7-1-2015

EFFECTS OF LOW-DOSE GAMMA RADIATION IN A MURINE MODEL OF BENZO[A]PYRENE-INDUCED LUNG CANCER

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by

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DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy

Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

July 2015

DEDICATION

II Timothy 2:15

Study to shew thyself approved unto God, a workman that needeth not to be ashamed, rightly dividing the word of truth.

I dedicate this dissertation to my parents, Ricardo S. Gonzales and Barbara J. Gonzales as well as my brother, Ricardo J. Gonzales. Mom and Dad, you are the most loving and caring parents a child can have. Thank you for raising me to love God and for teaching me how to be a person of integrity. Ricardito, I love the relationship we share. I am so blessed to be your sister and think you are awesome in every way!

I also dedicate this work to my husband, Jerry Bruce, a wonderful man of God. Jerry, you have been by my side for every part of this journey. Thank you for the love, encouragement, and support you have shown me since the day we met. I am so proud to be your wife and I love you beyond words.

ACKNOWLEDGEMENT

Thank you first and foremost to my mentor Dr. Julie Wilder for your support and guidance through this adventure we call graduate school. To past and present members of the Wilder lab, your hard work and dedication is top notch and I am very thankful to have worked alongside you all. Dr. Bobby Scott, your commitment to the field of radiation research especially in the arena of low-dose effects is to be admired. My deepest appreciation goes out to the scientists, technologists, and support staff at Lovelace Respiratory Research Institute who have supported the works included in this dissertation. And to all members of my graduate committee, Dr. Helen Hathaway, Dr. Judy Cannon, Dr. Todd Thompson, Dr. Bobby Scott, and Dr. Julie Wilder, thank you for helping me grow as a scientist. Finally, thank you Dr. Jerry Cox for your friendship and inspiration throughout the years.

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ABSTRACT

Lung cancer is the leading cause of cancer related mortality in the United States and in the world. One of the carcinogenic compounds found in cigarette smoke, Benzo[a]pyrene (B[a]P), at high levels elicits a number of inflammatory events consistent with those found in lung cancer patients. In our studies, the effects of low-dose gamma radiation (LDR) on carcinogenesis and tumor progression in a murine model of B[a]P–induced lung cancer were investigated. Two studies (46 weeks in duration) were conducted to assess the effects of fractionated doses of low-dose gamma rays on whole body irradiated A/J mice either before or following intraperitoneal injection with the carcinogen. Additionally, a series of short-term studies were performed to investigate the effects of LDR and B[a]P on Kras mutation in tumors, leukocyte cytotoxicity and function, and oxidative stress in mice. Treatment with fractionated doses of LDR (100 mGy) delivered post B[a]P injection suppressed carcinogen-induced lung adenoma formation and prevented the multiplicity of these tumors, however

exposure to LDR delivered prophylactically failed to prevent B[a]P-induced carcinogenesis or tumor progression. Radiation alone did not induce lung tumors in either study. B[a]P was cytotoxic to leukocytes and promoted inflammation through pro-inflammatory cytokine secretion (IL-1β, IL-6, IL-17, TNF-α), infiltration of inflammatory cells, and increased lipid peroxidation in lungs and spleens of mice. LDR increased anti-inflammatory cytokine secretion (IL-2, IL-4, IL-10) in the spleen but this did not mitigate the inflammatory response induced by B[a]P. In addition we found no evidence that LDR reduces B[a]P-mediated lipid peroxidation in either spleen or lung. We also observed no change in Kras mutation incidence between treatment groups. Therefore we believe that it is unlikely that LDR suppresses B[a]P-mediated tumor progression through modulating Kras mutation, the immune response, or by reducing oxidative stress. Herein we have demonstrated that exposure to radiation at low doses does not increase cancer risk and suppresses the progression of lung cancer when delivered as a treatment. Our studies support the continued research of LDR in animal models to identify the mechanisms behind its ability to inhibit cancer progression for the potential use of LDR in human treatment of cancer in the future.

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CHAPTER 1. INTRODUCTION

Radiation

Radiation (electromagnetic and particulate) possesses energy. As atoms attempt to achieve stability, they emit energetic particles (e.g. alpha and beta particles) and/or electromagnetic waves (e.g. X-rays and gamma rays). The electromagnetic spectrum, comprised of oscillating electric and magnetic fields, is made of up these and other waves of energy varying in wavelength and frequency. This spectrum ranges from radio waves and microwaves to X-rays and gamma rays. X-rays and gamma rays exhibit shorter wavelengths with increased frequencies and energies compared to radio waves or microwaves. It is the energy and other features (e.g. charge for alpha and beta particles) of various types of radiation that determine how they will interact with matter. There are two types of radiation: ionizing and non-ionizing. Ionizing radiation is more energetic than non-ionizing radiation and has sufficient energy to ionize atoms resulting in charged atoms usually due to removal of one or more electrons. Alpha and beta particles as well as X-rays and gamma rays are considered ionizing radiation. Since alpha and beta particles have both mass and charge components, this limits their mobility and these can travel only a short distance in air. Alpha particles can be effectively shielded by skin or a single sheet of paper whereas beta particles require thicker barriers like a thin sheet of metal or a block of wood. Although easily shielded, they can possess a large amount of energy. In contrast to beta particles, high linear-energy transfer (LET) alpha particle

emitting radionuclides when taken inside the body are potentially dangerous (depending on the radiation dose) because of their high mass and energy deposition in tissues. Gamma rays or X-rays are considered low-LET energy waves which can travel longer distances in air and are more penetrating than radiation particles. X-rays and gamma rays require thick barriers of concrete or dense materials (i.e. lead) for shielding however this type of radiation does not deposit a large amount of energy as it traverses through thin slices of tissue. However a total-body lethal dose of this type of radiation can be achieved and this would require deposition of a very large amount of energy in tissue to exceed the lethal damage threshold (Scott & Guilmette 2005).

Radiation can be measured in terms of radioactivity, absorbed dose, equivalent dose, or effective dose each being represented by interrelated units. Radioactivity is defined as the amount of ionizing radiation released by a material as it decays over time. Radioactivity is most often represented by the Système International (SI) unit the becquerel (Bq) which is equivalent to a single disintegration per second. Absorbed dose is the amount of energy deposited in a medium (i.e. tissue) by ionizing radiation divided by the mass of the medium. The gray (Gy) is the SI unit for absorbed dose when energy is measured in joules and mass measured in kilograms (J/kg). Effective dose is evaluated as the sum of the weighted (with subjective linear-no-threshold-hypothesis-based weights) equivalent doses in all the tissues or organs of the body. Equivalent dose is defined by Border's Dictionary of Health Physics (2015) as the "product of the average absorbed dose in a specified organ or tissue and the radiation weighting

factor". The weighting factor used is specific to different qualities of radiation and are used to convert absorbed dose to equivalent dose. The SI unit for equivalent dose is the Sievert (Sv). For gamma and X-rays, equivalent dose is 1 Gy = 1 Sv (Nuclear Regulatory Commission 2014). To put these units into perspective, the Environmental Protection Agency (EPA) places an action limit at a concentration of 148 Bq/m^3 of the alpha emitter Radon-222 in homes. The average absorbed dose from a multiple full body computed tomography (CT) scans is 50 - 100 mGy (0.05 – 0.1 Gy) whereas a dose to a solid tumor through radiotherapy ranges from 20 to 80 Gy (Office of Biological and Environmental Research, DOE 2010). In the United States the average natural background radiation exposure to a person is 3.1 mSv per year.

Our exposure to natural background radiation comes from a variety of sources including cosmic rays, radon gas in soil and in the air, and potassium-40 found in certain plants and foods. Additionally we experience increased radiation exposure through man-made sources. We are exposed to radiation from these sources on a daily basis.

Lung Cancer

Lung cancer is the leading cause of cancer mortality in both men and women in the United States and will soon reach epidemic proportions worldwide. In 2014 it was estimated that 224,210 new cases of lung cancer would arise and an estimated 159,260 deaths due to this disease would occur (American Cancer Society 2014). Thus, lung cancer prevention is currently an active research area.

The most important risk factor for lung cancer is tobacco smoking. It is estimated to account for up to 87% of lung cancer deaths in men and 70% in women, with the risk of developing lung cancer being higher in men than in women (American Cancer Society 2014). Cigarette smoke contains approximately 5,000 reactive chemical compounds and carcinogens that can lead to the development of lung cancer by damaging DNA and inducing inflammation through the recruitment of inflammatory cells (Yao & Rahman 2009). Current literature supports evidence of a strong correlation between chronic inflammation, carcinogenesis, and tumor progression (Azad et al. 2008, Balkwill & Mantovani 2001). For example, lung cancer patients often exhibit higher blood serum levels of the pro-inflammatory cytokine Interleukin-6 (IL-6), which can also be associated with a poor prognosis (Yanagawa et al. 1995).

During carcinogenesis and tumor promotion, reactive chemicals from cigarette smoke can bind and create bulky adducts in DNA. This along with oxidative damage to DNA and inadequate DNA repair processes can all lead to increased rates of mutagenesis (U.S. Department of Health and Human Services 2010). Genes regulating cell proliferation are often mutated. In human lung cancer, mutations in the Kras, EGFR, and p53 genes have been well documented (Kim et al. 2013). These mutations can lead to gain of function (i.e. cell proliferation) or loss of function (i.e. inadequate suppression of proliferation) and are consistent with those in cancers induced by cigarette smoke carcinogens (You et al. 1989, Gray et al. 2001). For instance, Kras, a GTPase, normally functions in the regulation of cell growth, differentiation, and apoptosis (Riely et

al. 2009). When mutated this gene exhibits oncogenic properties which can lead to increased and dysregulated cell proliferation. Mutated Kras has been identified in up to 30% of human lung tumors (Reynolds & Anderson 1991). Point mutations such as G → T transversions or G → A transitions have been identified in Kras derived from tumor DNA of smokers and never-smokers (Le Calvez et al. 2005).

A/J mouse model of lung cancer

We have chosen the A/J mouse strain for our studies because of their wide use in cancer and immunological research. These mice have a predisposition for spontaneous lung adenoma formation and lung tumors can be easily induced in response to carcinogens (Hecht et al. 1994). In the lung, chemically induced lesions in treated mice bear morphological and histological similarities to human adenocarcinoma (Malkinson 1992). Lesions can be histologically identified by similar growth patterns in both species as well as in the same cells of origin. This property makes the use of this mouse model desirable to study lung tumor development.

