the in-life portion of the study using an unrestrained whole body plethysmograph (WBP; Buxco Electronics, Wilmington, NC).

A baseline measurement was recorded for each subject prior to irradiation. The WBP
allowed assessment of respiratory rate, enhanced pause (a proposed marker of air flow), relaxation time, minute volume, peak inspiratory flow, and peak expiratory flow, among others. As a number of studies have shown genetic differences among murine strains in airway mechanics, we first evaluated baseline differences in ventilation and respiratory parameters among strains. Although statistically significant differences were observed prior to radiation, none appeared to be associated with radiation tolerance of the lung. Moreover, retrospective analysis of respiratory changes over time found no single respiratory parameter to be useful as an early predictor for individual sensitivity or likelihood for developing lethal lung injury. However, there was a clear difference between survivors and non-survivors in several respiratory parameters including minute volume (Figure 7), enhanced pause (Figure 8), and relaxation time (Figure 9).
Figure 7: Strain-dependent changes in gas exchange (minute volume) between survivors and decedents during the course of disease progression.

The decrease in MV suggests a progressive impairment in gas exchange among moribund mice in comparison to their surviving age-matched, irradiated (7.5-17.5 Gy WTLI) counterparts (A-C). This is consistent with airway obstruction at the time of morbidity. A Student’s t-test was performed to determine whether the differences between survivors and decedents were statistically significant. At baseline (pre-irradiation), the MV was significantly lower in the mice that eventually succumbed to lethal lung injury when compared to the MV among survivors in all strains. *p < 0.05 decedents vs. survivors. Error bars represent standard error mean. D) Comparison of histologic damage after 10-15 Gy WTLI among strains at high magnification (40x) is consistent with the observation of impaired gas exchange. Images illustrate fibrosis in C57BL/6J (1), mononuclear inflammation (2), edema and congestion (3), and alveolar obstruction with giant macrophages with cholesterol clefts within the alveoli (4), and foamy macrophages (5 and 6).
Figure 8: Temporal changes in airflow (Penh) after thoracic irradiation

Penh rises sharply during the peak phase of injury suggesting impairment in airflow. Temporal analysis of changes in Penh demonstrates an acute increase (~2.5 fold) in Penh approximately 9-14 days prior to mortality among individual mice. This data suggests Penh may be an excellent indicator of morbidity/mortality in mice. Linear regression of Penh vs. time post-radiation showed a positive correlation in C57BL/6J mice (p=0.0075 vs. pre-irradiation baseline), CBA/J mice (p=0.0076 vs. pre-irradiation baseline), and C57L/J mice (p<0.0059 vs. pre-irradiation baseline). A Students t-test showed a significant difference between survivors and dece...
Figure 9: Changes in relaxation time between the time of irradiation and onset of lung disease

The decrease in relaxation time among decedents is consistent with the histopathologic onset of lung damage and morbidity/mortality. Linear regression showed a strong correlation between relaxation time and time post-radiation across all groups within each strain (p<0.01; A-C). The difference in relaxation time before irradiation and at the time of death among individual mice was determined (D). There was a statistically significant difference in relaxation time between survivors vs. decedents at the time of death (** p< 0.01; **** p< 0.0001). Error bars represent standard error mean.
3.2.4 Gross morphology and histopathologic damage

At the time of necropsy, wet lung weights were recorded as an indicator of edema and congestion (Figure 10). Lung weights in C57L/J were approximately three to four times the lung weight of controls indicating significant edema and inflammation. A less significant change was observed in C57BL/6J and CBA/J mice reflecting the differences in severity of inflammation among strains. Histopathologic damage and cause of morbidity was dependent on both strain and radiation dose (Figure 11). As with humans, the lungs of C57L/J mice demonstrated a heavy mononuclear cell infiltrate particularly around the major vessels, congestion of the airways, increase in acidophilic alveolar macrophages and foamy lipid-laden macrophages, type II cell hyperplasia, and hyaline membranes. Inflammation appeared most severe near the airways and disseminated towards the subpleura. Severe injury was observed even at the lower doses of 7.5 and 10 Gy in this strain with a strong likelihood that many of the surviving mice receiving 10 Gy or even 7.5 Gy would have eventually succumbed to acute pneumonitis if follow-up time had been extended past 180 days (Figure 12). Collagen deposition was observed in Masson’s trichrome stained slides. There was an observed increase in alveolar wall thickness and fibrosis. Comparison of perivascular and alveolar inflammation, fibrosis, and lung weights demonstrate C57L/J mice exhibit a more severe inflammatory reaction and greater extent of fibrosis than either CBA/J or C57BL/6J strain.
Evaluation of histopathologic damage at the time of death suggested a reduction in air space required for sufficient pulmonary gas exchange. This was due to accumulation of giant, multinucleated cells and macrophages within the alveoli, gross distortion of the alveoli due to contracted fibrosis, and blockage of airways with an acidophilic crystalline material and mononuclear inflammatory cells. In moribund C57L/J mice, almost all airways appeared to be congested with significant interstitial edema. This was less severe in C57BL/6J mice. While a similar increase in alveolar macrophage accumulation was observed in CBA/J mice (C), a similar reduction in MV was not seen. This may be due in part to the difference in type of histopathologic damage. Whereas C57L/J and C57BL/6J demonstrated gross architectural distortion and honeycombing fibrosis in parts of the lung, the CBA/J mice showed thickening of alveolar walls and massive inflammation, but little to no contracted fibrosis.
Perivascular and alveolar inflammation and fibrosis were evaluated in lung tissue from decedents across all radiation doses (7.5 to 17.5 Gy). Perivascular inflammation was scored on a scale of 0-4, alveolar inflammation on a scale of 0-5, and fibrosis on a score of 0-8 by three independent observers. Statistically significant differences were not observed among strains with respect to perivascular and alveolar inflammation, although there was greater variance around the mean in C57BL/6J mice. C57L/J mice displayed more severe fibrosis than C57BL/6J or CBA/J mice. Wet lung weights were recorded at the time of necropsy as a marker for edema and congestion. The wet lung weights among irradiated, moribund C57L/J mice were significantly higher than moribund C57BL/6J (p < 0.001) and CBA/J (p<0.001). Overall, C57L/J and CBA/J exhibit a more salient pneumonitis with edema and congestion than the C57BL/6J strain. Bars represent the mean ± SEM.
Figure 11: Comparison of histopathologic damage (Masson’s Trichrome stain) among strains at radiation doses ranging from 7.5 to 17.5 Gy WTLI

There is significant variation in pathology among strains at the time of death. In the majority of C57BL/6J mice, significant portions of the lung appear to be relatively normal (A). Severe inflammation in the airways (B) and around blood vessels (C) is seen in CBA/J and C57L/J strains. Foamy macrophages (C) and alveolar inflammation (E, F, G) are observed across strains. In contrast, interstitial pneumonia is seen in all most all C57L/J mice with a dose-dependent increase in severity. C57BL/6J mice predominantly display tissue scarring/contracted fibrosis (J). CBA/J mice displayed an inflammatory reaction consistent with interstitial pneumonia and organized exudate, but limited contracted fibrosis.
Figure 12: Obliterative bronchiolitis and interstitial pneumonia in C57L/J mice 26 weeks following 7.5 Gy to the whole thorax

A) Masson’s Trichrome stain of lung tissue collected from C57L/J mice 26 weeks after thoracic irradiation with a single dose of 7.5 Gy; 5x magnification. B) Higher magnification (10x magnification) shows complete alveolar consolidation with foamy-macrophages (→) and perivascular lymphocytic cuffing (*). C) Fine, eosinophilic needle like structures within the terminal bronchioles (→). D) Breakdown of the vessel wall (^), inflammation (*), and fibroproliferation (→). E) Giant, eosinophilic alveolar macrophages characteristic of alveolar inflammation (→). C-E, 40x magnification.
3.3 Discussion

There have been a number of therapeutic modalities over the years that displayed strong efficacy in pre-clinical models, but for unknown reasons, were ineffective in the clinic\textsuperscript{121}. One of the explanations for the poor translation of promising therapeutics has been the use of pre-clinical models that fail to adequately represent the pathophysiology of the disease in humans. One of the most commonly used organisms in pre-clinical research is the mouse due to its genetic similarity to humans (~2.5\% of the genome differs between mice and humans), small size, and cost-effectiveness. Therefore the purpose of this study was to identify the murine strain (s) that best emulates the clinical pathophysiology of radiation-induced lung disease\textsuperscript{122}.

The U.S. government is currently funding the development of medical countermeasures (MCM) against radiation-induced lung toxicity for future FDA licensure under the “Animal Rule”. The current study was designed to develop a research platform that meets the FDA Animal Rule criteria for a robust and reliable pre-clinical model of radiation-induced lung injury in the context of supra-therapeutic whole-thorax exposure.

The experimental design was based on our previous studies evaluating the pulmonary pathology associated with thoracic irradiation among six of the most commonly used rodent strains in pre-clinical radiobiology research\textsuperscript{90,97}. Based on those studies\textsuperscript{97,116}, we identified the CBA/J and C57L/J mouse strains, and to a lesser extent, the
C57BL/6J strain, to represent the full spectrum of pulmonary pathology associated with acute radiation exposure to the thorax. As a result, these three strains were chosen for further characterization in the current study.

Here we systematically characterized the pulmonary response to thoracic irradiation over a dose range between 7.5 and 17.5 Gy and compared functional, histopathologic, and survival outcome to a non-human primate model at the University of Maryland and historical evidence from humans. As the primary endpoint for MCM efficacy is improvement in survival, the first aim was to determine the dose-response for survival among strains. The mean survival time in this study was 120 (± 17) days in C57L/J mice, 125 (± 12 days) in CBA/J mice, and 162 (± 14) days in C57BL/6J mice. The long latency period in C57BL/6J contrasts with the mean survival time in humans (100 days) and non-human primates (144 days) (Table 4).

The difference in the lethal dose for 30-70% of mice within 180 days (LD30-70/180) was striking. In C57L/J mice, the dose-response was steep with an LD30/180 of 10.14 Gy and an LD70/180 of 10.33 Gy. In contrast, for C57BL/6J mice, the LD30-70/180 was 12.37-14.59. The incidence for radiation-pneumonitis in C57L/J mice was most closely analogous to that observed in non-human primates (NHP) and humans following thoracic irradiation. In NHPs, the LD30-70/180 is approximately 9.79-10.72 Gy and in humans, approximately 9.30-10.50 Gy after wide-field thoracic irradiation.
Another criteria for successful adherence to the FDA Animal Rule is clear understanding of the natural history of pulmonary pathogenesis following radiation. In the clinic, a decline in pulmonary function following radiotherapy for tumors in and around the thorax appears to be correlated with symptomatic lung damage\textsuperscript{123}. Therefore, in this study, respiratory function was assessed non-invasively using an unrestrained, whole body plethysmograph (WBP) system (Buxco Electronics, Wilmington, NC). The overall goal was to establish a pre-clinical correlate for pulmonary function tests in the clinic. The advantage of the unrestrained WBP over other, more invasive techniques is the ability to monitor changes in pulmonary function among individual, freely moving mice during the course of disease development. The disadvantage of the WBP is a lack of precision when compared to more invasive techniques\textsuperscript{124}. As a result, values obtained with WBP are considered to be estimates of lung mechanics rather than absolute values\textsuperscript{125}. Here, we found changes in gas exchange (Figure 7), airflow (Figure 8), and relaxation time (Figure 9) first occurred two to four weeks prior to animal mortality. The temporal onset was inversely proportional to dose among all strains and was earliest in the more “sensitive” C57L/J strain.

One of the most interesting parameters evaluated was “enhanced pause” or Penh. Penh is a dimensionless quantity derived from pressure changes within the plethysmograph chamber\textsuperscript{126}. There is a great deal of controversy over the reliability of using Penh as a marker for airway hyperreactivity\textsuperscript{127}. Numerous studies have
alternatively suggested Penh to be a measurement for changes in respiratory patterns rather than pulmonary mechanics. Regardless, in this study, Penh was a reliable and robust predictor for mortality. A sharp rise in Penh occurred consistently 9-14 days prior to mortality among individual mice (Figure 8). In our opinion, this makes Penh useful as a marker for euthanasia as it is an excellent indicator for imminent mortality.

Although there was a clear divergence in pulmonary function between survivors and decedents in the weeks to months following radiation, no single parameter could predict individual risk for developing lethal pneumonitis prior to symptomatic onset.

Pathologic exam was performed on lung specimens collected either at the time of morbidity/mortality or at the pre-determined endpoint of 10, 14, or 26 weeks (data from 10-14 weeks not shown). In humans, acute interstitial pneumonitis develops 2-3 months after radiation exposure and may range from mild inflammation to fulminant organ failure. The pneumonitis reaction is followed by a progressive fibrosis that can result in significant tissue scarring and contraction, impaired gas exchange, and organ dysfunction. Histopathologic exam of tissue collected from individuals succumbing to pneumonitis show epithelial cell hyperplasia, thrombosis and edema, accumulation of foamy, lipid-laden macrophages and mononuclear cellular infiltrates, increased alveolar septa thickness, hyaline membranes, and increased connective tissue density. Histopathologic damage from individuals succumbing to lung injury six months after
exposure is dominated by contracted fibrosis, gross architectural distortion, and reduced perfusion\textsuperscript{a}.

In this study, diffuse interstitial pneumonitis was observed in the lungs of C57L/J and CBA/J mice at all radiation doses (7.5-17.5 Gy). In contrast, a milder pneumonitis was observed in C57BL/6J and only at the highest radiation doses (≥12.5 Gy) (Figure 11). Pathologic findings from C57L/J lungs indicate severe airway obstruction, alveolar edema, and moderate to extensive fibrosis likely contribute to the decline in lung function and mortality in this strain (Figures 11 & 12). Fine, eosinophilic crystalline structures and inflammatory cells of mixed type were observed within almost all of the bronchiolar airways (Figure 12C). Perivascular lymphocytic cuffing that progressed to widespread interstitial inflammation and patchy consolidation of tissue was a prominent feature in the lungs of moribund C57L/J mice. Alveolar inflammation characterized by eosinophilic and lipid-and hemosiderin-laden alveolar macrophages and giant multinucleate cells was also seen (Figure 12D). This led to complete congestion/consolidation of the alveoli. Organized exudate and parenchymal scarring were evident; severity increased with dose. Most importantly, in this strain, the entirety of the lung appeared to be severely damaged in moribund mice.