Classic examples of chemical carcinogens commonly used in lung cancer research are polycyclic aromatic hydrocarbons (PAH) such as Benzo[a]pyrene (B[a]P), and nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK is found in tobacco products and has been implicated specifically in lung carcinogenesis whereas B[a]P, a byproduct of incomplete combustion (i.e. cigarette smoking, auto exhaust), has been known to

cause lung cancer primarily as well as cancers in other tissues. Both NNK and B[a]P are very effective at inducing lung lesions in A/J mice. In one side by side comparison, Hecht et al. (1994) showed a 100% (NNK) versus 80% (B[a]P) lung adenoma-bearing mouse incidence at 26 weeks post carcinogen treatment (i.p.). Although NNK is a stronger pulmonary carcinogen, we chose to utilize B[a]P in our in vivo studies. B[a]P, is the most extensively studied PAH, and is often used as the prototypical PAH in cancer research. Our decision to utilize B[a]P was also partly influenced by our collaboration with the laboratory of Dr. Yong Lin which was currently utilizing one of the reactive metabolites of B[a]P, benzo[a]pyrene diol epoxide (BPDE), in in vitro lung cancer studies in a project funded on the same Program Project as that funding our own work (DOE). Additionally, the time course for development and progression of carcinogen induced lung cancer (hyperplastic foci progressing to adenoma and finally carcinoma) is well known in this mouse strain (Malkinson 1992, Lyon et al. 2009). The presence of B[a]P-induced lesions has been observed in A/J mice occurring within 21 weeks of carcinogen exposure and have been measured up to 46 weeks (Anderson et al. 2008).

Benzo[a]pyrene (B[a]P)

The use of B[a]P in the A/J mouse is an established model used in lung cancer research. B[a]P-induced cancer has been documented using various routes of administration: intraperitoneal injection (i.p.), intragastric gavage (i.g.), orally or topically (Stoner et al. 1984). I.p. and i.g B[a]P treatment have been

shown to produce the highest number of lung lesions in mice (Hecht et al. 1994). In a comparison between these two routes, a higher percentage of lung tumor bearing mice were found when B[a]P was administered i.g. However one drawback to i.g. was a much higher incidence of forestomach tumor bearing mice. In light of this, administration of B[a]P via the i.p. route was chosen for our studies in that it was anticipated that it would result in high numbers of lung tumors while reducing non-pulmonary lesions.

B[a]P is a pro-carcinogen which is metabolized by cytochromes P-450 (CYP1A1, CYP1A2, and CYP1B1) to form mutagenic compounds (e.g. BPDE) and reactive intermediates (e.g. B[a]P quinones, Burdick et al. 2003, Burchiel et al. 2007). B[a]P causes DNA damage in lung cells consistent with DNA damage observed in malignant lung cancers (Denissenko et al. 1996). Several studies have shown that B[a]P induces mutations in the Kras gene consistent with those found in patients with lung cancer (You et al. 1989, Gray et al. 2001). Cells that cannot repair B[a]P-induced damage often undergo programmed cell death: apoptosis. B[a]P can induce apoptosis of cells through increasing the expression of pro-apoptotic genes and decreasing the expression of anti-apoptotic genes (i.e. Bax and Bcl-2, respectively, Salas & Burchiel 1998).

B[a]P in sufficient quantities is known to be cytotoxic to lymphocytes and a promoter of inflammation through pro-inflammatory cytokine secretion (Wojdani et al. 1984, Umannova et al. 2008, 2011). Pro-inflammatory cytokines are important during an immune response however when dysregulated or chronically produced, they can lead to immune-regulated pathogenesis, progression of

cancer, and other inflammatory diseases (e.g. arthritis). In addition to inducing pro-inflammatory cytokines, B[a]P is known to increase oxidative stress as evidenced by lipid peroxidation in rat tissues as early as 24 hours post treatment (Emre et al. 2007, Aktay et al. 2011). This can result in further DNA damage and cellular transformation modulated by reactive oxygen- and/or nitrogen- species (ROS/RNS) in addition to adducts created by B[a]P metabolites (Reuter et al. 2010).

Potential uses for LDR in a lung cancer model

Radiation exposure at low doses has been shown to produce biological effects that contrast with those induced by high levels of B[a]P. For example, Bogdandi et al. (2010) reported that exposure to 0.01 up to 0.1 Gy of X-rays is protective against spontaneous apoptosis of splenic B cells, natural killer (NK) cells, dendritic cells (DCs), and T regulatory cells (Tregs, Bogdandi et al. 2010). It is thought that exposure to LDR regulates apoptosis through the modulation of anti-apoptotic, *Bcl-2*, and pro-apoptotic, *Bax*, gene expression in an opposing way to B[a]P (Long 2012, Azimian et al. 2015). Also, LDR has been shown to selectively remove neoplastically transformed cells in vitro under suitable conditions and alleviate symptoms and improve the health of both rodents and humans with inflammatory diseases such as arthritis, diabetes, and cancer (Bauer 2007, 2011). The establishment of an anti-inflammatory phenotype and the reduction of oxidative damage through the induction of antioxidant defenses may be responsible for these outcomes (Yamoaka 2000, Pathak et al. 2007,

Farooque et al. 2011). In addition, LDR has been shown to boost DNA damage repair (Dauer 2010, Grudzenski et al. 2010, Gharib et al. 2012). Adaptive responses to LDR, such as modifications in immune response, reactive oxygen and nitrogen species, and DNA damage and repair are further discussed in the "Review of Related Literature" chapter.

Summary

Lung cancer is a very serious and deadly disease which can be studied utilizing the pro-carcinogen B[a]P in mice. The mutagenic effects and inflammatory profile elicited in mice by B[a]P are similar to those found in lung cancer patients. Although high-dose radiation exposure is known to cause cancer and is detrimental, recent studies have shown that low-dose radiation exposure can cause diverse and potentially beneficial biological effects. B[a]P is known to induce inflammation and cytotoxicity while LDR reportedly relieves inflammatory symptoms while protecting against spontaneous apoptosis of normal cells and enhancing apoptosis of neoplastically transformed cells. In theory, exposure to LDR may mitigate the harmful effects of B[a]P.

Purpose and Hypotheses

The purpose of our study was to examine the biological effects of low-dose gamma radiation alone and the effects of a known cigarette smoke carcinogen, delivered alone or in combination with low-dose gamma radiation, on the development of lung cancer in the A/J mouse.

We hypothesized that treatment with low-dose gamma rays would suppress B[a]P-induced lung cancer when given as a treatment or inhibit B[a]P-induced lung cancer when LDR was delivered prophylactically. We also hypothesized that the suppression or inhibition of B[a]P-induced lung cancer by LDR is mediated by radiation driven adaptive responses: reductions in Kras mutation, protection against cytotoxicity of normal cells, and reduction of inflammatory processes through decreased pro-inflammatory cytokines and oxidative stress.

Significance of Dissertation Study

Man-made sources of radiation such as X-rays or gamma rays are increasingly being utilized in medical diagnostic testing and procedures (e.g. radiological imaging, radiation treatment of tumors) and certain occupations (e.g. nuclear plant workers, miners, etc.) can also increase exposure. With increased use of ionizing radiation, especially in the medical field, it is of great interest to study and understand the health risks and/or benefits associated with exposure. It is well established that exposure to high radiation doses (> 2 Gy) is detrimental to health; ranging from cell killing to whole organ failure and mortality (UNSCEAR 1986, Pacific Northwest National Laboratory 2003). However the biological effects of exposure to low dose, below 100 – 200 mGy, or low-dose rate, below 0.1 – 0.2 Gy/min, remain under investigation and have yet to be fully understood (UNSCEAR 1986, BEIR VII 2006).

In our studies we employed an in vivo mouse model, while using a high dose of benzo[a]pyrene (B[a]P) as a tool to induce primary lung tumors. This design provided a physiologically relevant model to study the effects of low dose gamma rays in the context of lung cancer. This study also permitted the investigation of the impact of radiation exposure alone in carcinogenesis and tumor progression as well as facilitated the description of adaptive responses in our model.

Limitations

Going into this study we were aware that certain limitations may impact our findings. Our model is very useful in studying lung cancer, however the doses of B[a]P and the method in which it was administered is not physiologically relevant. Nonetheless, this is a relatively quick method of primary tumor induction in an in vivo model for the comparisons between treatment groups.

CHAPTER 2. REVIEW OF RELATED LITERATURE

Health risk and radiation models

It is well known that exposure to high doses of ionizing radiation can be detrimental and is associated with significant health risk. The most popular examples of this effect can be seen in the atomic bomb survivors at Hiroshima and Nagasaki, Japan and in the fruit fly studies performed in the 1920s by Hermann Muller (Calabrese & O'Connor 2014). Data collected from A-bomb survivors indicated an increased cancer incidence at high radiation doses while Muller showed that high doses of X-rays led to lethal genetic mutations in fruit flies. These studies concluded that risk is directly proportional to dose without a threshold, although this is quite controversial. This model has been deemed the linear no-threshold (LNT) model. Interestingly, these data exist mainly for high doses but little if any data was collected at low doses (none was collected in the Muller experiments). Consequently, risk has been extrapolated in a linear fashion in the low dose region (below 100 - 200 mGy) to fit the LNT-model. Regulatory bodies have adopted the use of the LNT model for radiation risk assessment to determine radiation safety policies and regulations. This use of the LNT model has been debated and criticized because there is evidence that radiation at low doses is not harmful and in some instances might provide beneficial biological effects (Brenner & Raabe 2001, Doss 2012, Calabrese & O'Connor 2014). To this end, alternative models such as the threshold model and hormetic model have been proposed (Calabrese 2005, Ricci & Sammis

2012). These models aim to describe the dose-response relationship between radiation and biological responses. Depending on the end parameters measured, one model may better describe this relationship than another. The threshold model assumes that a response (i.e. risk) increases only after a threshold dose has been achieved. The hormetic model is biphasic in nature where detrimental biological effects occur at high doses however protective or beneficial effects occur at low doses (Figure 1).