In contrast, lungs from the C57BL/6J strain had large areas that appeared normal. In the majority, less than 30\% of the lung was damaged. This included the presence of cholesterol clefts, patchy consolidation and inflammation that are characteristic of
interstitial lung disease, as well as extensive tissue scarring and retraction mainly around the subpleura (Figure 1).

While CBA/J mice displayed clear evidence for an organizing pneumonia with thickened alveolar walls, there was very little contracted fibrosis. The lungs of CBA mice showed extensive perivascular and alveolar inflammation, with edema and congestion.

Although the mouse lung differs from human in a number of ways including differences in innate and adaptive immunity\textsuperscript{121}, lobularity, septa and pleural thickness, and blood supply to the pleura\textsuperscript{129}, it is one of the best models for human disease. Developing a robust and reproducible model that can be standardized across institutions will help guide our ability to better define the complex interrelationships between molecular and cellular biology, physiologic effects, and pathologic outcomes associated with radiation-induced lung disease. Establishing a superior research platform for MCM development requires a constant flow of information between clinicians and researchers to ensure data can be extrapolated from pre-clinical models to human disease. In the current study, C57L/J, CBA/J, and C57BL/6J mice were followed for up to six months after thoracic irradiation to determine the lethal dose for 30-70% of subjects, identify the temporal onset for morbidity/mortality, and discern the histopathologic sequelae that contribute to morbidity/mortality after radiation. This study found significant strain-related differences in radiation-induced lung damage. These differences can be exploited to better qualify the molecular mechanisms
associated with radiation-induced lung injury and efficiently screen medical countermeasures to meet the criteria laid out within the FDA Animal Rule.
4. Gene expression profiling with microarrays identifies unique gene patterns associated with radiation pneumonitis in C57L/J and CBA/J mice and not to radiation fibrosis in C57BL/6J mice.

4.1 Introduction

Intrinsic variations in the development of lung injury among patients undergoing radiotherapy for tumors in the thoracic region suggest there are one or more genes that influence the development and severity of radiation pneumonitis and/or fibrosis. Murine strain differences in pulmonary response to radiation may offer a novel method to extract the specific genes and their protein products that participate in the pathogenesis of radiation injury.

Microarray technology is being used in the field of radiation research to identify gene expression signatures in peripheral blood that correlate with physical radiation dose and to identify gene signatures that influence the pulmonary response to radiation. In previous studies, Haston et al. used a backcross between the pneumonitis prone C3H and fibrosis prone C57BL/6J mice to map fibrosis susceptibility to two loci on chromosomes 17 and 1, named Radpf1 and Radpf2 for radiation-induced pulmonary fibrosis. In separate studies, Paun et al., performed gene expression profiling on lung tissue from pneumonitis (A/J, C3H) and fibrosis (C57BL/6) prone mice at the time of peak injury to differentiate genes involved in the early versus late tissue response. While it is important to understand the myriad of mechanisms involved in the acute
tissue response at the time of major morbidity, these differences are post factum and cannot tell us the underlying pathways that influence the divergence of pathologic progression prior to symptomatic onset of disease. Identifying novel targets for early therapeutic intervention may be beneficial for reducing morbidity and improving survival.

In these studies, gene expression profiling with microarrays was performed twenty-four hours after sham- or single dose irradiation of the lungs of mice with well-characterized phenotypes of radiation damage consistent with the “pneumonitis” and/or “fibrosis” phenotype. Genes identified as segregating with the pneumonitis or fibrosis phenotypes were validated by real-time PCR and their protein products evaluated by western blot. Differences in ultrastructural pathology among strains were also observed at twenty-four hours. The results suggest there is an immediate divergence in tissue response among the strains at the time of irradiation and prior to overt histopathologic damage.

Of the few therapies that have successfully made the transition from the laboratory to the clinic, most were based on sound scientific rationale that the biologic targets were important in human disease. Therefore, in this study, a literature search was performed to compare genes identified in this study with published data from patients undergoing clinical radiotherapy. The results allowed us to develop a list of priority gene products deemed relevant to radiation pneumonitis and/or fibrosis in both
pre-clinical models and in human patients. Data from these studies will enable identification of novel therapeutic targets and careful selection of combined therapies to more efficiently mitigate the pathogenesis of radiation injury using a multi-drug approach.

4.2 Results

4.2.1 Ultrastructural changes in lung tissue twenty-four hours after thoracic irradiation

Hematoxylin and eosin (H&E) staining was performed on tissue sections collected from mice twenty-four hours after sham-irradiation or irradiation with 15 Gy. No overt differences were observed between strains upon histologic exam (data not shown). We next sought to determine whether there were differences between strains at the ultrastructural level. Ultrastructural pathology showed significant swelling of endothelial and epithelial cells resulting in vascular occlusion, evidence of lethal cell injury, bronchial epithelial cell damage, and inflammation in the lungs of C57L/J mice. The major ultrastructural pathology in the lungs of CBA/J mice was severe bronchial epithelial cell damage (Figure 13). C57BL/6J showed mild endothelial and epithelial cell swelling and significant interstitial edema. Bronchial epithelial damage was not observed in the evaluated sections from C57BL/6J mice.
Figure 13: Transmission electron micrographs of lung tissue twenty-four hours after 15 Gy irradiation to the whole thorax of C57L/J, CBA/J and C57BL/6J mice

Prominent ultrastructural differences among C57L/J, CBA/J, and C57BL/6J mice are observed twenty-four hours after thoracic irradiation with a single dose of 15 Gy. In C57L/J mice, there is prominent endothelial (EC) and epithelial cell (P1/PII) swelling. In some instances, EC swelling results in capillary occlusion that likely affects blood flow to the tissue. Interstitial cell (IC) necrosis and inflammatory cell infiltrates are also seen. There is abundant lethal cell injury and apoptosis characterized by cytoplasmic blebbing, nuclear membrane invagination, mitochondrial (M) and endoplasmic reticulum (ER) swelling. Bronchial epithelial cells are severely swollen and apoptotic in the lungs of both C57L/J and CBA/J mice. The primary ultrastructural features in C57BL/6J lungs are significant interstitial edema and mild epithelial and endothelial cell swelling. Inflammation is not as prominently observed in C57BL/6J mice as in C57L/J.
4.2.2 Gene expression profiles in “pneumonitis-prone” mice are distinct from “non-pneumonitis” prone mice

For the purposes of this study, CBA and C57L were categorized as “acute responders” based on the mean survival time (<140 days) and presence of acute interstitial pneumonitis at the time of morbidity/mortality. C57BL/6J mice were categorized as “delayed responders” due to a survival time in excess of 140 days following radiation. In this strain, morbidity/mortality were primarily associated with a mixture of severe fibrosis and mild pneumonitis.

5088 genes were differentially expressed between acute and delayed responders (p < 0.01; 20% change in expression). Of these, 1445 genes were upregulated and 3642 genes were downregulated in acute responders in contrast to delayed responders. 3781 genes were differentially expressed after 15 Gy single dose irradiation to the thorax between C57BL/6J versus CBA and C57L/J mice (p<0.01; 20% change).

To better understand the unique gene expression patterns among murine strains both before and after radiation, unsupervised hierarchical cluster analysis was performed. Cluster analysis was performed in a blinded fashion without a priori knowledge of the data. **Figures 14 and 15** shows the clustering of samples according to their similarity in gene expression.
Hierarchical clustering analysis demonstrates segregation of samples by (A) strain and (B) anticipated onset of mortality.

C57BL/6J, CBA/J, and C57L/J mice were irradiated to the whole thorax with a single dose of 12.5 or 15 Gy. Twenty-four hours post-irradiation, lung tissue was harvested, RNA extracted, and microarray analysis performed to determine differential gene expression among strains. Sham-irradiated age- and sex-matched mice were included as controls. The raw fluorescent intensities were normalized by Robust Multichip Average (RMA) and unsupervised analysis of the normalized microarray data was performed. A) Hierarchical cluster analysis of gene expression shows clustering of samples according to strain. The radiation fibrosis-prone C57BL/6J strain is furthest away from the non-fibrosing, pneumonitis-prone CBA/J strain suggesting these strains had the largest differences in gene expression. C57L/J mice, which are both pneumonitis and fibrosis prone, lie in the middle. B) Early responders (CBA and C57L/J; average mortality <140 days) clustered together and separate from late responders (C57BL/6; average mortality > 140 days).
Figure 15: Unsupervised principal component analysis (PCA) of lung tissue samples demonstrates clustering of genes according to A) strain, B) expected pathology (Pneumonitis vs. Fibrosis), C) response time for mortality, and D) radiation dose and strain.

A) PCA of acute responders [red circles (C57L/J, CBA/J, average mortality <140 days)] compared to delayed responders [blue circles (C57BL/6J, average mortality >140 days)]. Grouping of individual tissue samples between the two groups suggests distinct differences in gene expression between acute vs. delayed responders. The close clustering of individual samples notes the biological reproducibility of the results. B) PCA shows similarity in gene expression between samples from fibrosis-prone mice (blue/C57BL/6J), pneumonitis-prone mice (green/CBA/J), and strains with a mixed pathology of fibrosis and pneumonitis (red/C57L/J).
4.2.3 Functional network analysis reveals differential activation of cellular response to DNA damage after irradiation between “pneumonitis-prone” and “non-pneumonitis prone” mouse strains

Genes of interest were imported into the Ingenuity Pathways Knowledge Base. Overlay of the genes of interest with data contained in the IPKB identified the top enriched molecular networks based on the ratio between the number of genes mapped to a given pathway and the number of genes within the pathway itself. P-values were calculated using Fischer’s exact test based on the relatively likelihood that the genes were expressed by chance. Network connectivity maps were generated using the IPA “pathway designer” tool (data not shown).

Three molecular networks were constructed by IPA software based on gene expression data. The top network was p53 -centered and contained the maximum number of molecules, thirty-five, that function in cellular development, growth, and proliferation. The second network was NR3C1-centered. NR3C1 is a molecule that participates in inflammation and cellular differentiation. The pathway contained thirty-five molecules related to cell-to-cell signaling and interaction and hematopoiesis. The third pathway was p73 centered with thirty-four molecules expressed that participate in cell cycle, DNA replication, recombination and repair.

The top three networks are shown in Table 5. Genes up-regulated are in red and those downregulated are in green. The comparison was made between C57L/J and CBA/J mice versus C57BL/6J mice.
Table 5: Top 3 IPA networks with associated genes and functions

<table>
<thead>
<tr>
<th>Network</th>
<th>Molecules</th>
<th>Top Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I: TP53 centered</td>
<td>ACSL3, BLZF1, CSNK1D, CUL7, CUL9, DUT, ETHE, GAK, GART, HIPK1, ING5, JMJD1C, LATS2, MAP2K2, MSL2, MTDH, PAK3, PANK1, PLK2, POLG, PPM1A, PPP4R2, PRIM1, PSMC3, PVRL3, SCN3B, SON, SORBS1, SPATA18, TMSB10/TMSB4X, <strong>TP53</strong>, TP53R, TPRKB, UNC5B, ZFP36L1</td>
<td>Cellular Development, Cellular Growth and Proliferation, Renal and Urological System Development and Function</td>
</tr>
<tr>
<td>II: NR3C1 centered</td>
<td>AKTIP, ATG4B, ATP1B1, BRCC3, BRE, BRWD1, CDCA7L, CORO1C, CSRNP2, DAG1, DAPK3, DPF2, FASTKD5, MAEA, MAGI3, NMT1, NOL3, <strong>NR3C1</strong>, POCK, PPP2R1B, PSMB1, PTMS, RNF38, RRAGC, SAP30BP, SGPP1, SIAH2, THAP1, TNFAIP1, TNFAIP8, TRAF4, UBE2K, WDR3, WDR26, WDR37</td>
<td>Cell-To-Cell Signaling and Interaction; Hematological System Development and Function, Hematopoiesis</td>
</tr>
<tr>
<td>III: <strong>p73</strong> centered</td>
<td>ABI2, AUH, CCNG2, CCNH, CDC42EP2, CDKN2C, CLMN, COL18A1, CyclinD, DLG1, DR1, DUSP8, DUSP11, EFNB2, ERCC2, ERCC3, HUS1, IRF3, KAT2B, KDM1A, KIF13B, MAPK8, MFAP3, PHF21A, PIN1, PUF60, RAD1, RAD17, RNF144B, TBL1X, TIRAP, <strong>TP73</strong>, TREX1, TRIM32, TSPYL1</td>
<td>Cell Cycle, DNA Replication, Recombination and Repair, Dermatological Diseases and Conditions</td>
</tr>
</tbody>
</table>
4.2.4 Canonical pathway analysis shows Protein Ubiquitination Pathway and PI3K-Akt pro-survival signaling are among the top pathways significantly altered twenty-four hours after radiation between “pneumonitis prone” and “non-pneumonitis prone” mice.

To derive biological meaning from the given data sets (acute vs. delayed responders), 5088 genes were analyzed for enrichment of functional annotation using IPKB. The significance of the association between the genes from the dataset and the functional pathway is calculated by IPA using a right-tailed Fisher exact test.

Figure 16 shows fifteen functional pathways with the highest gene enrichment as determined by IPA software. Three pathways significantly enriched in the acute vs. delayed responders data set, PI3K/Akt signaling, Nrf2 oxidative stress response, and nucleotide excision repair pathway, have previously been reported to participate in radiation pathogenesis\(^7\)\(^9\),\(^\,\)\(^1\)\(^4\)\(^2\). Nrf2 pathways have also been found to play a role in radiation fibrosis in both C57BL/6J mice\(^1\)\(^4\)\(^3\) and in NHPs (personal communication, Dr. Tom MacVittie).
Figure 16: Top 16 IPA functional pathways differentially expressed among acute vs. delayed responders to thoracic irradiation

Shown above are the most enriched pathways differentially expressed between acute (C57L/J and CBA/J) and delayed (C57BL/6J) responders. Pathways include those involved in DNA synthesis (purine/pyrimidine metabolism), DNA damage/repair (nucleotide excision repair pathway), cell survival/cell death (PI3K/Akt signaling, ERK/MAPK signaling, Myc mediated apoptosis signaling), and oxidative stress (Nrf-2 mediated oxidative stress response). The nucleotide excision repair pathway is one of the most commonly expressed pathways induced by radiation regardless of total dose, dose rate, species/strain, or cell type.
4.2.5 Top differentially expressed genes between acute (C57L/J, CBA/J) and delayed responders (C57BL/6J) mice

In this study we identified the top differentially expressed genes between acute and delayed responders using an ANOVA-based approach. First, we identified the top genes differentially expressed between groups using an established cut-off of $p < 0.01$ and greater than 20% change in relative expression. Lists were then imported into IPA and the top differentially expressed genes identified. The top molecular and cellular functions were related to RNA Post-Transcriptional Modification (112 molecules), Post-Translational Modification (246 molecules), Cell Cycle (265 molecules), DNA Replication, Recombination, and Repair (223 molecules), and Gene Expression (408 molecules).

Next, a gene search was performed using the Gene Ontology database (www.geneontology.org) to identify the biological process, cellular component, and molecular function (not shown) associated with each of the top 20 differentially expressed genes. Differences in gene expression were found between acute phase response (Serpina1 and ORM1, 2), cell migration (VEGFC and FEZ1), and angiogenesis (ANG, VEGFC). Tables 6 and 7 list the top ten upregulated and top ten down regulated genes, their biological process, cellular component, and p-value for significance.
Table 6: Top genes upregulated in pneumonitis-prone mice (acute responders = CBA/J, C57L/J) compared with non-pneumonitis prone mice (delayed responders = C57BL/6J)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Biological Process</th>
<th>Cellular Component</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serpina1</td>
<td>Serine (or cysteine proteinase inhibitor, clade A, member 1 (aka. Alpha-1 antitrypsin)</td>
<td>Response to cytokine stimulus</td>
<td>Extracellular</td>
<td>$1.4 \times 10^{-10}$</td>
</tr>
<tr>
<td>ORM1,2</td>
<td>Orosomucoid 1,2 (aka. Alpha-1 acid glycoprotein)</td>
<td>Acute phase response</td>
<td>Extracellular</td>
<td>$1.12 \times 10^{-4}$</td>
</tr>
<tr>
<td>Mtap2</td>
<td>Microtubule associated protein 2</td>
<td>Negative regulation of microtubule polymerization</td>
<td>Cytoplasm</td>
<td>$9.46 \times 10^{-11}$</td>
</tr>
<tr>
<td>ALAD</td>
<td>Aminolevulinate, delta dehydratase</td>
<td>Heme biosynthetic process</td>
<td>Extracellular</td>
<td>$3.17 \times 10^{-18}$</td>
</tr>
<tr>
<td>AFP</td>
<td>Alpha fetoprotein</td>
<td>SMAD protein signal transduction</td>
<td>Cytoplasm/ Extracellular</td>
<td>$3.12 \times 10^{-5}$</td>
</tr>
<tr>
<td>VEGFC</td>
<td>Vascular endothelial growth factor-C</td>
<td>Angiogenesis</td>
<td>Extracellular</td>
<td>$6.82 \times 10^{-6}$</td>
</tr>
<tr>
<td>PAIP1</td>
<td>Polyadenylate binding protein- interacting protein 1</td>
<td>Positive regulation of translation</td>
<td>Cytoplasm</td>
<td>$9.05 \times 10^{-3}$</td>
</tr>
<tr>
<td>FEZ1</td>
<td>Fasciculation and elongation protein zeta 1</td>
<td>Mitochondrial localization</td>
<td>Cytoplasm</td>
<td>$5.61 \times 10^{-3}$</td>
</tr>
<tr>
<td>TSHB</td>
<td>Thyroid stimulating hormone, beta subunit</td>
<td>Hormone Activity</td>
<td>Extracellular</td>
<td>$2.15 \times 10^{-8}$</td>
</tr>
<tr>
<td>FAM20B</td>
<td>Family with sequence similarity 20, member B</td>
<td>Phosphorylation; metabolic process</td>
<td>Integral to Membrane</td>
<td>$7.71 \times 10^{-21}$</td>
</tr>
</tbody>
</table>
Table 7: Top 10 genes downregulated in strains with acute pneumonitis reaction (acute responders=CBA/J, C57L/J) compared to non-pneumonitis prone C57BL/6J mice (delayed responders)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Biological Process</th>
<th>Cellular Component</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC15A2</td>
<td>Solute carrier family 15 (H+/peptide transporter)</td>
<td>Dipeptide transport</td>
<td>Integral to membrane</td>
<td>2.21 x 10^{-14}</td>
</tr>
<tr>
<td>RPS9</td>
<td>Ribosomal Protein S9</td>
<td>Positive regulation of cell proliferation</td>
<td>Cytoplasm</td>
<td>6.63 x 10^{-26}</td>
</tr>
<tr>
<td>GALC</td>
<td>Galactosylceramidase</td>
<td>Galactosylceramide catabolic process</td>
<td>Mitochondrion/Lysosome</td>
<td>1.83 x 10^{-25}</td>
</tr>
<tr>
<td>TRIP11</td>
<td>Thyroid hormone receptor interactor 11</td>
<td>Transcription from Pol II promoter</td>
<td>Nucleus</td>
<td>2.19 x 10^{-22}</td>
</tr>
<tr>
<td>PTTG1</td>
<td>Pituitary tumor transforming gene 1</td>
<td>DNA Repair</td>
<td>Nucleus</td>
<td>3.60 x 10^{-20}</td>
</tr>
<tr>
<td>ANG</td>
<td>Angiogenin</td>
<td>Activation of phospholipase C</td>
<td>Extracellular</td>
<td>6.18 x 10^{-03}</td>
</tr>
<tr>
<td>MID1</td>
<td>Midline 1</td>
<td>Positive regulation of stress-activated MAPK cascade</td>
<td>Cytoplasm</td>
<td>1.15 x 10^{-20}</td>
</tr>
<tr>
<td>SOX11</td>
<td>SRY-box containing gene 11</td>
<td>Positive regulation of transcription from RNA polymerase II promoter</td>
<td>Cytoplasm/Nucleus</td>
<td>1.33 x 10^{-07}</td>
</tr>
<tr>
<td>HFE</td>
<td>Hemachromatosis</td>
<td>Antigen processing and presentation; iron homeostasis</td>
<td>MHC class 1 protein complex</td>
<td>7.84 x 10^{-09}</td>
</tr>
<tr>
<td>Erdr1</td>
<td>Erythroid differentiation receptor-1</td>
<td>Somatic stem cell maintenance</td>
<td>Unknown</td>
<td>2.91 x 10^{-05}</td>
</tr>
</tbody>
</table>
4.2.6 Quantitative real-time PCR confirms differential gene expression in 15 of the 20 top genes identified by IPA

In order to validate the microarray results, quantitative real-time PCR (qRT-PCR) was performed using Assays on Demand primers for the top 20 genes identified as differentially expressed between acute and delayed responders. Quantitative RT-PCR was performed on the same RNA previously used for microarray analysis. All data was normalized to the C57BL/6J control. Each sample was run independently in quadruplicate. GAPDH was used as the reference gene. Samples from only one radiation dose (15 Gy) and their age-matched, sham-irradiated controls were chosen due to the similarity in gene expression between 12.5 and 15 Gy.

Figure 17 shows the relative mRNA expression of the top twenty genes in irradiated (15 Gy) lungs from each strain. One-way ANOVA was performed to determine whether there was a difference in means among groups. When the differences were statistically significant, we then performed Tukey’s multiple comparison test to determine the differences among each strain and radiation dose. Of the six genes shown in Figure 18, four showed significant differences across strains at p < 0.05 (one-way ANOVA). These are MTAP, ALAD, AFP, and PTTG1.
Figure 17: Gene expression among the top 20 genes identified by IPA as being differentially expressed between acute (C57L/J, CBA/J) vs. delayed (C57BL/6J) responders.

Quantitative real-time PCR (qRT-PCR) was performed using mRNA from the same tissue samples collected for microarray analysis. Relative mRNA expression across strains (C57BL/6J, CBA/J, C57L/J) and doses (0 or 15 Gy) was normalized to the C57BL/6J sham-irradiated controls. Fifteen of the top twenty genes correlated with the results from the microarray data. Four genes (RPS9, SLC15A2, ANG, and PAIP1) showed no difference between strains or radiation doses. Samples were run in quadruplicate.
Figure 18: Comparison of relative mRNA expression among select genes in C57BL/6J, C57L/J, and CBA/J strains before and after radiation (* p<0.05, ** p<0.01, *** p<0.001)
4.2.7 Differences in messenger RNA and protein expression of acute phase proteins in early tissue response to radiation

Quantitative-real time PCR was performed on samples to determine changes in Serpina1, the gene encoding alpha-1 antitrypsin. Samples were run independently and normalized to the C57BL/6J non-irradiated control. GAPDH was used as the reference gene. There was a wide variation in gene expression that trended toward a significant difference in gene expression between CBA and C57BL/6J after irradiation (F-test p-value <0.1) and was significant at p <0.05 when comparing C57L/J vs. C57BL/6J after radiation (Figure 19A).

Alpha-1 antitrypsin (A1AT) protein levels in the lungs of non-irradiated and irradiated mice were analyzed by western blot in a separate group of mice undergoing thoracic irradiation with a single dose of 15 Gy (n = 5/group). Here, we found no difference in protein expression in the lungs of mice after irradiation, although there were higher basal levels in C57L/J when compared to C57BL/6J or CBA/J (Figure 20A).

The gene encoding for alpha-1 acid glycoprotein (ORM1) was significantly increased in the lungs from CBA and C57L/J after radiation when compared to their non-irradiated controls (p<0.01 and p < 0.05, respectively). In comparison, no change in ORM1 expression was found to occur in response to thoracic irradiation in the lungs of C57BL/6J mice. The difference in ORM1 expression between CBA and C57L/J when
compared to C57BL/6J after irradiation was also significant (p < 0.01 and p < 0.05, respectively) (Figure 19B).

In a separate group of mice receiving thoracic irradiation (n =5/group), western blot analysis was performed to determine changes in alpha-1 acid glycoprotein (AAG) expression. Higher basal levels of AAG were found in C57L as compared to both CBA/J and C57BL/6J (Figure 20B). Twenty-four hours after radiation, AAG was not increased; however, this may be due to the time needed for translation of protein from mRNA. A time point study to evaluate AAG expression after irradiation might offer a clearer picture of alterations in AAG levels in the lungs of C57L/J, CBA/J, and C57BL/6J mice. The higher basal levels of both A1AT and AAG in C57L/J mice may indicate this strain is predisposed to inflammation.
Figure 19: Relative expression of the genes encoding for alpha-1 antitrypsin (Serpina1) and alpha-1-acid glycoprotein (ORM1)

Shown is the relative mRNA expression (qRT-PCR) for Serpina1 (A) and ORM1 (B). Both genes were identified by one-way ANOVA as being differentially expressed in C57L/J & CBA/J vs. delayed C57BL/6J mice. While differences in Serpina1 were not statistically significant at \( p<0.05 \) using qRT-PCR, ORM1 showed a statistically significant increase after radiation in CBA/J (\(*p<0.05\)) and C57L/J (\(**p<0.001\)).
Figure 20: Protein expression of alpha-1 antitrypsin (A1AT) and alpha-1 acid glycoprotein (AAG) in a separate cohort of mice

Protein expression was evaluated in lung tissue twenty-four hours after sham-irradiation or thoracic irradiation in C57BL/6J, CBA/J, and C57L/J mice (n = 5 per strain and dose). The lack of correlation between mRNA and protein expression may be due to the lag time between transcription and translation. ** p< 0.01; ***p<0.001.
4.2.8 Phenotypic expression of lung injury differs significantly among rodent strains fourteen weeks post-irradiation in a murine model of radiation-induced lung injury

Histopathologic damage in irradiated lungs was consistent with that reported previously. At 14 weeks post-irradiation, lungs from C57L/J and CBA/J mice showed evidence of severe perivascular and alveolar inflammation, foamy-macrophages within the alveoli, increased alveolar wall thickness, and atelectasis. C57L/J also had signs of tissue scarring and retraction, similar to that seen in C57BL/6J mice, but more severe. The lungs of C57BL/6J mice on the other hand exhibited less severe inflammation and limited collagen deposition and fibrosis. Figure 21 shows representative images stained with Masson’s Trichrome.

IPA pathway analysis was performed at 24 hours to identify individual differences for each murine strain compared to the others (i.e. B6 vs. CBA & C57L; CBA vs. B6/C57 & C57 vs. CBA & B6). The predominant pathways expressed in each strain correlated well with the phenotypic expression of lung disease. In the top 10 enriched pathways in C57L/J mice, almost all were related to inflammation (TLR signaling, TNFR signaling). In contrast, pathways upregulated in C57BL/6J pathway were mostly related to cell proliferation, reorganization, and oxidative stress. CBA/J mice were related to cell proliferation/survival (ERK/MAPK; ER-stress pathway), the coagulation cascade (tissue factor), and inflammation (chemokine signaling, TLR signaling).
Figure 21: Histopathologic damage among murine strains 14 weeks following whole thorax lung irradiation.