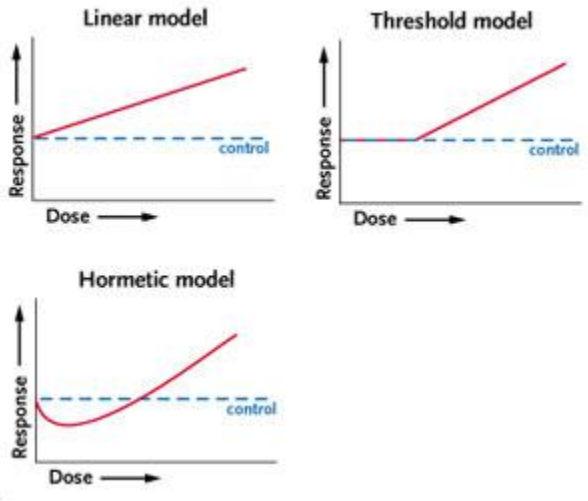

Figure 1. Linear, Threshold, and Hormetic Model Curves illustrating proposed dose-response relationships (Calabrese 2005).

Low-dose radiation: epidemiological studies in areas of high natural background radiation

To investigate the effect of low-dose radiation (LDR) in the human population, epidemiological studies in areas of high background radiation (HBR) have been performed. These areas include but are not limited to regions in

India, Iran, Poland, China, and the United States. The major source of radiation in these areas comes from terrestrial sources. Exposure to natural radiation in the United States averages about 3 mSv/year, however individuals residing in areas of high background radiation, for instance in Ramsar, Iran, are exposed to upwards of 260 mSv/year (Ghiassi-nejad et al. 2002, Nuclear Regulatory Commission 2014). In places such as Iran, Poland, and the United States the source of increased radiation is Radon-222 but radioactive thorium is also present in high background areas of India and China. The results of these epidemiological studies suggest that radiation levels delivered to residents living in HBR areas are not harmful and in some cases may provide protection against certain cancers. Studies performed in Kerala, India and Yangjiang, China concluded that high background radiation was not responsible for cancer occurrence or mortality as these data were not statistically different from data collected in areas with lower background radiation levels (Nair et al. 1999, Tao et al. 2000,2012, Tubiana et al. 2009). Ghiassi-nejad et al. (2002) reported that decreased lung cancer rates in Ramsar, Iran were associated with high background radon levels. Similar findings were reported when comparing provinces with low versus high radon levels in Poland (Fornalksi & Dobryzynski 2012). In Worcester County, Massachusetts, a case-control study of lung cancers was performed and modeled resulting in a predicted 65% reduction in the odds of cancer if residents were exposed to a hypothetical dose of 70 Bq/m^3 of radon. A significant decrease in cancer risk was found for radon exposure between 4.4 and 157 Bq/m^3, the latter dose being slightly higher than the 148

Bq/m^3 (4.0 pCi/L) action limit set by the US Environmental Protection Agency (EPA). In the same study it was also suggested that exposure to 0 Bq/m^3 versus 4.4 Bq/m^3, the lowest exposure seen in this study, would lead to a 7% increase in cancer risk. The findings of this research demonstrate the importance of further investigation into the mechanisms behind these protective effects of radiation.

Low-dose radiation: effects on inflammatory disease

Therapeutic uses for low level radiation have a long history dating back for centuries. In Europe, certain areas, usually tunnels or pools of water, with "healing properties" were highly sought out by those suffering from inflammatory ailments. In 1917, Marie Curie identified radon as the source of radioactivity in Lacco Ameno, on the Italian island of Ischia, a site renowned for its therapeutic properties (Becker 2004). It was later recognized that high levels of radon were common amongst these tunnels and pools. Despite the proposed increased cancer risk, radon therapy remains popular today. Although they remain an unpopular notion in the United States and the UK, radon therapy spas can be found in central European countries, Russia, and Japan. People who frequent these sites claim to find relief from pain associated with rheumatoid arthritis, asthma, psoriasis, ankylosing spondylitis, and other inflammatory diseases (Falkenbach et al. 2005, Erickson 2007).

In a recent publication by Charles Sanders, several human cases were discussed in which β and γ-emitting mudpacks, derived from pulverized rock from an abandoned uranium mine, were successfully utilized to treat warts, seborrheic

keratosis, and breast adenocarcinoma (Sanders 2012). Total dose ranged from 15-150 mGy and dose rates ranged from 70-250 μGy/hr, varying between cases. The cases of warts and seborrheic keratosis were resolved after 4-6 weeks of localized mudpack treatment and had not reoccurred after 24 or 44 months post treatment, respectively. Breast adenocarcinoma was continuously treated with a mudpack and a 40% size reduction of the primary tumor was noted after seven weeks of treatment.

The effects of LDR on inflammatory disease have also been studied in animal models of arthritis and diabetes. In inducible arthritis mouse models, overall inflammation and relief of symptoms in LDR-treated mice are believed to be regulated by reducing oxidative stress and modulating the immune system by reducing inflammatory cell recruitment and up-regulating anti-inflammatory cytokines (Hildebrandt et al. 2003, Arenas et al. 2006, Calabrese & Calabrese 2013a,b). Similarly, increased antioxidant response and reduction of oxidative stress seem to play a major role in the relief of symptoms of disease in diabetic mouse models. Relief in insulin resistance, inflammation, and oxidative stress in diabetic mice has been reported after receiving multiple whole body irradiations of 50 or 75 mGy X-rays for up to 8 weeks (Wang et al. 2008, Shao et al. 2014). These data, collected from both human cases and animal studies suggest a role for LDR as a treatment for inflammatory disease.

Low-dose radiation: effects on cancer

Cancer has long been associated with inflammation and it is believed that DNA mutation accompanied by deregulated inflammatory processes contributes to carcinogenesis. For instance, chronic inflammation can result in increased reactive oxygen or nitrogen species (ROS/RNS) which can then directly bind to DNA and induce mutations leading to cellular transformation (Weitzman & Gordon 1990, Azad et al. 2008, Reuter et al. 2010). Given the evidence that radiation at low doses does not contribute to, and is associated with decreased cancer incidence in areas of high background radiation, along with studies where LDR treatment is beneficial against inflammatory disease, it seems plausible to hypothesize that it plays a role in cancer prevention or suppression.

Recently, investigators have studied the effects of LDR, given as a single dose or fractionated and protracted over time, with regard to altering cancer development or resolution (Liu 2003a,b, Sakai et al. 2003). In humans, therapy with 0.1 - 0.25 Gy fractions for a total dose of 1.5 - 2 Gy has been successfully used against Non-Hodgkin's Lymphoma (NHL), with reported response rates ranging from 70 - 90% for nodular, and 50 - 80% for diffuse lymphomas (Safwat 2000). In a separate NHL study, tumors significantly regressed in response to low dose X-rays and patient survival increased compared to patients receiving only conventional chemotherapy (Sakamoto 1997, Pollycove & Feinendegen 2003, Farooque et al. 2011).

Animal studies have indicated that gamma or X-rays provide protection against the progression of cancerous tumors. Hashimoto et al. (1999)

demonstrated suppression of lung and lymph node metastasis of implanted hepatoma cells when rats were whole-body exposed to a single low dose (200 mGy) of radiation 14 days post implantation (Hashimoto et al. 1999). In mice, studies have indicated that pre-conditioning with a single dose of 100 or 200 mGy X-rays followed by i.v. injection of tumor cells reduced the amount of lung tumors in these animals (Nowosielska et al. 2011). Likewise, mice irradiated with 75 mGy X-rays prior to tumor cell injection were found to have a reduced lung tumor burden, reduced tumor size, and increased survival (Liu 2007).

Although adaptive-response related beneficial effects of LDR exposure have been clearly demonstrated and are published, the lack of mechanistic understanding limits the potential for its therapeutic use against cancer and other inflammatory diseases. Thus, it is critical for all LDR-associated biological mechanisms to be thoroughly investigated and well documented. Certain factors such as increased immune function, reduced oxidative stress, selective elimination of aberrant cells via apoptosis, and increased DNA repair are hypothesized to drive anti-cancer effects and are currently an active area of research.

Radiation adaptive response: immune stimulation

The immune system plays a crucial role in the prevention and suppression of cancer by identifying and eliminating neoplastic cells. Although some cells can be identified by the immune system as being cancerous and destroyed, an immune response sufficient for their total eradication is often not mounted and

cancerous cells can escape. In the early stages of tumorigenesis, cancerous cells can become antigenic, expressing proteins that are recognized by surveying immune cells, and can be destroyed. As carcinogenesis progresses, the cancerous cells escape detection by ceasing to express these recognizable proteins and can employ multiple mechanisms to evade the immune system. Tumors may influence the secretion of inhibitory molecules (i.e. TGF-β, IL-10, VEGF) in tumor cells or from nearby tissues, and/or down-regulate major histocompatibility complex (MHC) I proteins, co-stimulatory molecules, or adhesion factors, which can regulate the immune system's ability to recognize, infiltrate, and kill tumor cells (Igney & Krammer 2002, Stewart & Abrams 2008). Tumors can also upregulate proteins such as PD-1 or CTLA-4 that can suppress effector T cell activity while maintaining T regulatory cell activity (Zhou et al. 2004, Akbay et al. 2013). To give the boost the immune system needs, immunotherapy involving antibodies, cytokines, drugs, or vaccines has been employed to gain an edge on growing tumors (Scott et al. 2012, Vanneman & Dranoff 2012).

Recent studies in rodent models have shown that exposure to < 200 mGy X-rays or gamma rays stimulate the immune response by increasing cytotoxic potential, proliferation, activation, and cytokine secretion by leukocytes. Reduction in growth and metastasis of tumors has also been described after use of these exposure doses (Hosoi & Sakamoto 1993, Hashimoto et al. 1999). Increased killing of tumor cell lines by natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) has been observed in rodents at exposure doses < 200 mGy

(Nowosielska et al. 2010). The upregulation of co-stimulatory molecules found on antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs) may be a contributing factor to this effect (Liu 2003a). Additionally, the immune system is modulated by the secretion of cytokines: cell-derived molecules that influence the behavior of other cells in an autocrine fashion. Cytokines aid in directing cell proliferation, survival, growth, and differentiation. Several studies have reported the increase of IFN-γ, IL-2, IL-12, IL-18, TNF-α, and IL-1β cytokines after LDR exposure (Liu 2007, Nowosielska et al. 2011). These cytokines have anti-tumorigenic properties and can stimulate T cell proliferation, phagocytosis, cellular maturation, and inflammation. Exposure to LDR increases T cell proliferation with the exception of the T-regulatory (Treg) subset which is negatively affected by irradiation and this effect is potentially mediated by cytokines (Liu 2003b). IL-10, which negatively regulates the immune system by suppressing leukocytes, is downregulated in response to irradiation at low doses. In accordance with the aforementioned evidence, immune stimulation by LDR may provide protection against neoplastic cells and be an effective treatment against tumor growth and metastasis (Farooque et al 2011).

Radiation adaptive response: DNA damage and repair

Carcinogenic transformation of a cell occurs when a damaging agent such as radiation or a chemical creates single strand breaks (SSBs) or double strand breaks (DSBs) in the DNA. DSBs can be lethal, lead to mutation or

transformation if repaired incorrectly. High doses of radiation can cause irreparable DSBs leading to elimination of damaged cells and is the mechanism utilized to kill tumor cells in radiation therapy (Baskar et al. 2012). In contrast, LDR exposure above a threshold effectively induces DNA repair, hypothesized to provide protection against carcinogenesis, yet has also been implicated in the apoptosis of transformed cells while not affecting healthy cells (Azzam et al. 1996, Redpath et al. 2001, Rothkamm & Löbrich 2003, Bauer 2007).

DNA repair is an early response that occurs when repair proteins are recruited to the site of the DSBs and SSBs. The main activators of this response are ATM for DSBs and ATR for SSBs, proteins that headline a cascade of phosphorylation events that lead to the recruitment and activation of a number of downstream proteins involved in DNA repair, cell cycle arrest, or apoptosis. Two radiation-induced ATM targets are the histone variant H2AX and p53 which become phosphorylated and are involved in the DNA repair process or apoptosis, respectively. Active H2AX (γH2AX) binds directly to the lesion and facilitates the recruitment of repair proteins via non-homologous end joining (NHEJ) or homologous recombination (HR) pathways. Immunofluorescence analysis has revealed that the number of γH2AX foci is proportional to the amount of DNA damage. Resolution of these foci is associated with repair (Sedelnikova et al. 2003). According to Grudzenski et al. (2010), γH2AX foci at damaged sites is resolved by 72 hours in human fibroblast cells post irradiation with X-rays at doses > 10 mGy up to 200 mGy. There seems to be an optimal

exposure dose for repair since γH2AX foci was not resolved at doses less than 10 mGy or greater than 200 mGy.

When DNA damage is too extensive to repair, a cell will often undergo apoptosis, programmed cell death. This is a normal function that balances cell proliferation and can be mediated by a number of proteins. One of the more famous proteins involved in the tumor suppressive function related to apoptosis is p53 (protein 53 kilodalton, Lowe & Lin 2000). Deemed the "guardian of the genome", p53's responsibilities include regulating cell cycle checkpoints and inducing cell cycle arrest to allow for DNA repair or directing apoptosis in cells where DNA has been damaged beyond repair (Lane 1992). It has recently been found that LDR induces hyperradiosensitivity (HRS) in tumor cell lines mediated by p53 driven apoptosis. Exposure of human A459 (lung adenocarcinoma), human T98G (glioma), and transformed rat fibroblasts to < 500 mGy gamma rays resulted in increased HRS caspase-3 activation and Annexin V binding, markers of apoptosis. These results are believed to be p53 dependent as these effects were abolished when p53 was inhibited (Enns et al. 2004, Krueger et al. 2007).

The fate of cells with DNA damage depends on the extent of the damage. There are at least two ways previously discussed in which irradiation at low doses deter cellular transformation: inducing DNA repair through the increased recruitment of DNA repair proteins (i.e. γH2AX) or by inducing apoptosis.

Radiation adaptive response: balancing ROS/RNS

ROS/RNS is a double-edged sword. ROS/RNS including the superoxide anion ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\bullet OH$), nitric oxide ($\bullet NO$), and peroxynitrite ($ONOO^-$) are products of natural cellular metabolism. These short-lived products play essential roles in regulating several cell signaling pathways including those regulating cell proliferation and survival, the DNA damage response, and anti-oxidant responses (Azad et al. 2008, Reuter et al. 2010, Ray et al. 2012). Alternatively, chronic ROS/RNS has been implicated in promoting carcinogenesis, tumor progression, and inflammatory disease through repeated tissue and DNA damage and by promoting cell proliferation and inflammation. These deleterious effects are associated with oxidative stress, an imbalance in ROS/RNS production and insufficient detoxification by anti-oxidants. This makes ROS/RNS a therapeutic target against inflammatory diseases, including cancer. There is an ongoing debate on how modulating the ROS/RNS response should be used depending on the type and stage of disease as well as the cell types affected. Both increasing ROS/RNS to induce tumor cell death and decreasing ROS/RNS by modulating antioxidant levels to limit damage have been tested in various contexts. A thorough review of these processes has been published by Gupta et al. (2012).

Exposure to LDR has been shown to relieve symptoms of inflammatory disease and prevent cancer progression, both states which are associated with oxidative stress, and it is suggested that LDR modulates ROS/RNS to yield these outcomes. One mechanism by which LDR acts is through ROS-mediated

apoptosis. LDR induces intercellular apoptosis through ROS and transforming growth factor beta (TGF-β) signaling, a non-targeted effect. Non-targeted effects are the result of cell signaling between irradiated and neighboring unirradiated cells (Kadhim et al. 2012). Studies by George Bauer and colleagues have shown that LDR enhances apoptosis of transformed cells while not affecting healthy cells (Bauer 2007, 2011). Maximal apoptosis (40%) of transformed cells was observed when co-cultured with healthy cells irradiated with 50 mGy (Portress et al. 2007). This phenomenon is dependent on increased ROS signaling mediated by TGF-β secreted by irradiated cells. This stimulates the production of oxygen radicals from non-transformed cells which in turn interact with $\bullet O_2^-$ generated from transformed cells to induce apoptosis in an autocrine fashion (Temme & Bauer 2013). Additionally, exposure to radiation at doses between 100 mGy – 500 mGy of X rays or gamma rays increase the production of the antioxidants superoxide dismutase (SOD) and glutathione peroxidase (GPx, Yamaoka 2000). SOD detoxifies $\bullet O_2^-$ to H_2O_2 which is then further reduced to H_2O and O_2 by either Catalase or GPx. LDR-induced reduction of oxidative stress, as measured by decreased lipid peroxidation, along with concurrent increases in antioxidants have been shown in brain, liver, kidney, spleen, and testes in rats. These effects were noted when radiation was administered before or after treatment with Trichloroethylene (TCE), carbon tetrachloride (CCl_4) or cisplatin to induce oxidative damage (Gharib et al. 2012, Fahmy et al. 2013, Kataoka 2013). Thus the modulation of RNS/ROS by LDR exposure may be beneficial depending on the disease context.

CHAPTER 3. METHODOLOGY

ANIMAL PROCEDURES

Mice

Female, 6 week-old, A/J mice purchased from Jackson Laboratories (Bar Harbor, ME), were quarantined for 2 weeks prior to each study. Mice were group housed in ventilated cages (4 mice/cage) with access to water bottles and food and exposed to a 12 hour light/dark cycle. Mice were separated into the following groups: B[a]P only, B[a]P + radiation, radiation only, and untreated, non-irradiated controls. Mice were humanely euthanized by intraperitoneal (i.p.) injection of a lethal dose of Euthasol. All experimental procedures were approved by and complied with the Institutional Animal Care and Use Committee (IACUC) of the Lovelace Respiratory Research Institute.

Chemicals

Benzo[a]pyrene, B[a]P (Sigma), at a concentration of 100 mg/kg, 25 mg/kg, or 12.5 mg/kg body weight was dissolved in 0.2 mL of tricaprylin vehicle (Glyceryltrioctanoate, Sigma) prior to i.p. injection.

Irradiation

Mice were whole-body irradiated (WBI) using an absorbed radiation dose of 10.3 ± 2.1 mGy, at a dose rate of 1.2 ± 0.2 mGy/s (10 mGy target dose) or 106.9 ± 14.6 mGy at a dose rate of 1.6 ± 0.3 mGy/s (100 mGy target dose) gamma rays from the Gammacell 1000 irradiator with a Cesium-137 source (Best Theratronics). Mice were exposed individually in polypropylene tubes set inside

a stainless steel canister lined with lead foil (7.4 mm thickness). Experimental doses received were evaluated using nanoDots (Optically Stimulated Luminescence Technology, 1 cm^2, Landauer) adhered to the outside of the tubes containing the mice.

STUDY DESIGNS

Cancer treatment

Ten week old mice were given a single dose of B[a]P (100 mg/kg) four weeks prior to the initiation of gamma radiation exposures. Fractionated whole-body gamma ray target doses of 10 mGy or 100 mGy were then given once every 2 weeks until week 14 post-B[a]P injection for a cumulative dose of 60 mGy and 600 mGy, respectively. At 46 weeks, lungs were harvested and lesions were enumerated grossly. In addition, lungs were fixed and tissue sections were prepared for histological examination (Figure 2).

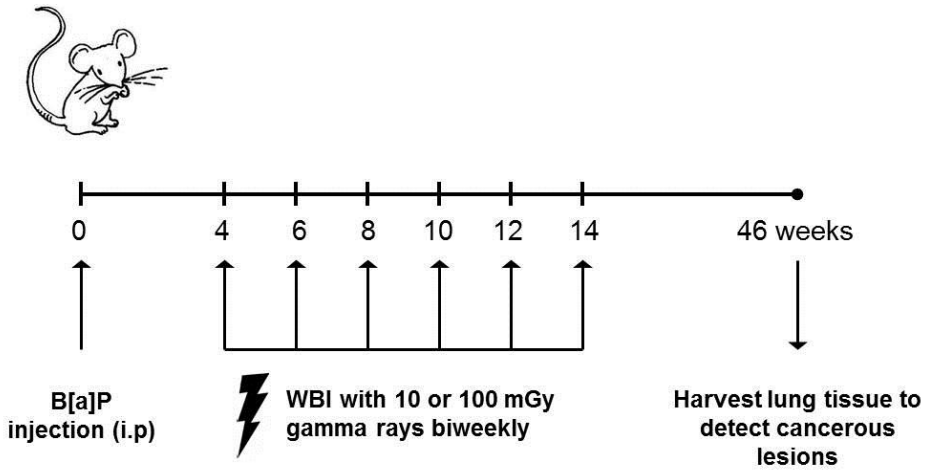

Figure 2. Cancer treatment study design.