A separate cohort of C57BL/6J, CBA/J, and C57L/J mice were irradiated to the whole thorax with 0, 12.5, or 15 Gy irradiation and followed for 14 weeks. Histopathologic damage and fibrosis were assessed in hematoxylin and eosin (H&E) and Masson’s trichrome stained lung tissue sections. Pathways enriched within individual strains are consistent with the phenotypic expression of injury at 14 weeks. In the pneumonitis prone C57L/J mice, pathway enrichment is consistent with a pro-inflammatory phenotype. In fibrosis-prone C57BL/6J mice, pathway enrichment is consistent with cell growth and proliferation. IPA pathway analysis of microarray data at twenty-four hours post-WTLI suggests pathologic outcome is genetically pre-determined.
4.2.9 Inflammatory cytokines in lung tissue differ among strains 14 weeks after radiation

A 23-multiplex cytokine assay was used to compare intrastrain variations in cytokine expression in plasma and lung tissue at 14 weeks post-radiation with a single dose of 0, 12.5, or 15 Gy delivered to the thorax. No significant differences were observed in plasma cytokine levels. Data shown in Figure 22 is a comparison of cytokine profiles in the lungs of each strain after 15 Gy WTLI (n = 4/group). Only cytokines with a significant difference are shown. The most significant differences were seen in IL-1α, IL-1β, IL-12 and keratinocyte chemoattractant (KC; IL-8 in humans). A significant increase in IL-1α expression levels was observed in C57L/J and CBA/J (p < 0.05 vs. C57BL/6J), but expression levels were below the limits of detection in C57BL/6J. IL-1β was equally elevated in all three strains at 14 weeks. IL-12 increased in a dose-response manner in both C57L/J and CBA/J mice (p < 0.05 vs. C57BL/6J). KC was more significantly elevated in C57BL/6J when compared to C57L/J (p < 0.01). The differences in cytokine expression are consistent with the observed influx of inflammatory cell subsets (see Figure 23). MCP-1 increased in all three strains in a dose-response fashion.
Figure 22: Inflammatory cytokines in whole lung tissue 14 weeks after 15 Gy thoracic irradiation

C57L/J, CBA/J, and C57BL/6J received 12.5 Gy (not shown) or 15 Gy single dose irradiation to the whole thorax and were followed for fourteen weeks. Non-irradiated, age-matched animals were included as controls (not shown). At fourteen weeks, mice were euthanized, lung tissue harvested, and 23-plex cytokine analysis performed. Statistically significant differences in cytokine expression were observed between strains in IL-1α, IL-12 (p40), and KC. IL-1b, GCSF, MCP1, and MIP-1beta also showed differences but were not statistically significant. Taken together, the differences in cytokine profiles are consistent with greater cell inflammation in the lungs of C57L/J mice when compared to C57BL/6J or CBA/J mouse lungs. *p < 0.05 vs. C57BL/6J (15 Gy); ** p< 0.01 vs. C57BL/6J (15 Gy)
4.3 Discussion

In this study, differences in gene expression patterns were analyzed twenty-four hours after radiation among three murine strains using microarray profiling. Data interpretation in this study was strengthened by the utilization of three mouse strains with well-characterized pulmonary tissue responses to radiation\textsuperscript{89, 90, 114}. In previous studies, we found organizing pneumonitis and bronchiolitis in the lungs of C57L/J mice approximately 14-20 weeks post-radiation with evidence of tissue scarring and retraction. The dose-response relationships for functional damage and survival were similar among C57L/J mice, non-human primates and humans\textsuperscript{114}. CBA/J mice developed an organizing pneumonitis as well, albeit at moderately higher doses than that to cause the same level of injury in C57L/J and without contracted fibrosis. The “fibrosis prone” C57BL/6J mice were the furthest from NHPs and humans in dose-response. They developed a mild pneumonitis, but only at higher doses (12.5 Gy or greater).

In this study, a subset of mice were irradiated at 12.5 and 15 Gy and followed for 14 weeks after which pathologic exam and cytokine analysis was performed on the tissue. The observed pathology was consistent with that previously reported (Figure 21)\textsuperscript{89, 114, 131, 132}. IPA pathway analysis of gene expression at 24 hours within individual strains found pro-inflammatory pathways such as TNFR1 and TLR signaling to be the predominant pathways activated in C57L/J mice. Endoplasmic reticulum-stress and MAPK signaling were among the top pathways in CBA/J mice. In C57BL/6J mice,
activated pathways were primarily involved in cell cycle regulation and cell proliferation. Pathway activation at 24 hours is consistent with ultrastructural changes at the same time period and the anticipated pathologic response based on tissue collected at 14 weeks from another set of similarly irradiated animals.

As the latency period between radiation exposure and symptomatic injury is inversely proportional to dose, the use of multiple radiation doses were chosen to provide valuable knowledge towards understanding wide-scale variations in dose-dependent gene expression. Although there were large variations in inter and intrastrain gene expression before and after radiation, principal component analysis (PCA) showed very little dose-dependent difference in gene expression using a combined cut off value of $p<0.01$ and 1.2 fold change.

The feasibility of using gene expression analysis to identify novel targets for pharmacologic intervention has been described$^{144}$. As mentioned previously, the most successful translation of pharmacologic interventions have been based on substantial biological rational that the targeted pathways were important in human disease$^{100}$. In this study, a serine protease inhibitor, alpha-1 antitrypsin, and a serine protease carrier, alpha-1 acid glycoprotein, were shown to be the top two differentially expressed genes among acute versus delayed responders ($p < 1.4 \times 10^{-10}$ and $p < 1.12 \times 10^{-4}$, respectively) (Table 6).
There is accumulating evidence to suggest acute phase proteins/serine protease inhibitors, specifically alpha-1 antitrypsin and alpha-1 acid glycoprotein, may play a role in the development of radiation injury (Table 8). Zherbin et al. found an increase in alpha-1 antitrypsin at the peak of radiation illness following total body irradiation in a non-human primate model\textsuperscript{145}. More recently, Jakobsson et al. found an increase in both alpha-1 antitrypsin and alpha-1 acid glycoprotein in the serum of patients with GI toxicity following pelvic irradiation for anal or uterine cancer\textsuperscript{146}. In another set of studies, Oh et al.\textsuperscript{147} used a bioinformatics approach and found a correlation between alpha-2 macroglobulin, also an acute phase protein, and radiation pneumonitis in non-small cell lung cancer patients following fractionated radiation.

These NHP and human data are interesting as in an unpublished study by our own group, delivery of a serine protease inhibitor, camostat mesilate, prior to and after irradiation worsened radiation-related GI toxicity including ulceration, fibrous adhesions, and vascular adenopathy when compared to the non-treated, irradiated controls. The severity of GI toxicity and increased lethality among camostat-treated animals led to an early completion of that study (Hart et al. unpublished data).
Table 8: Studies associating acute phase proteins with radiation-induced injury

<table>
<thead>
<tr>
<th>Gene/Protein</th>
<th>Species</th>
<th>Radiation</th>
<th>Endpoint</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-1 antitrypsin</td>
<td>Non-Human Primates</td>
<td>LD100/45, total body irradiation</td>
<td>Increase in alpha 1 antitrypsin at the peak of radiation illness (7-10th day)</td>
<td>Zherbin et al. Radiobiologiia. 1987; 27 (2):25-3145</td>
</tr>
<tr>
<td>Alpha-1 acid glycoprotein</td>
<td>Rats</td>
<td>LD10/30-LD100/30 total body irradiation</td>
<td>Six fold increase in serum; increase, in part, was attributed to production by organs other than the liver</td>
<td>Magic et al. Radiation Res. 1995; 143(2):187-93148</td>
</tr>
<tr>
<td>Alpha-1 acid glycoprotein</td>
<td>Rats</td>
<td>6.7 Gy, 12 Gy, and 3 x 6 Gy TBI</td>
<td>2 fold increase in liver six hours after IR</td>
<td>Trutic et al. Comp Biochem Physiol Toxicol Pharmacol 2002; 133(3):461-70149</td>
</tr>
<tr>
<td>Alpha-1 acid glycoprotein, Alpha-1 antitrypsin</td>
<td>Humans</td>
<td>Pelvic irradiation for anal or uterine cancer</td>
<td>Increase in patient serum associated with fatigue and diarrhea 3 and 5 weeks after RT</td>
<td>Jakobsson et al. Oncologist 2010; 15(9):1009-15146</td>
</tr>
<tr>
<td>Alpha-2 macroglobulin</td>
<td>Humans</td>
<td>Fractionated RT for non-small cell lung cancer (NSCLC)</td>
<td>Association between A2M and radiation pneumonitis in NSCLC patients at 3 and 6 months following Fx RT</td>
<td>Oh et al. J Proteome Res 2011; 10 (3):1406-15147</td>
</tr>
</tbody>
</table>
The data presented here demonstrate the reliability of modern technology to elucidate the mechanism by which radiation alters gene expression patterns. We and others have investigated a number of therapeutic regimens to treat radiation-induced lung injury based on our current understanding of the molecular mechanisms associated with radiation pathogenesis. These include small molecule inhibitors of TGF-β1 and components of its signaling pathway, angiotensin-converting enzyme (ACE) inhibitors, statins to reduce inflammation, and potent antioxidants to mitigate oxidative stress and modulate redox-regulated signaling. None have proven to be one hundred percent effective. Due to the likelihood that radiation injury is a multifactorial process, gene expression studies, such as these, can help elucidate the pathophysiologic mechanism(s) underlying acute vs. delayed radiation injury and aid in identifying targets for therapeutic intervention. However, it is highly unlikely that modification or elimination of any single event will change the outcome of tissue damage due to the complexity of radiation pathogenesis. One solution to achieving sufficient reduction in morbidity/mortality is a multi-drug approach based on sound biologic rationale that the pathways being targeted facilitate radiation-induced lung injury. The rationale for a multi-drug approach is supported by the ability of broad-spectrum therapeutic interventions to achieve better overall reduction in lung damage when compared to single-target agents.
It should be noted that unlike previous studies that have evaluated gene expression at the time of peak injury\textsuperscript{141}, this study evaluated the differences in early tissue response. The way a cell perceives the initial radiation damage and communicates the injury to neighboring cells may play an important role in determining the tissue response and pathophysiological outcome\textsuperscript{156-158}. Therefore, we sought to better understand the gene expression patterns that occurred in response to the radiological insult to determine whether these were similar or dissimilar between strains. Pulling apart the differences in early versus late tissue injury has implications for development of medical countermeasures as well as determining the window of opportunity after exposure where treatment could mitigate or prevent the full development of the disease.

One of the limitations of a 24-hour time point is that it precedes the time when the overt expression of injury among murine strains appears to diverge (>8 weeks post-IR). However, transmission electron micrographs show an immediate difference in tissue response and severity of cell injury between early responding and late responding strains (\textbf{Figure 13}). Interestingly, gene expression profiles at twenty-four hours were remarkably distinct between the “pneumonitis prone” CBA and C57L/J strains and the non-pneumonitis prone C57BL/6J strain. The correlation between TEM and gene expression suggest tissue fate may be a result of pre-determined genetic influences.

One disadvantage of assessing gene expression at twenty-four hours, is that this time point does not allow for elucidation of other potential differences in the development of
late lung injury such as genetic differences in leukocyte recruitment $^{159}$ or differences in innate tissue kinetics$^{160}$. Therefore, evaluating gene expression differences during the clinically latent period and at the peak of injury should be the subject of future studies.
Inflammatory cell subtypes in whole fresh lung tissue at 14 weeks was determined using flow cytometry by Emily O’Koren in the laboratory of Dr. Michael Dee Gunn. Mice (n =3/group) were irradiated to the whole thorax with a single dose of 12.5 (not shown) or 15 Gy. Non-irradiated, age-matched mice were included as controls (not shown). Mice were followed fourteen weeks at which time they were humanely euthanized and the whole lung harvested for analysis of inflammatory cell subsets within the tissue. Differences in inflammatory cell subtypes among strains are consistent with cytokine profiles at the same time point shown in Figure 22. PMN= polymorphonuclear cells/neutrophils; NK = natural killer cells; *P<0.05
5. Natural history of disease progression in lung tissue is associated with the development of tissue hypoxia

5.1 Introduction

It was over a decade ago that the progressive development of tissue hypoxia was first recognized as a mechanism perpetuating the pathogenesis of radiation-induced lung toxicity. Prior to this groundbreaking work by Vujaskovic et al., it was well known that a dynamic, biocontinuum of cytokine activity underscored the pathogenesis of radiation-injury. However, it was unclear what mechanisms perpetuated the chronic cycling of pro-inflammatory and pro-fibrogenic cytokines during disease development.

The observation of tissue hypoxia in irradiated lung altered the accepted dogma of molecular and physiological events facilitating the development of normal tissue injury. Tissue hypoxia develops as a result of vascular dysfunction including capillary blockage due to endothelial cell swelling, microvessel loss due to endothelial cell damage, and fibroobliteration of alveoli and capillaries. As tissue becomes more hypoxic over time, macrophages are recruited to the areas of tissue hypoxia where they are activated to produce reactive oxygen species, a process that results in further oxygen consumption. The development of chronic oxidative stress resulting from macrophage accumulation with hypoxic tissue, activation of oxidant-generating enzymes within the

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1 Data presented here are in Jackson et al. Free Radical Biology Medicine 2012, In Press. The study was conceived and designed by ILJ. Data analysis and manuscript preparation was performed by ILJ. Experiments were performed by Dr. X. Zhang, Dr. ZN Rabbani, and C. Hadley.
vessel walls, and mitochondrial leakage lead to activation of a number of transcription factors and pro-inflammatory mediators involved in radiation-induced lung injury \(^{46}\).

Due to these findings, a number of studies over the past decade have focused on the use of potent antioxidants to prevent, mitigate, or treat radiation-induced normal tissue toxicity\(^{80,162-166}\). Among the most well studied antioxidant based radioprotectors are a family of small molecular weight catalytic metalloporphyrin antioxidant mimetics of superoxide dismutase\(^{82-84,167,168}\). First discovered by Fridovich and McCord in 1969, superoxide dismutase (SOD) maintains cellular redox homeostasis by converting superoxide anion to hydrogen peroxide that can then be converted to oxygen and water via catalase and glutathione\(^{169}\).

The purpose of the current study was to better define the role of hypoxia and oxidative stress in the pathogenesis of radiation-induced lung injury. One of the FDA requirements for MCM licensure is a thorough understanding of the MCM effect on the natural history of radiation-induced lung injury. To meet that objective, the natural history of disease progression must be clarified.

In the current study, we hypothesized that tissue hypoxia develops soon after thoracic irradiation and progressively worsens over time. Furthermore, we hypothesized that hypoxia induces an environment of chronic oxidative stress that participates in the activation of redox-sensitive molecules that contribute to the pathogenesis of lung injury. We further hypothesized that intervening in the cycle of
chronic hypoxia/oxidative stress through use of a potent antioxidant can mitigate tissue injury.

### 5.2 Results

**5.2.1 Temporal changes in tissue hypoxia after 15 Gy thoracic irradiation**

Tissue hypoxia was visualized using an endogenous marker for hypoxia, carbonic-anhydrase IX, and the exogenous marker hypoxia marker, pimonidazole. Hypoxia was initially observed 72 hours post-radiation and was strongly increased at one week (Figure 24). Tissue hypoxia preceded the onset of histopathologic damage as seen at the light microscopy level (H&E stain, Figure 24).
Tissue hypoxia (brown pigment) is first observed three days (CAIX, Pimonidazole) after radiation. Hypoxia progressively increases throughout the follow-up period of 6 months. At six weeks, the first histopathologic lesions are seen (H&E). Lesions are focal in nature. They are characterized by thickening of the alveolar wall and increased inflammatory cell infiltrate. At six months, tissue damage has considerably worsened and a greater number of focal lesions are observed. Bars represent 100 µm.