Cancer prophylaxis

Ten week old mice were exposed to a fractionated whole-body gamma ray target dose of 100 mGy (total dose of 600 mGy) followed by a low (12.5 mg/kg) or high (25 mg/kg) dose of B[a]P within 24 hours. This was repeated once every 2 weeks for a total of 10 weeks (total doses of 75 mg/kg or 150 mg/kg, respectively, delivered over the course of 10 weeks). At 46 weeks, lungs were harvested, fixed, and tissue sections were prepared for enumeration of lesions by histological examination (Figure 3).

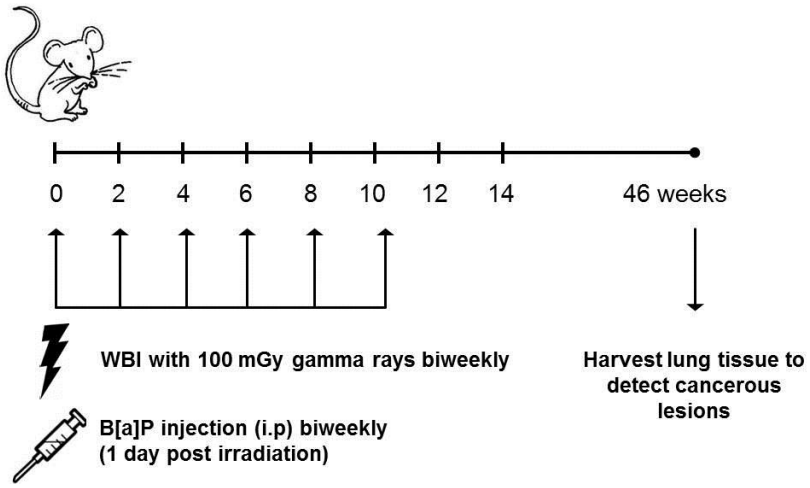

Figure 3. Cancer prophylaxis study design.

Mechanistic studies

Ten week old mice were exposed to a single whole body irradiation dose of 10 or 100 mGy gamma rays one day prior to a single i.p. injection with B[a]P (100mg/kg). Spleen and lung tissues were harvested on days 2 or day 7 for

analysis of cell number, cell phenotype, cytokine secretion/production, western blotting, or lipid peroxidation (Figure 4).

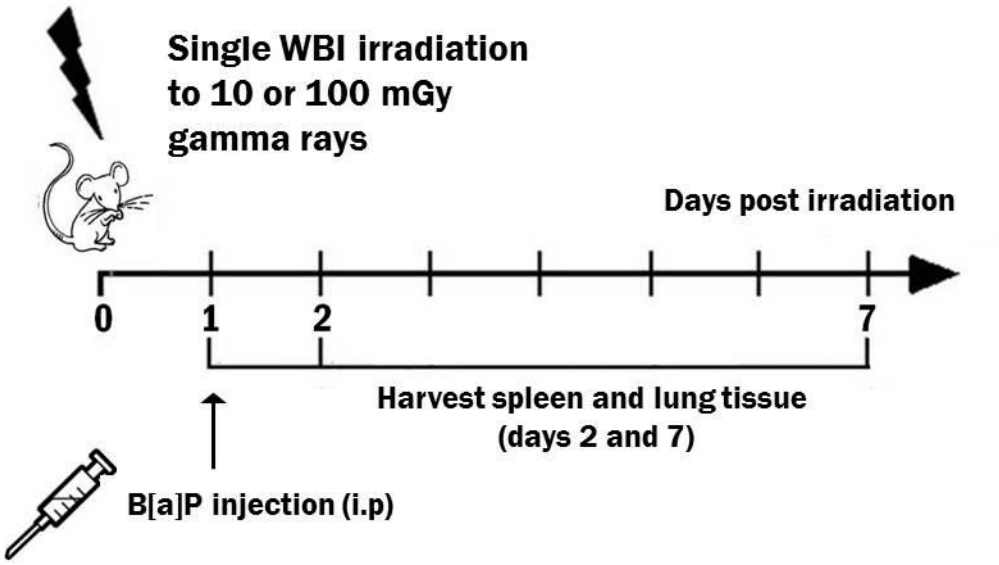

Figure 4. Mechanistic studies design.

HISTOLOGY

To estimate tumor burden, gross lung tumors were counted at necropsy. Lungs were inflated with neutral buffered formalin at a constant hydrostatic pressure of 25 cm for 6 hours and fixed further by immersion in formalin for >48 hours. Trimmed tissue was histologically processed and 5 μm-thick paraffin sections were mounted and stained with hematoxylin and eosin for light-microscopic identification and enumeration of hyperplastic foci, adenomas, and carcinomas.

Cancer treatment study lung trim

Left lung lobes were bread sliced at 3-4 mm intervals to yield 3-5 slices per lobe. A single slice along the axial airway of each of the right lung lobes was also made.

Cancer prophylaxis study lung trim

All lung lobes were bread sliced at 3-4 mm intervals to yield 3-5 slices per lobe.

KRAS

DNA amplification

DNA from formalin fixed paraffin embedded (FFPE) lung tumors was obtained using the E.Z.N.A. FFPE DNA Kit (Omega Bio-Tek). 200 ng DNA in a final volume of 50 µL was PCR amplified using the primer sequences 5'GTATAAACTTGTGGTGGTTGGAGGT3' and 5'GTTACCTCTATCGTAGGGTCATAC3' to generate a 103 base pair product for use with the BstN1 restriction enzyme or 5'GTATAAACTTGTGGTGGTTGGAGGT3' and 5'GTTACCTCTATCGTAGGGTCATAC3' to generate a 108 base pair product for use with the Hph1 restriction enzyme. This was followed by 40 cycles of amplification (94˚C for 30 s, 62˚C for 30 s, 72˚C for 30 s).

Restriction fragment length polymorphism identification of Kras mutation

To identify mutation in Kras from amplified DNA, the restriction enzyme BstN1 (New England Biosciences) was employed to identify GGT to GTT, GAT,

or GCT mutations in codon 12 of exon 1. The sense primer contains a C mismatch instead of a G in the third base from the 3' end, causing the formation of a restriction for the enzyme BstN1. When digested with BstN1, fragments containing wild type codon 12 sequences are cleaved resulting in 22 and 81 base pair fragments. Fragments containing mutations are not cleaved. Forty microliters of amplified DNA product was incubated with 2 µL BstN1 at 60°C for 12 hours. A 40 µL volume of reaction mixture along with 10 µL of dye containing bromophenol blue was loaded on a 3% agarose gel and run at 120V for 2 hours. The gel was then analyzed using a UV imaging machine.

To identify mutations in Kras from amplified DNA, the restriction enzyme Hph1 (New England Biosciences) was employed to identify a GGT to GAT mutation in codon 12 of exon 1. The sense primer contains a G mismatch instead of a C in the second base from the 3' end, causing the formation of a restriction site for the enzyme Hph1 only if the second base of codon 12 also contains the G to A transition mutation. Hph1 creates 35 and 73 base pair products upon recognition of the restriction site created when Kras contains this mutation. Twenty microliters of amplified DNA product was incubated with 1 µL Hph1 at 37°C for 1 hour. The reaction was stopped by incubating at 65°C for 20 minutes. A 20 µL volume of reaction mixture along with 10 µL of dye containing bromophenol blue was loaded on a 3% agarose gel and run at 120V for 2 hours. The gel was then analyzed using a UV imaging machine.

CYTOKINES

Secretion

Splenocytes were placed into culture at 2.0 x 10^6 cells/mL with either Concanavalin A (Con A, 5 µg/mL) or media for 48 hours at 37°C in a 5% CO_2 incubator. Supernatants were then collected and cytokines were measured using a Millipore Milliplex kit (EMD Millipore).

FLOW CYTOMETRY

Single-cell suspensions obtained from lungs or spleens of mice were analyzed via flow cytometry (FACSCanto; BD Biosciences). Datasets were analyzed using FlowJo software (Tree Star).

Cell counts and phenotyping

Total spleen and lung cells were counted manually using a hemocytometer. Cells were stained with antibodies specific for cell surface markers to identify T cells (CD4, CD8), B cells (CD19), macrophages and dendritic cells (CD11c) and neutrophils and monocytes (Gr-1). Auto-fluorescent$^{(High)}$ macrophages and auto-fluorescent$^{(Low)}$ dendritic cells were identified from CD11c+ populations (Osterholzer et al. 2008). Cytospin slides were prepared from lung cells and macrophages, lymphocytes, eosinophils and polymorphonuclear cells (PMNs) were manually counted.

Apoptosis

Spleen and lung cells were stained with cell surface marker antibodies followed by the Annexin V Apoptosis Detection Kit I (BD Sciences) and analyzed via flow cytometry within one hour.

Intracellular detection of cytokines

Lung cells were adhered for 1 hour at 37°C in a 5% CO_2 incubator to remove macrophages known to have suppressive properties when placed in culture. Splenocytes (2.0 x 10^6 cells/mL) and non-adherent lung cells (5 x 10^6 cells/mL) were cultured in the presence of Con A (5 µg/mL) for 16 hours. A protein transport inhibitor (Brefeldin A, eBioscience) was added for the last 4 hours of culture. Cells were then stained with surface marker antibodies, fixed and permeabilized (Intracellular Permeabilization Buffer, eBioscience) and stained with antibodies specific for intracellular cytokines: (pro)IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IL-17, and TNF-α.

LIPID PEROXIDATION

Lipid peroxidation was approximated using the thiobarbituric acid reactive substances (TBARS) assay. Spleen and lung tissues (30 – 50 mg) were homogenized at 1:10 in buffer (PBS + 1mM EDTA + 0.01mM Butylatedhydroxytolulene,BHT) using a Tissuelyzer with stainless steel beads (QIAGEN). An aliquot was diluted 1:25 in deionized water prior to measuring total protein levels using a Bradford protein assay. Homogenate was mixed in equal volume with a mixture of thiobarbituric acid (Sigma) and tricholoacetic acid

(Sigma) and incubated at 95°C for 20min then cooled to room temperature. 1-Butanol (300 µL) was added to the mixture and TBARS were measured spectrophotometrically. Samples were read at 532nm with a correction at 700nm on a Spectra max 340PC (Molecular Devices) to obtain nmol TBARS per milliliter volume. 1,1,3,3-Tetramethoxypropane, (Sigma) was used as a standard for calibration of the curve. Results were reported as TBARS nmol/mg of total protein.