Figure 24: Development of tissue hypoxia in lung after radiation
5.2.2 Dynamic alterations among hypoxia-inducible factor subunits at the mRNA and protein levels

Total RNA from the lungs of control and irradiated mice (n=7) were pooled and changes in mRNA expression for HIF-1α, 2α, 2β, and 3α determined by RT-PCR. There were no observed changes in HIF-1α mRNA expression at the examined time points. In contrast, HIF-2α mRNA expression was elevated in irradiated mice at all time points. HIF-2β expression was upregulated in irradiated animals starting 1 week after radiation. HIF-3α mRNA expression was not upregulated until 6 months post-radiation (Figure 25).

Western blot analysis suggests a dynamic role for HIF-1α, -2α, and -3α. Although no increase in HIF-1α mRNA was observed, assessment of protein expression showed an increase in stabilization of the alpha subunit 24 hours after radiation. However, no increase in HIF-1 α stability was seen among all other time points examined until 6 months post-irradiation. Interestingly, at six months, HIF-1α expression is reduced relative to untreated controls (Figure 26A). HIF-2α expression was elevated 24 hours post-irradiation and again 6 months following radiation (Figure 26B). No difference in HIF-3α protein was observed at any point following radiation (not shown).
Figure 25: Temporal changes in hypoxia-inducible factor mRNA expression after sham- or 15 Gy thoracic irradiation in C57BL/6J mice

The amplified PCR fragments were visualized on 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. The GAPDH and HIF genes were amplified in the same reaction. The top band shows a 1 kb GAPDH fragment and the bottom band shows the HIF gene fragment. C = Control, R = Radiation (15 Gy to the whole thorax); Time points shown are one day (1 d), three days (3 d), one week (1 wk), three weeks (wk), six weeks (wk), and six months (6 m). M is the molecular ladder.
Figure 26: Dynamic changes in HIF-1α (A) and HIF-2α (B) proteins after radiation.

C57BL/6J mice were irradiated to the whole thorax (WTLI) with a single dose of 15 Gy. Sham-irradiated mice were included as controls. Protein levels for HIF-1α (A), HIF-2α (B), and HIF-3α (not shown) were assessed between 1 day and 6 months post-WTLI. Western blot bands were normalized to α-tubulin expression. The relative intensities were averaged within groups. Irradiated animals were compared to age- and time-matched controls. * p < 0.05 vs. time-matched control; ** p < 0.01 vs. time matched control.

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5.2.3 Temporal expression of hypoxia associated genes

Tissue hypoxia and hypoxia-associated gene expression was assessed in lung tissue collected at multiple pre-determined time points between twenty-four hours and six months following thoracic irradiation with a single dose of 15 Gy in C57BL/6J mice. Age-matched, non-irradiated mice were included as controls.

Twenty-four hours after radiation, messenger RNA expression of Adm, Agpat2, Ard1, CTGF, Eno1, HIF-2α, Gna11, Prkaa1, Sdh1, and Tubα3 was elevated in irradiated animals relative to age-matched, non-irradiated controls. Expression of these genes increased throughout the six-month follow-up period. An increase in Agtpbp1, Angptl4, HIF-2β, Nmyc1, Ppar-α, and Th expression was observed one week following irradiation and remained elevated in all later time points. Messenger RNA expression of two genes, Cygb and Dapk3, demonstrated a biphasic increase beginning one day after radiation that briefly returned to normal before peaking again 6 weeks and 6 months after radiation. Dctn2, Gap43, Man2b1, Rora, and Rps7 transcript levels were elevated 3 days and 6 weeks post-radiation (during the “clinically latent period”). At six weeks post-irradiation, increased transcription of Car12, Cdc42, Eef1a1, Htatip, IL-1β, Lep, Lipe, Mmp14, Plod3, and TGF-β1 was observed in all irradiated animals. Expression of Dr1, Fabp4, Fnbp3 and Slc2α1 were not found to be elevated during the latent period, but instead were expressed only once histopathologic damage became apparent (6 months after radiation) (Table 9).
Table 9: Temporal expression of selected hypoxia associated genes

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Timing of Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Damage/Repair /Cell Death</td>
<td>Dapk3</td>
<td>Death associated kinase 3</td>
<td>Early, Late</td>
</tr>
<tr>
<td></td>
<td>Cdc42</td>
<td>Cell division 42 homolog</td>
<td>Intermediate; Late</td>
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<tr>
<td></td>
<td>Dr1</td>
<td>Downregulation of transcription 1</td>
<td>Late</td>
</tr>
<tr>
<td>Oxidative Stress</td>
<td>Cygb</td>
<td>Cytoglobin</td>
<td>Early, Late</td>
</tr>
<tr>
<td></td>
<td>Sdh1</td>
<td>Succinate dehydrogenase 1</td>
<td>Early, Intermediate, Late</td>
</tr>
<tr>
<td></td>
<td>Plod3</td>
<td>Procollagen-lysine, 2-oxoglutarate 5 dioxygenase 3</td>
<td>Late</td>
</tr>
<tr>
<td></td>
<td>Th</td>
<td>Tyrosine hydrolase</td>
<td>Intermediate, Late</td>
</tr>
<tr>
<td>Fibrogenesis</td>
<td>TGF-beta</td>
<td>Transforming growth factor beta</td>
<td>Intermediate, Late</td>
</tr>
<tr>
<td></td>
<td>MMP14</td>
<td>Matrix metalloproteinase 14</td>
<td>Intermediate, Late</td>
</tr>
<tr>
<td></td>
<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
<td>Early, Intermediate, Late</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Il1b</td>
<td>Interleukin 1 beta</td>
<td>Intermediate, Late</td>
</tr>
<tr>
<td></td>
<td>Lep</td>
<td>Leptin</td>
<td>Intermediate, Late</td>
</tr>
<tr>
<td></td>
<td>Ppara</td>
<td>Peroxisome proliferator activated receptor-alpha</td>
<td>Early, Intermediate, Late</td>
</tr>
</tbody>
</table>

* Modified from Jackson et al.170; Early = 1 & 3 days, 1 week; Intermediate= 3 & 6 weeks; Late = 6 months.
5.3 Discussion

Tissue hypoxia plays a central role in the progression of radiation-induced lung injury. However, the pathogenic mechanisms that are driven by the development of tissue hypoxia in the irradiated lung are not clear. In this study, we evaluated time-dependent changes among genes whose expression is influenced by tissue hypoxia. We then sought to determine whether the introduction of a potent antioxidant, AEOL10150, during the first four weeks post-irradiation could mitigate expression of those genes six weeks into the course of disease development (results not shown).

One of the most interesting findings in the current study was the contrast in mRNA expression between hypoxia-inducible factor-1 alpha (HIF-1α) and HIF-2α following radiation. Hypoxia-inducible factors (HIF) are one of the most prominent gene families regulated by hypoxia. The HIF proteins are transcriptional complexes composed of alpha and beta subunits. When stabilized, they can activate more than 40 downstream genes involved in cellular response to stress. The alpha subunit of the HIF complex is the main hypoxia sensor and is rapidly degraded (half-life <5 minutes) by the proteasome under normoxic conditions. However, the alpha-subunit is stabilized in the absence of oxygen or the presence of oxidative/nitroxidative stress. Once stabilized the alpha subunit is free to associate with its binding partner, HIF-1β to form an activated transcriptional complex.
HIF-1α and HIF-2α are structurally similar and both bind hypoxia response elements in their target genes\textsuperscript{174}. However, whereas HIF-1α is ubiquitously expressed, HIF-2α expression is cell-specific\textsuperscript{174}. Although no increase in HIF-1α mRNA was observed in irradiated lung tissue at any of the time points evaluated, HIF-2α mRNA was strongly increased as early as 24 hours post-radiation. An increase in protein stabilization of both HIF-1α and HIF-2α subunits was observed at twenty-four hours. However, whereas HIF-1α returned to basal levels until six months post-radiation when expression declined, HIF-2α remained elevated at multiple time points throughout the follow-up period.

HIF-2α has been associated with endothelial cell ultrastructural changes, increased vascular permeability with focal inflammation, and hypertension\textsuperscript{175}. In addition, HIF transcription factors have also been reported to regulate iron homeostasis during times of low oxygenation\textsuperscript{176}. This is important as in previous studies (Figure 18) we found differential expression of genes involved in iron homeostasis (Hfe) and heme biosynthesis (ALAD). In those studies, Hfe was elevated after radiation in C57BL/6J mice whereas ALAD remained near or below basal levels.

Mastrogiannaki et al have found HIF-2α facilitates high iron absorption rates through upregulation of genes involved in intestinal iron import using a murine model of hemachromatosis\textsuperscript{176}. If this is true, then this would seem to support the hypothesis that chronic upregulation of HIF-2α in the lungs of C57BL/6J mice after irradiation leads to a disruption in the iron equilibrium. How this influences the development of lung damage
after irradiation is unclear. However, studies by Chandler et al. found iron-deficient mice did not exhibit collagen accumulation or develop fibrosis in response to bleomycin treatment in comparison to their iron-replete counterparts\(^1\). Moreover, significantly elevated iron levels have been found in patients with idiopathic pulmonary fibrosis\(^3\) and in bilateral lung transplant patients\(^4\). Moreover, Zakynthinos et al. reported iron overload and interstitial fibrosis in a patient with thalassaemia major\(^5\). Taken together, this would suggest that modulation of iron homeostasis by HIF-2\(\alpha\) after radiation may contribute to the development of pulmonary fibrosis in this strain.

It is well known that macrophages accumulate around hypoxic regions where they are activated to secret HIF-1\(\alpha\) and HIF-2\(\alpha\)\(^6\), as well as pro-inflammatory cytokines, chemokines, and growth factors\(^7,\ 8,\ 9\). Interleukin-1\(\beta\) (IL-1\(\beta\)), a cytokine produced by activated macrophages, is an important mediator of cell proliferation, differentiation, and apoptosis as part of the inflammatory response\(^10\). The gene encoding IL-1\(\beta\), as well as those encoding fatty acid binding protein 4 (Fabp4) and Leptin, both of which are involved in inflammatory response, were all upregulated at six weeks after radiation. TGF-\(\beta\)1 and matrix metalloproteinase-14 (MMP-14), which are involved in collagen deposition and fibrogenesis were also upregulated in this time frame. These later changes are indicative of initiation of late injury disease processes.

While tissue hypoxia can influence the expression of a number of genes, the cellular and physiological effects that lead to tissue hypoxia are not well known. Using a
hemithoracic model of irradiation in rats (28 Gy), Fleckenstein et al. found a transient decrease in perfusion three days after radiation followed by a progressive and steep decline between two to 10 weeks\textsuperscript{70}. In that study, hypoperfusion was accompanied by an increase in the number of activated macrophages, oxidative DNA damage, and tissue hypoxia\textsuperscript{70}.

It is plausible to hypothesize that these early changes in tissue perfusion develop as a result of endothelial cell damage and capillary obstruction. At the ultrastructural level, persistent endothelial cell swelling and cytoplasmic vacuolation leading to obstruction of the capillary lumen has been observed in the irradiated rat lung during the first week post-exposure (20 Gy)\textsuperscript{187}. Furthermore, during the first few hours to days after radiation, there is an increase in edema, fibrin deposition, and epithelial denudation\textsuperscript{44}. A number of other studies have also shown endothelial cells to be a primary target of radiation\textsuperscript{188,189}.

At later times, tissue hypoxia likely arises from a combination of congestion, obstructive bronchiolitis, and the complete fibroobliteration of the alveoli and small vessels. When small volumes of lung are irradiated, the tissue compensates through shunting of the blood resulting in hyperperfusion in the non-irradiated portions.

The timing of alveolar epithelial cell and endothelial cell damage appears to be species/strain and radiation-dose-specific. Overall, the C57BL/6J strain appears to be an “exceptional” model in a number of disease types, including the development of
pulmonary toxicity after radiation exposure\textsuperscript{89, 90, 100, 114}. In a comparison of pulmonary radiation tolerance among C57BL/6J, CBA/J, and C57L/J mouse strains, we found the radiation dose to cause morbidity/mortality in the C57BL/6J strain was significantly higher than the dose to cause similar injury in C57L/J and CBA/J mice (Table 4). The question is then, what makes the lungs of C57BL/6J mice exceptional?

Few studies have looked at ultrastructural changes in the irradiated mouse lung. In our recent study (Figure 13), we found evidence of interstitial edema and mild endothelial and epithelial cell swelling after radiation in the C57BL/6J mouse strain twenty-four hours after radiation. However, this was far less severe than that observed in more sensitive murine strains (CBA and C57L). Reportedly, the C57BL/6J mouse strain demonstrates a greater resistance to ischemia/reperfusion injury than other strains\textsuperscript{122}. This may be due to their ability to maximize oxygen efficiency under hypoxic conditions that allows them to live 10 times longer than their outbred counterparts when exposed to acute hypoxia\textsuperscript{190}.

Ward et al. evaluated pulmonary endothelial dysfunction and collagen deposition among four murine strains exposed to radiation, including the C57BL/6J strain\textsuperscript{191}. In those studies, the CBA and C3H strains, neither of which develop extensive pulmonary fibrosis, had significantly higher levels of angiotensin converting enzyme (ACE) and plasminogen activator (PLA) in comparison to the fibrosis-prone C57BL/6J and C57BL/10J strains both before and after radiation. The authors concluded that
vascular dysfunction played a role in radiation pneumotoxicity and higher levels of ACE activity and plasminogen activator likely resulted in protection against fibrosis and hyaline membrane formation\textsuperscript{191}.

Moulder, Medhora and colleagues have evaluated the ability of ACE inhibitors, including captopril and enalapril, to attenuate fibrosis primarily in rat models of radiation-induced nephropathy, but also in irradiated lung\textsuperscript{119, 152, 192-194}. The investigators have recently shown captopril can successfully mitigate tachypnea in irradiated rats when given up to one week after radiation\textsuperscript{119}. However, Wang and Anscher performed a retrospective study in 213 patients treated for lung cancer with thoracic irradiation from 1994-1997\textsuperscript{195} and found the incidence and onset of radiation pneumonitis among patients taking ACE inhibitors (captopril, benazepril, enalapril, lisinopril, quinapril or fosinopril) for hypertension was not significantly different than those who were not on ACE inhibitors\textsuperscript{195}. Thus, it remains unclear whether the mitigating effect of ACE inhibitors in preclinical models will translate to the clinic.

In conclusion, upregulation of hypoxia-associated genes begins following radiation and continues at least throughout the first six-months post-exposure. These genes participate in a variety of physiological and pathological functions, including hypoxia response, inflammation, oxidative stress, transcriptional regulation, signal transduction, cell metabolism, proliferation and differentiation, and apoptosis. Radiation-induced overexpression of 30 of 31 hypoxia-related genes was suppressed six
weeks after radiation following four weeks of post-radiation administration of AEOL10150, a potent catalytic antioxidant. It has been previously demonstrated that AEOL10150 can attenuate development of late radiation-induced pulmonary injury in rats following single dose or fractionated radiation. A relationship between early signaling changes and late injury would support the initiation of therapeutic interventions in the first few weeks post-exposure in order to protect the lungs from developing acute and chronic lung damage. This may not be the only therapeutic option, however, as the biphasic wave of gene expression observed in this study and in previous studies suggests that there may be a larger window of opportunity for intervening in the progression of disease processes. More importantly to this study, however, was that the observed reversal of radiation-induced gene expression following treatment with AEOL10150 suggests that these genes may be involved in the cellular response to oxidative stress or the generation of ROS/RNS. Identifying the roles of these genes in pulmonary radiation injury could provide insight into the role of hypoxia-associated genes in radiation lung injury, widen the temporal window for therapeutic interventions, and introduce novel therapeutic targets.

2 ILJ is a consultant for AEOLUS Pharmaceuticals Inc., Mission Viejo, CA
6. Summary

In this proposal, we developed a pre-clinical research platform to evaluate the biologic effects of radiation on healthy lung tissue. Initially we characterized and validated the pulmonary dose-response to thoracic irradiation using three murine strains (Figure 6). The dose-response for survival, functional injury, and histopathologic damage varied among strains. We found the lethal dose for 50% of C57L/J mice within the first 180 days post-thoracic irradiation (LD50/180) was quite similar to the LD50/180 for non-human primates (NHP) and the tolerated dose for 50% of individuals (TD50/180) in humans exposed to thoracic irradiation (Table 4). In contrast, the LD50/180 for CBA and C57BL/6J strains was approximately 3 Gy higher than that for C57L/J mice, NHPs, and humans (Table 4).

The next step was to compare ultrastructural damage and differential gene expression in the lung tissue of C57L/J, CBA/J, and C57BL/6J mice twenty-four hours after thoracic irradiation with one of three doses, 0, 12.5 or 15 Gy. Epithelial denudation of the airways was observed in C57L/J and CBA/J mice, but was not seen in C57BL/6J mice (Figure 13). Epithelial and endothelial cell swelling were observed in C57L/J and CBA/J mice and to a lesser extent in C57BL/6J mice. Swelling of the endothelial cells resulted in vascular occlusion in C57L/J mice. Lethal cell injury, necrosis, and inflammation were observed in C57L/J mice. Interstitial edema was the prominent feature in C57BL/6J mice. Ultrastructural changes were consistent with differential gene
expression. For the purposes of microarray data analysis, C57L/J and CBA/J mice were classified as “acute” responders due to their early onset of lethal pneumonitis (18-22 weeks) and C57BL/6J mice were classified as “late” responders due to their long latency period and development of only mild pneumonitis and fibrosis in approximately 5-10% of the lung by twenty-six weeks post-radiation. Ingenuity pathway analysis identified the top functional pathways differentially expressed between acute and delayed responders to be related to DNA repair/cell survival/death and oxidative stress (Figure 16). The most significant differences in gene expression were related to acute phase response and iron homeostasis (Tables 6 and 7). Quantitative real-time polymerase chain reaction (qRT-PCR) showed considerable correlation with the microarray results, although not all differences were statistically significant. Only four genes showed no changes in gene expression before or after radiation or between strains. These were RPS9, SLC15A2, ANG, and PAIP1.

Quantitative RT-PCR showed a significant increase in ORM1 (gene encoding alpha-1 acid glycoprotein) mRNA expression in pneumonitis-prone C57L/J and CBA/J mice in comparison to C57BL/6J mice. Protein expression of ORM1 did not correlate with mRNA expression but this may be due to the delay in translation of the protein. Further studies are warranted at later time points to clarify the results. Since the ultimate goal is translation of these findings to radiation effects on human lung, a literature search was performed. Jakobsson et al. found alpha-1 acid glycoprotein levels were
elevated in patients experiencing GI toxicity after radiotherapy for anal or uterine carcinomas (Table 8).

In a separate cohort of mice, tissue inflammation (inflammatory cell subsets and cytokines in whole lung) was evaluated and found to be significantly different among strains fourteen weeks post-WTLI (Figures 22 and 23). Interleukin (IL)-1alpha and IL-12 (p40) were significantly elevated in C57L/J mice compared to C57BL/6J. In contrast, C57BL/6J mice displayed higher expression of keratinocyte chemoattractant (KC; IL-8 in humans) in whole lung tissue. C57L/J had an increase in almost all inflammatory cell types evaluated in whole lung tissue, but the most significant differences were observed in B and T-cells (both CD4+ and CD8+) when compared to C57BL/6J mice. CBA/J mice had a significant increase in natural killer (NK) cells when compared to C57BL/6J and CD8+ T-cells. Although the highest number of NK cells was observed in C57L/J mice, the variability was too large to be significant when compared to either CBA/J or C57BL/6J.

The development of an exaggerated inflammatory response in C57L/J mice fourteen weeks post-radiation is consistent with IPA pathway analysis of strain-dependent differences in functional pathways within one-day post-radiation. C57L/J mice displayed differences in pro-inflammatory pathways such as toll like receptor and tumor necrosis factor-receptor signaling. C57BL/6J and CBA/J mice also showed consistencies between gene expression at twenty-four hours and the phenotypic of lung damage at fourteen weeks post-exposure.
Lastly, the natural history of radiation progression was evaluated in fibrosis-prone C57BL/6J mice\textsuperscript{170}. Hypoxia was observed in lung tissue as early as three days post-WTLI using exogenous and endogenous markers of hypoxia. As hypoxia (as well as oxidative stress) is known to stabilize the alpha subunit of the hypoxia-inducible factor (HIF) family of transcription factors, protein and mRNA expression were evaluated in lung tissue at 1 day, 3 days, 1 week, 3 weeks, 6 weeks, and 6 months post-exposure. Dynamic fluctuations were observed in protein stabilization. No differences were observed in HIF-1\(\alpha\) mRNA expression at any of the time points evaluated, whereas HIF-2\(\alpha\) mRNA remained elevated at all time points post-radiation.

HIF is known to participate in regulation of iron homeostasis. In the microarray experiments performed above, two of the top twenty genes differentially expressed between acute and delayed responders are involved in iron homeostasis (HFE, ALAD). Furthermore, iron deficiency has been shown to correspond to a reduction in pulmonary fibrosis in fibrosing pathologies (e.g. bleomycin-induced fibrosis)\textsuperscript{177}.

As C57BL/6J mice have much more delayed pulmonary response to radiation and require higher doses to cause lethal lung injury, it is important to understand the differences between this relatively radioresistant strain and other, more radiosensitive strains (e.g. CBA/J; C57L/J). C57BL/6J mice have been shown to better adapt to hypoxic conditions than other strains of mice. A pathway-focused oligo array was used to evaluate temporal changes in hypoxia-associated gene expression at each of the
aforementioned time points. Many of the genes upregulated in response to radiation are known to be involved in maintenance of cellular redox balance (Cygb, Sdh1, Plod3, Th), DNA/repair (Dapk3, Cdc42, Dr1), inflammation (Il1b, Ppara), and fibroproliferation (TGFb, MMP14, CTGF) (Table 9). Many of these genes may be important to evaluate in the future, including Cygb (gene encoding Cytoglobin), which has been shown to be protective against fibrosis\textsuperscript{198}.

Treatment with AEOL10150, a potent antioxidant mimetic, mitigated the expression of many of the genes found to be differentially expressed when treatment was started two hours after radiation and given thereafter for four weeks. However, gene expression in this cohort of mice was evaluated only at one time point, six weeks. Therefore, it would be interesting to see if this effect is continued throughout the time course to disease progression\textsuperscript{170}.

In the end, these studies developed a pre-clinical research platform that was utilized to evaluate the effects of radiation on gene expression among three murine strains with heterogeneous latency and phenotypic expression of lung injury. The platform was then used to characterize the pathogenesis of radiation-induced lung injury in fibrosis-prone C57BL/6J mice.

Designing a well-characterized and validated pre-clinical model of radiation-induced lung injury is critical for ensuring the successful translation of scientific knowledge to improvement in clinical outcomes. Understanding the mechanisms of
radiation-induced lung injury will help to identify novel therapeutic targets, design new treatment strategies, and select a multi-drug approach to successfully prevent, mitigate, and/or treat lung injury following accidental or deliberate radiation overexposure.
7. Future directions and concluding remarks

The purpose of the current project was to develop a research platform for the study of radiation effects on healthy lung tissue. The platform was designed to comply with the FDA “Animal Efficacy Rule” for radiation countermeasure (MCM) licensure.

In the first part of the project, we compared the pulmonary radiotolerance among three commonly used murine strains for pre-clinical radiobiological research. The radiation dose-response for survival, functional, and histopathologic damage among strains was compared to data generated from a identically treated non-human primate (NHP) model at the University of Maryland-Baltimore and published literature for human lung. The dose-response, temporal onset, and pathology associated with acute thoracic irradiation in C57L/J mice recapitulated the pathogenesis of radiation-induced lung injury in NHP and human lungs. In contrast, the lungs from C57BL/6J mice were found to display exceptional radiotolerance when compared the lungs of C57L/J mice, NHP, and human.

Although the lungs of C57L/J mice closely reflect the radiotolerance of NHP and human lung, there is considerable heterogeneity in the development of radiation-induced lung injury among individuals exposed to thoracic irradiation. Due to the lack of genetic variability among inbred mice, it would be prudent to use more than one strain in pre-clinical radiobiological research studies.
In the second part of this project, we exploited the phenotypic differences in pulmonary injury among murine strains to identify genes and pathways likely involved in either radiation pneumonitis or fibrosis. Using gene expression profiling, we identified two acute phase proteins to be significantly differentially expressed between pneumonitis prone (CBA/J & C57L/J) and non-pneumonitis prone (C57BL/6J) mice. There is ample data from both clinical experience as well as non-human primate models to suggest acute phase proteins may in fact play a role in directing the early tissue response to radiation. Using serine protease inhibitors and/or gene-directed mutagenesis, we can explore the relationship between acute phase proteins and the development of radiation pneumonitis in future studies.

In the third part of this study, we evaluated temporal changes in tissue hypoxia and hypoxia-associated genes between twenty-four hours and six months post-radiation in “fibrosis-prone” C57BL/6J mice. For MCM approval, the FDA would like to see MCM effects on specific biological markers associated with the development of radiation injury. In this study, we found scavenging reactive oxygen/nitrogen species with a potent antioxidant during the first four weeks post-radiation could ameliorate hypoxia-associated gene expression six weeks after radiation and two weeks after treatment was stopped. Interestingly, dynamic changes were observed in HIF-1α and HIF-2α. HIF proteins are known to participate in cellular adaption to hypoxia and regulate iron homeostasis. Two of the genes found to be differentially expressed in the gene
expression studies were associated with regulation of iron uptake and tissue
distribution.

C57BL/6J mice are unique in their radiotolerance. The dose-response for lethality
is significantly higher than other murine strains and they have evolved compensatory
mechanisms to improve survival during times of low oxygenation. Disruption of iron
homeostasis has been shown to correlate with idiopathic pulmonary fibrosis and other
fibrotic lung disorders. Therefore, evaluating iron regulation among these three murine
strains may help to elucidate the mechanisms associated with differential tissue
response to the radiation insult. Studying the unique mechanisms underlying C57BL/6J
radioresistance to pneumonitis may enable identification of novel therapeutic targets.

In the broad picture, gene expression profiles may assist in identification of
biomarkers for organ-specific injury. The attempt to identify reliable biomarkers of
radiation pneumonitis/fibrosis in the clinical setting has not been successful and
currently there is no consensus on whether any of the cytokines or growth factors
previously identified correlate to development of lung injury\textsuperscript{130,199}. Gene expression
profiling may identify novel genes with secreted products that can be used as
biomarkers to predict individual risk for development of radiation-induced lung injury.
Such biomarkers may be used as “triggers-to-treat” following acute radiation exposure
or for patient-stratified dose escalation in clinical radiotherapy. One of the primary
impediments to successful eradication of tumors and improved survival in cancer
patients is the risk for development of normal tissue injury. Therefore, genes identified in this study with secreted products, such as VEGFC or TSHB, may be useful in clinical radiotherapy. To be sure, MCMs developed under the FDA Animal Efficacy Rule for use in radiation emergencies can be further developed for clinical use.

Lastly, the pre-clinical model developed in this project is an organ-specific model. It will be utilized to screen medical countermeasure efficacy for preventing, mitigating, and/or treating radiation-induced lung injury following thoracic irradiation without the complications of hematopoietic, GI, or other organ system toxicity. A “real world” model of total body irradiation with 5% bone marrow shielding (PBI/BM5) is being developed outside of the scope of this project. In a radiation accident or attack, individual exposure will be heterogeneous in the majority of cases. In such cases, the thorax may receive the highest radiation dose. The PBI/BM5 model allows enough bone marrow survival for the animal to proceed through each of the organ-specific sequelae following acute radiation exposure. This model can be used to assess drug interactions and effects of candidate MCMs on other organ systems. It can also be used to develop a treatment regimen involving a multi-drug approach for use in a radiation emergency.