WESTERN BLOTTING

Spleen and Lung tissues were placed in lysis buffer containing protease inhibitors (Sigma) and homogenized using a Dounce homogenizer. Samples were placed on a rotating wheel for 30 minutes at 4˚C followed by a 5 minute centrifugation. Supernatants were first used to measure protein levels using a Bradford protein assay. A standard western blotting protocol was used with antibodies (Abcam) to detect γH2AX and Phospho-p53(Ser15) to assess presence of these proteins. Adriamycin treated A549 cells were used as positive controls.

STATISTICS

Cancer treatment and cancer prophylaxis

Statistically significant differences in tumors between groups were determined by Wilcoxson Rank Sum tests. Fisher Exact tests were used to determine statistically significant differences in adenoma and carcinoma

multiplicity between treatment groups. Statistical significance was defined as $p < .05$.

Mechanistic studies

Statistically significant biological effects by B[a]P treatment compared to no B[a]P (regardless of radiation dose received) as well as effects by 10 or 100 mGy gamma rays compared to non-irradiated mice (regardless of B[a]P) were determined via ANOVA. Unpaired t-tests were used to determine statistically significant differences between individual groups. Statistical significance was defined as $p < .05$.

Median Fluorescence Intensity

Statistically significant effects by B[a]P treatment compared to no B[a]P (regardless of radiation dose received) were determined by Wilcoxson Rank Sum tests. Statistical significance was defined as $p < .05$.

CHAPTER 4. RESULTS

Cancer treatment study

In order to ascertain if low-dose radiation (LDR) could effectively treat and inhibit lung tumor progression initiated by a carcinogen contained in cigarette smoke, we investigated the potential for suppression of B[a]P-induced lung tumors in mice exposed to repeated low doses of gamma radiation (Figure 2).

As expected, B[a]P treated mice exhibited a significantly higher number of lesions compared to control mice or mice receiving radiation only ($p < .05$, Figure 5A). The tumors observed grossly represented hyperplastic foci, adenomas, and carcinomas. These observations were proportional to those performed by histological analysis. In each group treated with B[a]P, the number of hyperplastic foci greatly exceeded adenomas which in turn exceeded carcinomas representing the natural progression of lung cancer (Figure 5B). In contrast, exposure of B[a]P treated mice to six fractions of 100 mGy doses of gamma radiation (600 mGy total) was associated with a significant reduction of adenomas/mouse compared to B[a]P alone ($p = .04$). Nine out of 12 B[a]P treated mice (75%) developed one or more adenomas compared to 8 out of 14 mice treated with six fractions of 100 mGy gamma rays in addition to B[a]P (~57%, Table 1). No adenomas or carcinomas were detected in the untreated control group or the group treated with six fractions of 100 mGy dose in the absence of B[a]P (Figure 5B).

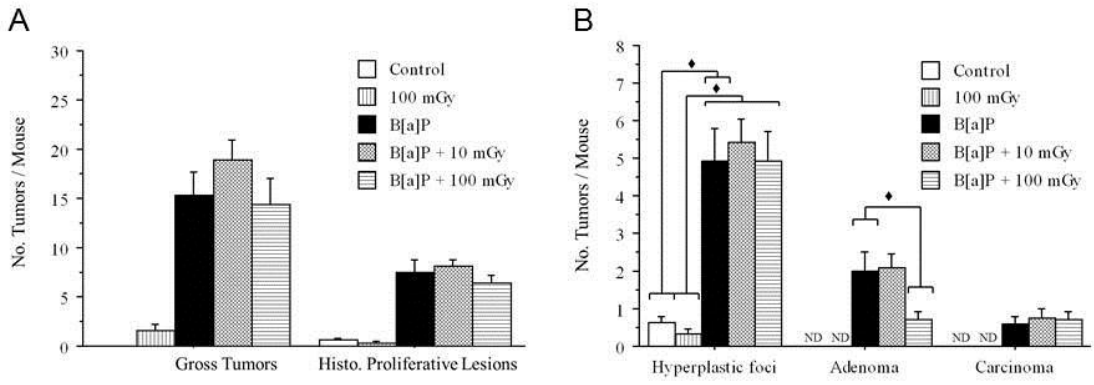

Figure 5. Cancer treatment lung tumor counts. Lung tumors from mice were grossly counted upon necropsy at 46 weeks post injection and histologically verified as proliferative lesions (A). Proliferative lesions (tumors) were further classified as hyperplastic foci, adenomas, or carcinomas (B). Mean values ± SE obtained from a single experiment is presented. Each experimental group contained 12-18 mice, and the control group contained 8 mice. ND = not detected; * = $p < 0.05$ with the indicated groups being significantly different than each other by Wilcoxson Rank Sum Test

We investigated the distribution of lung tumors (adenomas and carcinomas only) by exposure group. Repeated exposure to doses of 100 mGy provided protection against 2 or more and against 3 or more adenomas/mouse ($p < .05$) when compared to B[a]P treated mice (Figure 6). Treatment with the 100 mGy dose did not protect against carcinomas (Figure 7). There was no indication of a gamma ray protective effect by the six fractions of 10 mGy dose when considering either adenomas or carcinomas.

Although B[a]P primarily targets the lung when administered i.p., we also observed lesions not of pulmonary origin. In both scheduled and unscheduled deaths, mice were found to have proliferative lesions (hyperplasia, carcinoma, fibrosarcomas or other sarcoma) in gastric tissues as determined histologically (Tables 2 and 3). These observations are consistent with those previously

documented in which B[a]P induced injection site tumors and tumors in the abdomen and pancreas of mice injected i.p. (Hecht et al. 1994, Balansky et al. 2006).

Table 1. Cancer treatment tumor incidence (% of mice with indicated lesions ± SE).

Group	No. mice	% mice with 1 or more hyperplastic focus	% mice with 1 or more adenoma	% mice with 1 or more carcinoma
Control	8	62.5 ± 17.1	0.0	0.0
100 mGy	18	33.3 ± 11.1	0.0	0.0
B[a]P	12	91.7 ± 8.0	75.0 ± 12.5	41.7 ± 14.2
B[a]P+10 mGy	15	100.0	86.7 ± 8.8	46.7 ± 12.9
B[a]P+100 mGy	14	92.9 ± 6.9	57.1 ± 13.2	50.0 ± 13.4

Table 2: Incidence of non-pulmonary proliferative lesions in scheduled deaths.

Group	No. in scheduled group	No. with gastric squamous cell hyperplasia	No. with gastric squamous cell carcinoma	No. with fibrosarcoma or other sarcoma
100 mGy	18			
B[a]P	12	2		1
B[a]P+10 mGy	15	4		
B[a]P+100 mGy	14	5	3	

Table 3: Incidence of non-pulmonary proliferative lesions in unscheduled deaths.

Group	No. dead before schedule	No. with gastric squamous cell hyperplasia	No. with gastric squamous cell carcinoma	No. with fibrosarcoma or other sarcoma
100 mGy	1			1
B[a]P	3 [a]		2 [b]	1 [b]
B[a]P+10 mGy	2	1 [c]	1	1 [c]
B[a]P+100 mGy	3		2	1

[a] One animal had no non-pulmonary neoplasms /proliferative lesions.
[b] One animal had both squamous cell carcinoma and malignant fibrous histiocytoma.
[c] One animal had both squamous cell hyperplasia and malignant fibrous histiocytoma.

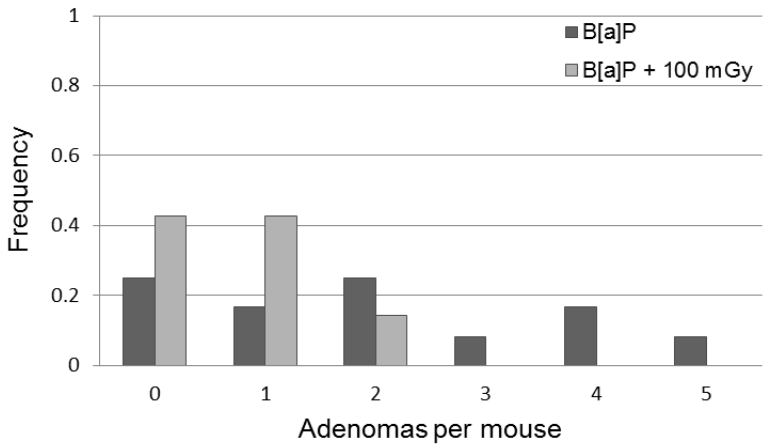

Figure 6. Cancer treatment frequency distribution of adenomas per mouse.

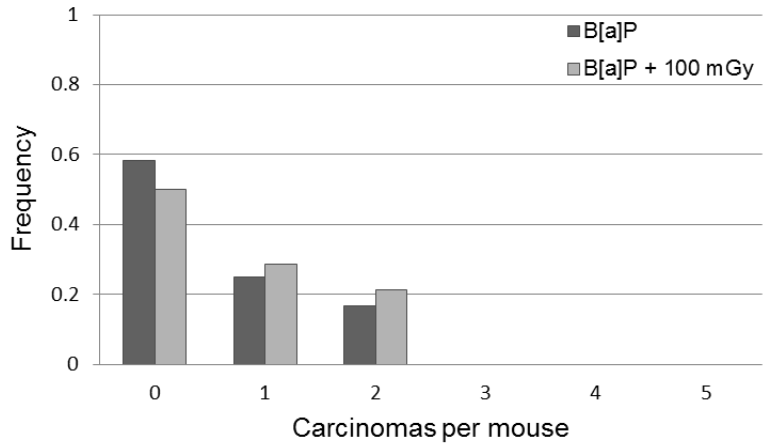

Figure 7. Cancer treatment frequency distribution of carcinomas per mouse.

Cancer prophylaxis study

In mice, studies have indicated that pre-conditioning with a single dose of 75, 100, or 200 mGy X-rays followed by i.v. injection of tumor cells has been effective in reducing pulmonary metastases by 25% and decreasing tumor size by 50% by two weeks post irradiation (Liu et al. 2007, Nowosielska et al. 2010).

In our cancer treatment study, we showed that irradiation with six fractions of 100 mGy four weeks after B[a]P treatment reduced the number of adenomas in mice (Bruce et al. 2012). We expected to see a similar decrease in carcinomas by LDR however due to the very low amount of tumors available by 46 weeks this effect was not seen. As a result we modified our study design to help answer this remaining question. In this experiment we utilized two doses of B[a]P in an attempt to manipulate lung tumor burden in mice after a 46 week period. B[a]P was fractionated over six injections in a multiple dosing strategy to achieve a cumulative high or low dose of 150 mg/kg or 75 mg/kg, respectively (Figure 3). Fractionation of lung cancer carcinogens has been successful in inducing tumorigenesis in mice (Hecht & Trushin 1994, Hecht 1997, Kassie et al. 2007). We chose to utilize a high B[a]P dose to increase tumor burden and carcinomas present at the end of 46 weeks. A lower B[a]P dose was used to examine any smaller protective effects by LDR that might otherwise be masked by an abundance of tumors at higher doses. Non-pulmonary proliferative lesions were not examined in this study.

In order to determine whether pre-conditioning with LDR could also inhibit lung carcinogenesis initiated by B[a]P, we investigated B[a]P-induced lung tumor development in mice exposed to repeated doses of gamma rays prior to receiving either high or low doses of B[a]P (Figure 3).

As expected, both high and low dose B[a]P treated mice exhibited a significantly higher number of lesions compared to control mice or mice receiving radiation only (Figure 8A). In our cancer treatment study we determined that

tumors observed grossly were proportional to histological proliferative lesion counts, therefore we have reported the latter for this experiment. Tumors were verified histologically as proliferative lesions and further classified as hyperplastic foci, adenomas, or carcinomas. The majority of lesions induced by B[a]P were hyperplastic foci while adenomas and carcinomas averaged below a single lesion per mouse (Figure 8B). Exposure to 100 mGy gamma ray fractions prior to B[a]P injections had no effect on total number of lesions nor did we observe a reduction of hyperplastic foci, adenomas, or carcinomas. Analysis of tumor multiplicity among adenomas and carcinomas showed no statistical differences between the irradiated and non-irradiated B[a]P-treated mice (Figures 9 and 10). Lung tumor counts from mice exposed only to radiation were not statistically greater than those found in non-irradiated, untreated control mice.

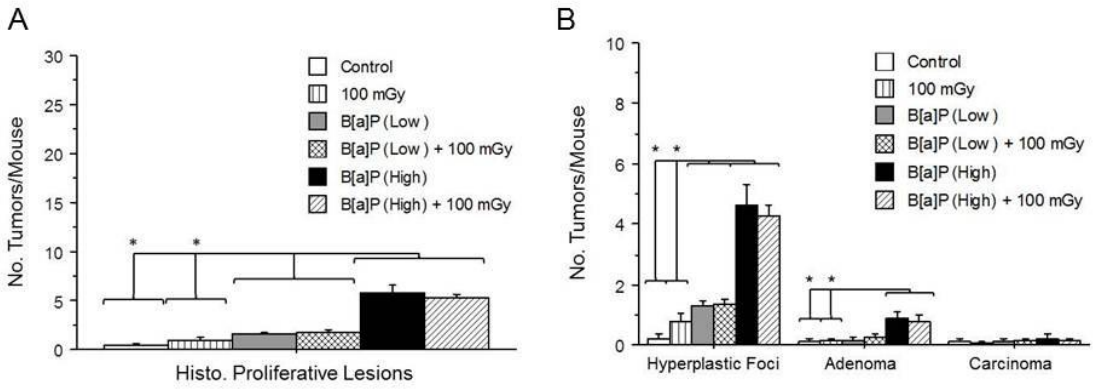

Figure 8. Cancer prophylaxis lung tumor counts. Lung tumors from mice were counted upon necropsy at 46 weeks post injection and histologically verified as proliferative lesions (A). Proliferative lesions (tumors) were further classified as hyperplastic foci, adenomas, or carcinomas (B). Mean values ± SE obtained from a single experiment is presented. Each experimental group contained 21-35 mice, and the control group contained 9 mice. * = $p < 0.05$ with the indicated groups being significantly different than each other by Wilcoxson Rank Sum Test.

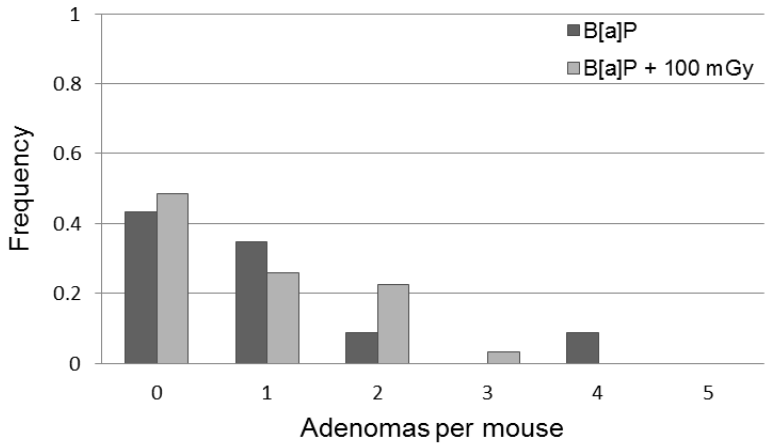

Figure 9. Cancer prophylaxis frequency distribution of adenomas per mouse.

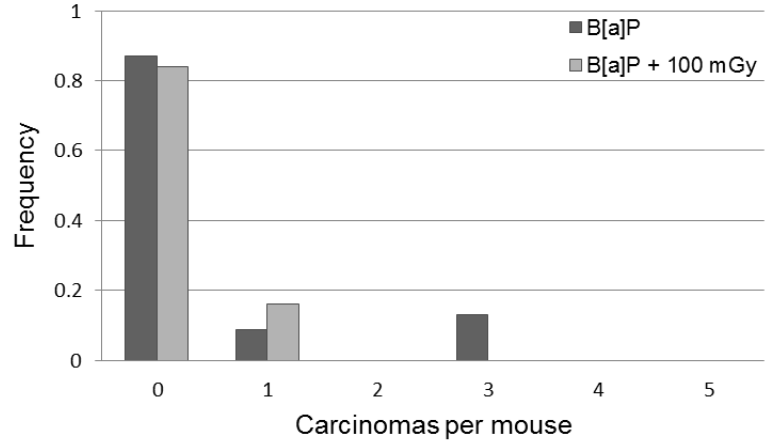

Figure 10. Cancer prophylaxis frequency distribution of carcinomas per mouse.

Kras

In our cancer treatment study we found that exposure to fractionated doses of 100 mGy gamma rays suppressed the progression of B[a]P-induced tumors. Because of this finding we sought for a mechanism to explain our observations. We hypothesized that the incidence of mutated Kras in tumor DNA of B[a]P treated mice would exceed that of B[a]P treatment with irradiation. To

test this hypothesis, existing lung tissue from our cancer treatment study was used to investigate the occurrence of this mutation. Tumors were extracted from lungs of mice treated with either B[a]P alone or with B[a]P + LDR (6 x 100 mGy gamma rays). We chose to investigate the incidence of Kras mutation in codon 12 and specifically the G → A mutation in Kras codon 12. Tumor DNA was extracted, combined, and amplified via PCR. Mutations in the Kras gene were identified by use of restriction enzymes BstN1 and Hph1.

Five of the 9 tumor DNA samples from the B[a]P treatment group and 4 of 8 from the B[a]P+LDR treatment group contained a mutation in codon 12 of the Kras gene (Table 4). The most common mutations that occur in this region are point mutations that change the GGT sequence to GTT, GAT, or GCT. We also identified a single sample in each treatment group which contained the G → A Kras mutation (Table 5).

Table 4. Kras codon 12 mutations in lung tumor DNA.

Treatment	No. samples	Codon 12	Mutation frequency (%)
		GTT, GAT, GCT	
B[a]P	9	5	55
B[a]P+100 mGy	8	4	50

Table 5. Kras codon 12 GGT→GAT mutation in lung tumor DNA.

Treatment	No. samples	Codon 12	Mutation frequency (%)
		GAT	
B[a]P	10	1	10
B[a]P+100 mGy	11	1	10

Cell number, phenotype, and function

To test our hypothesis that LDR protects against B[a]P-mediated cytotoxicity we investigated the effects of B[a]P on leukocytes in splenic and lung tissues of treated mice. A number of studies have shown beneficial effects of low-dose radiation exposure when administered prior to an insult (i.e. tumor cell injection, Liu 2007, Nowosielska et al. 2010). This is the classical adaptive-response study design. We adapted this study design to explore the effects of B[a]P alone or in combination with LDR on the immune system. Mice were exposed to a single dose of 10 or 100 mGy gamma radiation one day prior to injection with 100 mg/kg B[a]P (Figure 4). On days two (D2) and seven (D7) post irradiation, spleen and lung tissue were harvested and analyzed via flow cytometry for changes in cell number, phenotype, and cytokine production.

In the spleen, B[a]P treatment did not affect total cell number but CD4+ T cells were significantly reduced by D7 when compared with mice not treated with B[a]P (Figure 11). B[a]P treatment also increased Gr-1+ cells (monocytes and neutrophils, not shown) on both days. Irradiation with either dose of gamma rays had no significant effect on splenocyte populations.

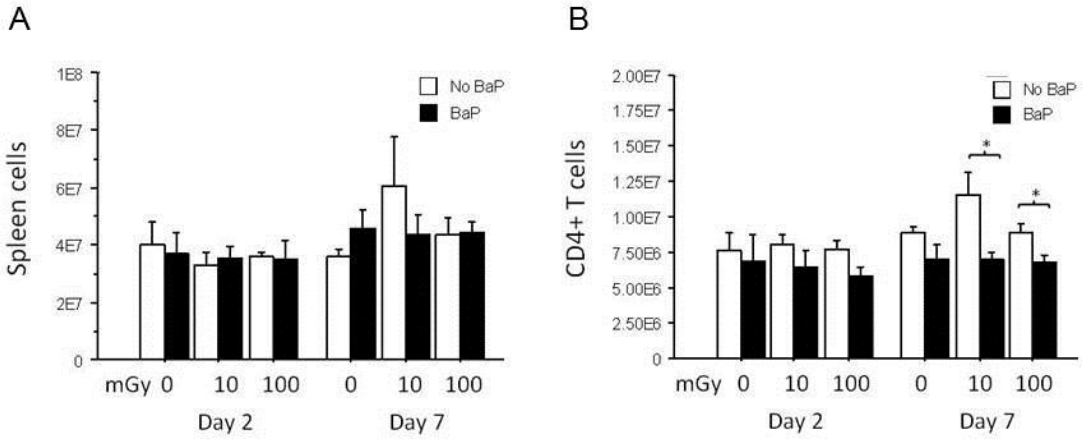

Figure 11. Spleen cell counts. A. Total spleen cell number was determined by counting using a hemocytometer. B. Number of splenic CD4+ cells was determined by flow cytometric analysis. Mean values ± SE obtained from three independent experiments are presented (n = 4/group in each experiment). * = $p < .05$ between indicated groups by ANOVA.