In the end, the current project has laid a foundation for future radiation research. It has characterized three murine models of lung injury that can be used consistently and reproducibly to elucidate the mechanisms underlying radiation-induced lung injury. It has been utilized in this project to identify novel pathways that can be further
evaluated for their role in the pathogenesis of radiation lung injury. Finally, the models will be used to screen medical countermeasures under the guidelines of the FDA Animal Rule in a Good Laboratories Compliant (GLP) facility. The model is applicable not only to acute radiation exposure but also in clinical radiotherapy where normal tissue injury continues to pose a major limitation to effective local control and overall survival.
## Appendix A

### Table 10: Radiation incidents resulting in fatalities from 1900-present

<table>
<thead>
<tr>
<th>Year, Location</th>
<th>Incident</th>
<th>No. Fatalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1945, Hiroshima (Japan)</td>
<td>Atomic bomb; military conflict</td>
<td>130,000*</td>
</tr>
<tr>
<td>1945, Nagasaki (Japan)</td>
<td>Atomic bomb, military conflict</td>
<td>30,000</td>
</tr>
<tr>
<td>1945, Los Alamos (USA)</td>
<td>Criticality accident (plutonium)</td>
<td>1 injured; 1 fatality</td>
</tr>
<tr>
<td>1946, Los Alamos (USA)</td>
<td>Criticality accident (plutonium)</td>
<td>2 injured; 1 fatality</td>
</tr>
<tr>
<td>1950-1968, Chelyabinsk/Mayak (USSR)</td>
<td>Multiple nuclear reactor accidents</td>
<td>60+ injured; 4+ fatalities</td>
</tr>
<tr>
<td>1954, Marshall Islands</td>
<td>Nuclear fallout, atmospheric nuclear test conducted by U.S.A.</td>
<td>93+ injured; 1 fatality</td>
</tr>
<tr>
<td>1954, Sarov (USSR)</td>
<td>Accidental exposure (polonium-210)</td>
<td>2 injured; 1 fatality</td>
</tr>
<tr>
<td>1954, Totsk (USSR)</td>
<td>Nuclear fallout, atmospheric nuclear test conducted by USSR</td>
<td>Unknown</td>
</tr>
<tr>
<td>1957, Chelyabinsk (USSR)</td>
<td>Explosion in stored nuclear wastes</td>
<td>Unknown</td>
</tr>
<tr>
<td>1958, Vinca (Yugoslavia)</td>
<td>Criticality accident (uranium)</td>
<td>5 injured; 1 fatality</td>
</tr>
<tr>
<td>1958, Los Alamos (USA)</td>
<td>Criticality accident (plutonium)</td>
<td>1 fatality</td>
</tr>
<tr>
<td>1960, USSR</td>
<td>Ingestion</td>
<td>1 fatality</td>
</tr>
<tr>
<td>1960, Moscow (USSR)</td>
<td>Intentional overexposure (suicide)</td>
<td>1 fatality</td>
</tr>
<tr>
<td>1961, Switzerland</td>
<td>Radiation contamination</td>
<td>1 fatality</td>
</tr>
<tr>
<td>1962, Mexico City (Mexico)</td>
<td>Lost Co&lt;sup&gt;60&lt;/sup&gt; industrial radiography source</td>
<td>1 injured; 4 fatalities</td>
</tr>
<tr>
<td>1963, Sanlian (China)</td>
<td>Orphaned source</td>
<td>4 injured; 2 fatalities</td>
</tr>
<tr>
<td>1964, Rhode Island (USA)</td>
<td>Criticality accident (uranium)</td>
<td>1 fatality</td>
</tr>
<tr>
<td>1964, F.R. Germany</td>
<td>Radiation contamination</td>
<td>3 injured; 1 fatality</td>
</tr>
<tr>
<td>1968, Barents Sea (USSR)</td>
<td>Reactor coolant leak; partial meltdown</td>
<td>83 injured; 9 fatalities</td>
</tr>
<tr>
<td>1968, Wisconsin (USA)</td>
<td>Radiotherapy accident</td>
<td>1 fatality</td>
</tr>
<tr>
<td>1969, Novaya Zemlya (USSR)</td>
<td>Underground nuclear test</td>
<td>Unknown</td>
</tr>
<tr>
<td>1970, Sormovo (USSR)</td>
<td>Radiation accident</td>
<td>2 injured; 3 fatalities</td>
</tr>
<tr>
<td>1972, Bulgaria</td>
<td>Intentional overexposure (suicide)</td>
<td>1 fatality</td>
</tr>
<tr>
<td>1974-1976, Ohio (USA)</td>
<td>Radiotherapy accident</td>
<td>78 injured; 10 fatalities</td>
</tr>
<tr>
<td>Year</td>
<td>Location</td>
<td>Event Description</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>1975</td>
<td>Sverdlovsk (USA)</td>
<td>Accidental exposure to source</td>
</tr>
<tr>
<td>1978</td>
<td>Setif (Algeria)</td>
<td>Lost Ir-192 radiography source</td>
</tr>
<tr>
<td>1978</td>
<td>Pacific Ocean (USSR)</td>
<td>Reactor accident aboard submarine</td>
</tr>
<tr>
<td>1980</td>
<td>Leningrad (USSR)</td>
<td>Radiation accident at sterilization facility</td>
</tr>
<tr>
<td>1980</td>
<td>Yuzho-Sakhalinsk (USSR)</td>
<td>Accidental exposure to source</td>
</tr>
<tr>
<td>1980</td>
<td>Setif (Algeria)</td>
<td>Lost Ir-192 radiography source</td>
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<tr>
<td>1980</td>
<td>Pacific Ocean (USSR)</td>
<td>Reactor accident aboard submarine</td>
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<tr>
<td>1980</td>
<td>Leningrad (USSR)</td>
<td>Radiation accident at sterilization facility</td>
</tr>
<tr>
<td>1980</td>
<td>Yuzho-Sakhalinsk (USSR)</td>
<td>Accidental exposure to source</td>
</tr>
<tr>
<td>1980</td>
<td>Houston, TX (USA)</td>
<td>Radiotherapy accident</td>
</tr>
<tr>
<td>1981</td>
<td>Tusla, OK (USA)</td>
<td>Intentional self exposure</td>
</tr>
<tr>
<td>1982</td>
<td>Kramatorsk (Ukraine)</td>
<td>Accidental exposure to Ce-137 source</td>
</tr>
<tr>
<td>1982</td>
<td>Kjeller (Norway)</td>
<td>Industrial irradiator accident</td>
</tr>
<tr>
<td>1982</td>
<td>Baku (Azerbaijan)</td>
<td>Orphaned Ce-137 source</td>
</tr>
<tr>
<td>1983</td>
<td>Constituyentes (Argentina)</td>
<td>Criticality accident</td>
</tr>
<tr>
<td>1983-1984</td>
<td>Ciudad Juarez (Mexico)</td>
<td>Orphaned Co-60 source</td>
</tr>
<tr>
<td>1984</td>
<td>Casablanca (Morocco)</td>
<td>Lost Ir-192 radiography source</td>
</tr>
<tr>
<td>1985</td>
<td>Chazhma Bay (USSR)</td>
<td>Reactor accident during refueling of submarine</td>
</tr>
<tr>
<td>1985</td>
<td>China</td>
<td>Radiotherapy accident</td>
</tr>
<tr>
<td>1986</td>
<td>Texas (USA)</td>
<td>Radiotherapy accident</td>
</tr>
<tr>
<td>1986</td>
<td>Chernobyl (Ukraine)</td>
<td>Explosion at nuclear power plant</td>
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<tr>
<td>1987</td>
<td>Yakima, WA (USA)</td>
<td>Radiotherapy accident</td>
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<tr>
<td>1987</td>
<td>Goiania (Brazil)</td>
<td>Lost Ce-137 radiography source</td>
</tr>
<tr>
<td>1989</td>
<td>El Salvador</td>
<td>Industrial irradiator accident (Co-60)</td>
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<td>1990</td>
<td>Shanghai (China)</td>
<td>Industrial irradiator accident (Co-60)</td>
</tr>
<tr>
<td>1990</td>
<td>Soreq (Israel)</td>
<td>Industrial irradiator accident (Co-60)</td>
</tr>
<tr>
<td>1990</td>
<td>Zaragoza (Spain)</td>
<td>Radiotherapy accident</td>
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<tr>
<td>1991</td>
<td>Nesvizh (Belarus)</td>
<td>Industrial irradiator accident (Co-60)</td>
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<td>1992</td>
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<td>1993</td>
<td>Moscow (Russia)</td>
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<td>Year</td>
<td>Location</td>
<td>Incident Type</td>
</tr>
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<td>-----------</td>
<td>-------------------</td>
<td>---------------------------------------------------</td>
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<tr>
<td>1994</td>
<td>Tammiku (Estonia)</td>
<td>Stolen source</td>
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<td>1995</td>
<td>Moscow (Russia)</td>
<td>Radiological homicide</td>
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<tr>
<td>1996</td>
<td>San Jose (Costa Rica)</td>
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<td>1997</td>
<td>Sarov (Russia)</td>
<td>Criticality accident (uranium)</td>
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<tr>
<td>1997</td>
<td>Republic of Georgia</td>
<td>Lost Co-60 source</td>
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<tr>
<td>1999</td>
<td>Tokai-mura (Japan)</td>
<td>Criticality accident (uranium)</td>
</tr>
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<td>1999</td>
<td>Leningrad (Russia)</td>
<td>Orphaned radioisotope source</td>
</tr>
<tr>
<td>2004</td>
<td>St. Petersburg (Russia)</td>
<td>Radiological homicide</td>
</tr>
<tr>
<td>2004</td>
<td>Lyon (France)</td>
<td>Radiotherapy overexposure</td>
</tr>
<tr>
<td>2004-2005</td>
<td>Epinal (France)</td>
<td>Radiotherapy overexposure</td>
</tr>
<tr>
<td>2006</td>
<td>London (UK)</td>
<td>Radiological homicide</td>
</tr>
<tr>
<td>2010</td>
<td>New Delhi (India)</td>
<td>Lost Co-60 source</td>
</tr>
</tbody>
</table>

*Information found in the Database of Radiological Incidents and Related Events*
### Appendix B

**Table 11: RT-PCR primers and product size**

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Primer Sequence</th>
<th>Fragment size (bp)</th>
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<tbody>
<tr>
<td>Adm-F</td>
<td>5’-AGCTGGTTTCCATCACCCTG-3’</td>
<td>502</td>
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<tr>
<td>Adm-R</td>
<td>5’-CCTGGGAGGACTCCACAGTC-3’</td>
<td></td>
</tr>
<tr>
<td>Angpt14-F</td>
<td>5’-GTGAGGACACAGCTACAGC-3’</td>
<td>333</td>
</tr>
<tr>
<td>Angpt14-R</td>
<td>5’-AGGCTGCTGTAGCCTCCATG-3’</td>
<td></td>
</tr>
<tr>
<td>CTGF-F</td>
<td>5’-CTGCCCTACCGACTGGAAGAC-3’</td>
<td>495</td>
</tr>
<tr>
<td>CTGF-R</td>
<td>5’-ACATCTTCCCTGTAGTAGCAGG-3’</td>
<td></td>
</tr>
<tr>
<td>Eno-1a-F</td>
<td>5’-GCCAGAGAGATCTTTGACTC-3’</td>
<td>443</td>
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<tr>
<td>Eno-1a-R</td>
<td>5’-CCGTTGATCACATTGAAAGC-3’</td>
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<tr>
<td>Gna11-F</td>
<td>5’-CAGAGGCAGGAAGGTGGATC-3’</td>
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<tr>
<td>Gna11-R</td>
<td>5’-ACTCCTTCAGGTTCAGCTGC-3’</td>
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<tr>
<td>HIF-1a-F</td>
<td>5’-CTCAGTTTTGAACTAACTGGAC-3’</td>
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<tr>
<td>HIF-1a-R</td>
<td>5’-GTCGTGCTGAATAATACCAC-3’</td>
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<td>HIF-Fa-F</td>
<td>5’-CAGTGGTGACCCCAAGACGGTGTA-3</td>
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<td>HIF-2a-R</td>
<td>5’-AGCATCCGGACTGAGGCCAGA-3’</td>
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<tr>
<td>HIF-2b-F</td>
<td>5’-CAGAAACATATCCCAGATATCTC-3’</td>
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<tr>
<td>HIF-2b-R</td>
<td>5’-GGTCAGCAAAGCTCTCGATG-3’</td>
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<tr>
<td>Ppara-F</td>
<td>5’-TGAAGCCCATCTTCAGGATGC-3’</td>
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<tr>
<td>Ppara-R</td>
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<td>Rora-F</td>
<td>5’-TCATTACGTTGGAAGGCTGC-3’</td>
<td>495</td>
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<tr>
<td>Rora-R</td>
<td>5’-CCAGATGCTGTGTAGTC-3’</td>
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<td>Sord-F</td>
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<td>Sord-R</td>
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<td><strong>GAPDH-F</strong></td>
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<td><strong>GAPDH-R</strong></td>
<td>5'-TGGAGCCATGTAGGCCATG-3'</td>
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### Appendix C

#### Table 12: Descriptive statistics for all comparisons

<table>
<thead>
<tr>
<th>Category</th>
<th>Comparison</th>
<th>Total Genes (p &lt; 0.01)</th>
<th>Up Genes (&gt;20%)</th>
<th>Down Genes (&lt;20%)</th>
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<tbody>
<tr>
<td><strong>Strain</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>B6 vs. Rest</td>
<td>6111</td>
<td>1574</td>
<td>4537</td>
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<tr>
<td></td>
<td>C57 vs. Rest</td>
<td>4730</td>
<td>1137</td>
<td>3393</td>
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<tr>
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<td>CBA vs. Rest</td>
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<td>1769</td>
<td>4365</td>
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<td>B6 vs. C57</td>
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<td>1179</td>
<td>3393</td>
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<td>C57 vs. CBA</td>
<td>5209</td>
<td>1828</td>
<td>3381</td>
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<td>B6 vs. CBA</td>
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<td><strong>Radiation</strong></td>
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</tr>
<tr>
<td></td>
<td>15 Gy vs. 0 Gy</td>
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<td>1464</td>
<td>3528</td>
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<tr>
<td></td>
<td>15 Gy vs. 12.5 Gy</td>
<td>233</td>
<td>21</td>
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<tr>
<td></td>
<td>12.5 Gy vs. 0 Gy</td>
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<tr>
<td></td>
<td>15 Gy. vs. Rest</td>
<td>2142</td>
<td>395</td>
<td>1747</td>
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<tr>
<td></td>
<td>12.5 Gy vs. Rest</td>
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<td>1237</td>
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<td>0 Gy vs. Rest</td>
<td>5631</td>
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<td><strong>Response</strong></td>
<td>Acute vs. Delayed</td>
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<tr>
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<td>B6 (15 Gy) vs. B6 (12.5 Gy)</td>
<td>133</td>
<td>33</td>
<td>100</td>
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<tr>
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<td>B6 (15 Gy) vs. B6 (0 Gy)</td>
<td>2789</td>
<td>675</td>
<td>2114</td>
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<td>B6 (12.5 Gy) vs. B6 (0 Gy)</td>
<td>2427</td>
<td>598</td>
<td>1829</td>
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<td>B6 15 Gy vs. [B6 (0 Gy) and B6 (12.5 Gy)]</td>
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<td>191</td>
<td>706</td>
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<td>B6 12.5 Gy vs. [B6 (0 Gy) and B6 (15 Gy)]</td>
<td>762</td>
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<td>B6 0 Gy vs. [B6 (12.5 Gy) and B6 (15 Gy)]</td>
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<td>2045</td>
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<td>C57 (15 Gy) vs. C57 (0 Gy)</td>
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<td>829</td>
<td>1471</td>
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<td>C57 (15 Gy) vs. [C57 (0 Gy) and C57 (12.5 Gy)]</td>
<td>1019</td>
<td>249</td>
<td>770</td>
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<td>C57 (12.5 Gy) vs. [C57 (0 Gy) and C57 (15 Gy)]</td>
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<td>250</td>
<td>400</td>
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<tr>
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<td>C57 (0 Gy) vs. [C57 (12.5 Gy) and C57 (15 Gy)]</td>
<td>2501</td>
<td>1086</td>
<td>1415</td>
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<tr>
<td>Strain-Radiation Response (cont'd)</td>
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<td>--------</td>
<td></td>
</tr>
<tr>
<td>CBA (15 Gy) vs. CBA (0 Gy)</td>
<td>2957</td>
<td>1636</td>
<td>1321</td>
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</tr>
<tr>
<td>CBA (15 Gy) vs. CBA (12.5 Gy)</td>
<td>1859</td>
<td>923</td>
<td>936</td>
<td></td>
</tr>
<tr>
<td>CBA (12.5 Gy) vs. CBA (0 Gy)</td>
<td>3040</td>
<td>1114</td>
<td>1040</td>
<td></td>
</tr>
<tr>
<td>CBA (15 Gy) vs. [CBA (0 Gy) and CBA (12.5 Gy)]</td>
<td>2159</td>
<td>1119</td>
<td>1040</td>
<td></td>
</tr>
<tr>
<td>CBA (12.5 Gy) vs. [CBA (0 Gy) and CBA (15 Gy)]</td>
<td>2396</td>
<td>702</td>
<td>1694</td>
<td></td>
</tr>
<tr>
<td>CBA (0 Gy) vs. [CBA (12.5 Gy) and CBA (15 Gy)]</td>
<td>3344</td>
<td>1330</td>
<td>2014</td>
<td></td>
</tr>
<tr>
<td>B6 (0 Gy) vs. C57 (0 Gy)</td>
<td>1420</td>
<td>646</td>
<td>774</td>
<td></td>
</tr>
<tr>
<td>B6 (0 Gy) vs. CBA (0 Gy)</td>
<td>2368</td>
<td>1238</td>
<td>1130</td>
<td></td>
</tr>
<tr>
<td>C57 (0 Gy) vs. CBA (0 Gy)</td>
<td>2923</td>
<td>1433</td>
<td>1490</td>
<td></td>
</tr>
<tr>
<td>B6 (12.5 Gy) vs. C57 (12.5 Gy)</td>
<td>2561</td>
<td>778</td>
<td>1783</td>
<td></td>
</tr>
<tr>
<td>B6 (12.5 Gy) vs. CBA (12.5 Gy)</td>
<td>4152</td>
<td>1913</td>
<td>2239</td>
<td></td>
</tr>
<tr>
<td>C57 (12.5 Gy) vs. CBA (12.5 Gy)</td>
<td>3438</td>
<td>1810</td>
<td>1628</td>
<td></td>
</tr>
<tr>
<td>B6 (15 Gy) vs. C57 (15 Gy)</td>
<td>2546</td>
<td>984</td>
<td>1562</td>
<td></td>
</tr>
<tr>
<td>B6 (15 Gy) vs. CBA (15 Gy)</td>
<td>4714</td>
<td>1409</td>
<td>3305</td>
<td></td>
</tr>
<tr>
<td>C57 (15 Gy) vs. CBA (15 Gy)</td>
<td>3541</td>
<td>1177</td>
<td>2364</td>
<td></td>
</tr>
<tr>
<td>B6 (0 Gy) vs. [C57 0 Gy) and CBA (0 Gy)]</td>
<td>1716</td>
<td>797</td>
<td>919</td>
<td></td>
</tr>
<tr>
<td>B6 (12.5 Gy) vs. [C57 (12.5 Gy) and CBA (12.5 Gy)]</td>
<td>3467</td>
<td>1241</td>
<td>2226</td>
<td></td>
</tr>
<tr>
<td>B6 (15 Gy) vs. [C57 (15 Gy) and CBA (15 Gy)]</td>
<td>3781</td>
<td>1110</td>
<td>2671</td>
<td></td>
</tr>
<tr>
<td>CBA (0 Gy) vs. [B6 (0 Gy) and C57 (0 Gy)]</td>
<td>2974</td>
<td>1144</td>
<td>1830</td>
<td></td>
</tr>
<tr>
<td>CBA (12.5 Gy) vs. [B6 (12.5 Gy and C57 (12.5 Gy)]</td>
<td>4219</td>
<td>1746</td>
<td>2473</td>
<td></td>
</tr>
<tr>
<td>CBA (15 Gy) vs. [B6 (15 Gy) and C57 (15 Gy)]</td>
<td>4567</td>
<td>2454</td>
<td>2113</td>
<td></td>
</tr>
</tbody>
</table>
Appendix D

Table 13: Signature genes in descending order for comparison between pneumonitis (C57L/J, CBA/J) vs. non-pneumonitis (C57BL/6J) prone strains after 15 Gy WTLI

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>C57BL/6J</th>
<th>C57L/J &amp; CBA/J</th>
<th>C57BL/6J vs. C57L/J and CBA/J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rps9</td>
<td>6.6753</td>
<td>-3.3377</td>
<td>6.38E-16</td>
</tr>
<tr>
<td>Galc</td>
<td>5.519</td>
<td>-2.7595</td>
<td>5.96E-16</td>
</tr>
<tr>
<td>Pisd-ps3</td>
<td>5.3093</td>
<td>-2.6547</td>
<td>8.15E-17</td>
</tr>
<tr>
<td>Pisd-ps3</td>
<td>5.0018</td>
<td>-2.5009</td>
<td>5.98E-13</td>
</tr>
<tr>
<td>Pttg1</td>
<td>4.4757</td>
<td>-2.2378</td>
<td>8.77E-16</td>
</tr>
<tr>
<td>Mid1</td>
<td>3.7466</td>
<td>-1.8733</td>
<td>8.19E-12</td>
</tr>
<tr>
<td>Fam20b</td>
<td>-3.2513</td>
<td>1.6257</td>
<td>6.68E-14</td>
</tr>
<tr>
<td>Trip11</td>
<td>3.1719</td>
<td>-1.5859</td>
<td>2.97E-13</td>
</tr>
<tr>
<td>Fam199x</td>
<td>3.0697</td>
<td>-1.5349</td>
<td>2.23E-09</td>
</tr>
<tr>
<td>D83002612Rik</td>
<td>2.9887</td>
<td>-1.4943</td>
<td>5.75E-13</td>
</tr>
<tr>
<td>Klra8</td>
<td>2.8812</td>
<td>-1.4406</td>
<td>1.28E-07</td>
</tr>
<tr>
<td>Tmsb15l</td>
<td>2.7019</td>
<td>-1.351</td>
<td>7.54E-10</td>
</tr>
<tr>
<td>Snhg1</td>
<td>2.6652</td>
<td>-1.3326</td>
<td>4.51E-08</td>
</tr>
<tr>
<td>Slc15a2</td>
<td>2.6597</td>
<td>-1.3298</td>
<td>1.34E-09</td>
</tr>
<tr>
<td>C920006O11Rik</td>
<td>2.6027</td>
<td>-1.3013</td>
<td>1.06E-11</td>
</tr>
<tr>
<td>Pisd-ps3</td>
<td>2.5593</td>
<td>-1.2796</td>
<td>2.59E-11</td>
</tr>
<tr>
<td>Mir1931</td>
<td>2.4428</td>
<td>-1.2214</td>
<td>8.94E-11</td>
</tr>
<tr>
<td>G530011O06Rik</td>
<td>2.4358</td>
<td>-1.2179</td>
<td>9.84E-08</td>
</tr>
<tr>
<td>Alad</td>
<td>-2.4192</td>
<td>1.2096</td>
<td>1.91E-13</td>
</tr>
<tr>
<td>Klra7</td>
<td>2.3045</td>
<td>-1.1523</td>
<td>6.03E-06</td>
</tr>
<tr>
<td>Wdfy1</td>
<td>-2.2837</td>
<td>1.1418</td>
<td>6.10E-12</td>
</tr>
</tbody>
</table>
Table 14: Signature genes in descending order for comparison between non-fibrosing (CBA/J) and fibrosing (C57BL/6J, C57L/J) strains

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>CBA</th>
<th>C57L/J and C57BL/6J</th>
<th>CBA vs. C57BL/6J and C57/J</th>
<th>Fold-Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Score</td>
<td>Score</td>
<td>P-Value</td>
<td></td>
</tr>
<tr>
<td>Pdxdc1</td>
<td>7.9109</td>
<td>-3.9555</td>
<td>6.89E-16</td>
<td>15.8926</td>
</tr>
<tr>
<td>E130309F12Rik</td>
<td>6.4752</td>
<td>-3.2376</td>
<td>1.78E-14</td>
<td>19.3573</td>
</tr>
<tr>
<td>H2-D1</td>
<td>6.4252</td>
<td>-3.2126</td>
<td>9.54E-14</td>
<td>31.4263</td>
</tr>
<tr>
<td>Mndal</td>
<td>-5.8798</td>
<td>2.9399</td>
<td>1.09E-16</td>
<td>-84.5791</td>
</tr>
<tr>
<td>Ang</td>
<td>-5.6265</td>
<td>2.8132</td>
<td>1.28E-16</td>
<td>-50.0804</td>
</tr>
<tr>
<td>Rpgrip1</td>
<td>-5.5848</td>
<td>2.7924</td>
<td>1.76E-17</td>
<td>-8.05873</td>
</tr>
<tr>
<td>Ifi203</td>
<td>-5.2889</td>
<td>2.6445</td>
<td>8.15E-15</td>
<td>-27.8248</td>
</tr>
<tr>
<td>Trim30d</td>
<td>-5.2632</td>
<td>2.6316</td>
<td>1.04E-16</td>
<td>-24.7809</td>
</tr>
<tr>
<td>Tmem87a</td>
<td>5.2507</td>
<td>-2.6254</td>
<td>1.86E-18</td>
<td>8.71164</td>
</tr>
<tr>
<td>Trim12a</td>
<td>-5.1207</td>
<td>2.5603</td>
<td>6.25E-17</td>
<td>-16.404</td>
</tr>
<tr>
<td>H2-D1</td>
<td>-4.9139</td>
<td>2.4569</td>
<td>7.31E-15</td>
<td>-24.4075</td>
</tr>
<tr>
<td>Fam126b</td>
<td>-4.9033</td>
<td>2.4516</td>
<td>5.54E-16</td>
<td>-10.7099</td>
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<tr>
<td>Pdxdc1</td>
<td>-4.6257</td>
<td>2.3129</td>
<td>3.64E-16</td>
<td>-10.6916</td>
</tr>
<tr>
<td>H2-K1</td>
<td>-4.3896</td>
<td>2.1948</td>
<td>1.41E-17</td>
<td>-7.79329</td>
</tr>
<tr>
<td>Mndal</td>
<td>-3.8494</td>
<td>1.9247</td>
<td>3.66E-12</td>
<td>-41.5968</td>
</tr>
<tr>
<td>Prdx2</td>
<td>-3.5954</td>
<td>1.7977</td>
<td>1.81E-10</td>
<td>-5.06063</td>
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<tr>
<td>Paip1</td>
<td>3.591</td>
<td>-1.7955</td>
<td>4.81E-14</td>
<td>10.5071</td>
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<tr>
<td>Skiv2l2</td>
<td>3.5481</td>
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<tr>
<td>H2-L</td>
<td>-3.5208</td>
<td>1.7604</td>
<td>5.41E-15</td>
<td>-12.1315</td>
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<tr>
<td>H2-Q7</td>
<td>-3.3967</td>
<td>1.6983</td>
<td>2.60E-15</td>
<td>-12.9824</td>
</tr>
</tbody>
</table>
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Biography

Date of Birth: January 21, 1982

Place of Birth: Concord, North Carolina

Education

2000  Cary Academy

2006  B.S. Microbiology, North Carolina State University (Raleigh, NC)

Research Experience

8/2010-1/2011  Visiting Scientist, University of Zagreb School of Medicine (Zagreb, Croatia), Institute of Pathology
                Advisor: Sven Seierweth, M.D.

8/2008-5/2012  Graduate Student, Duke University (Durham, N.C.)
                Advisor: Zeljko Vujaskovic, M.D., Ph.D.

                Advisor: Zeljko Vujaskovic, M.D., Ph.D.

                Advisor: Scott Lassiter, Ph.D.

Publications in peer-reviewed journals (chronological order)


Book Chapters


Selected Presentations

1. Down J, **Jackson IL**, Vujaskovic Z. Regional Thoracic Irradiation in Mice to Avoid Pleural Effusions and Resolve Genetic Differences in Radiation Lung Damage. *14th International Congress of Radiation Research*, Warsaw, Poland, August 28-September 1, 2011

2. Vujaskovic Z, **Jackson IL**, Down J. Getting to a Consensus on Selecting the Most Appropriate Mouse Models for Studying Medical Countermeasures against Radiation Injury to the Lung. *14th International Congress of Radiation Research*, Warsaw, Poland, August 28-September 1, 2011


18. Isabel L. Jackson, Ines Batinic-Haberle, Zeljko Vujaskovic. The synergistic effect of hypoxia and hyperthermia on macrophage activation and free radical production. Annual Society for Thermal Medicine Meeting, Washington, DC April, 2005

Honors

2011: Sponsored by the National Institutes of Allergy and Infectious Disease to attend the 14th International Congress of Radiation Research
2006: New Investigator Award from Society for Thermal Medicine
2004: North Carolina State University Undergraduate Research Award