In the lung, B[a]P had no effect on total cell number (not shown) but significantly decreased CD4+ and CD8+ T cells, and dendritic cells (DCs, not shown) overall when compared to mice without B[a]P treatment and regardless of radiation dose (Figure 12). CD4+ T cells were significantly decreased starting on D2 and remained at decreased levels on D7. Dendritic cells were decreased only at the early time point but returned to normal levels by D7 (not shown). CD8+ T cells were significantly decreased on both days. It is uncertain if any of

the reduced populations recover at a later time. In addition to reducing lung lymphocytes, B[a]P induced neutrophilia in the lung as indicated by an increase in polymorphonuclear cells (PMN) on both days at which it was analyzed (Figure 12).

Exposure to radiation significantly decreased total lung cell number transiently on day 2 (not shown). This observation was likely related to decreases in CD4+,CD8+, and CD19+ cells and dendritic cells (DCs) on D2 (not shown) when compared to non-irradiated groups (Figure 12). All cell numbers affected by radiation returned to normal levels by D7.

Finally we examined the effects of radiation in combination with B[a]P on cell number. Exposure to 10 mGy in combination with B[a]P increased the number of lung CD8+ and CD19+ cells compared to B[a]P treatment alone on D7 (Figure 12). We did not observe this effect in any cell type with the 100 mGy dose.

We wanted to know if the reductions in cell populations in spleen or lung were due to apoptosis driven by either the carcinogen or by radiation exposure. Current literature points to apoptosis as a means by which B[a]P is both cytotoxic and immunosuppressive (Salas & Burchiel 1998, Revel et al. 2003, Xiao et al. 2007). Using a flow cytometric method with Annexin V and 7-AAD, we measured apoptosis on day 1 (4 hours post B[a]P injection, Bogdandi et al. 2010). We observed no increase in apoptosis of these cell types due to either B[a]P or LDR above that of levels measured on D0 from untreated controls (Figure 13).

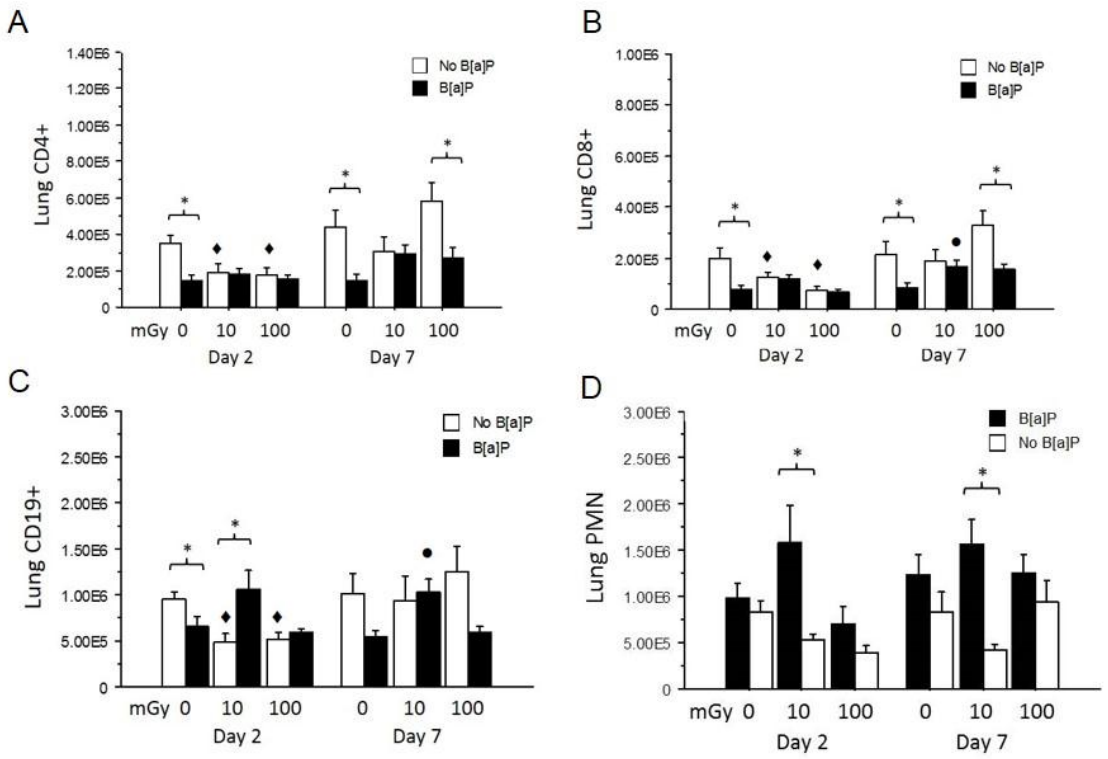

Figure 12. Lung cell counts. Number of lung CD4+ (A), CD8+ (B), and CD19+ (C) cells were determined by flow cytometric analysis. Cytospins from lung cells were created and stained to determine lung neutrophils (PMNs, D). Data shown is representative of significant trends observed in at least 2 of 3 independent experiments. Mean values ± SE obtained from a single experiment is presented (n = 4/experiment). * = $p < .05$ between indicated groups by ANOVA. ● = $p < .05$ compared to B[a]P, 0 mGy by unpaired t-test on that particular day, ♦ = $p < .05$ compared to No B[a]P, 0 mGy by unpaired t-test on that particular day.

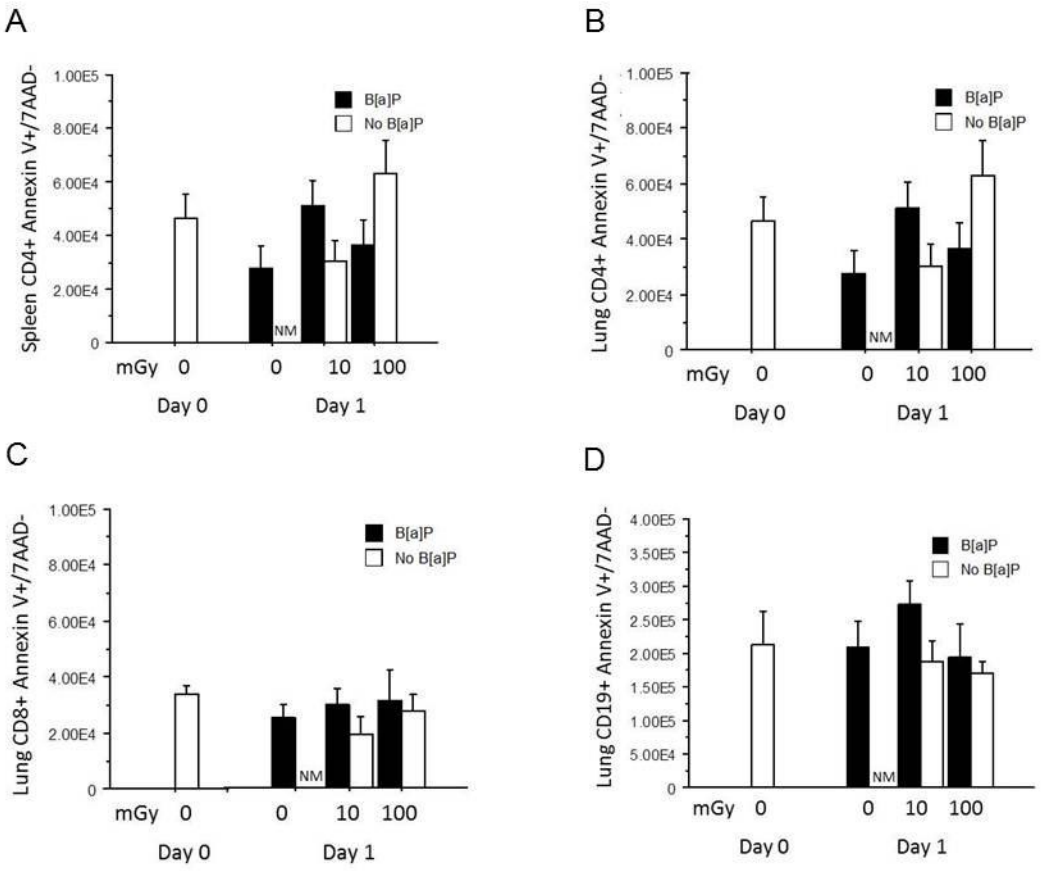

Figure 13. Apoptosis of spleen CD4+ (A) cells and lung CD4+ (B), CD8+ (C), and CD19+ (D) cells were determined by flow cytometric analysis. Mean values ± SE obtained from a single experiment is presented (n = 4 mice/group). NM = not measured.

Cytokine secretion and production

Establishing that B[a]P and LDR had effects on certain immune cell populations, we investigated changes in cell function as measured by mitogen-induced cytokine secretion. Mice were treated in the same manner as previously described and cytokines were measured from supernatants collected from splenocytes harvested on D2 or D7 after culture for 48 hours either in the presence or absence of the mitogen, Con A. Very little cytokine secretion was observed in unstimulated samples regardless of treatment (not shown).

Regardless of radiation dose, B[a]P significantly increased the secretion of IL-1β and IL-17 on D2 and D7, and IL-6 on D7 only (Figure 14). In contrast, B[a]P decreased secretion of anti-inflammatory cytokines IL-13 on days D2 and D7 (not shown) while IL-4 was decreased on D7 (Figure 15).

On the other hand, irradiation with 10 mGy gamma rays in the absence of B[a]P transiently increased IL-4 and IL-10 on D2, and IL-2 was elevated on both D2 and D7. The 100 mGy dose also increased IL-2 and IL-4 (D2) but not IL-10 (Figure 15). Increases were significant when irradiated mice were compared to non-irradiated mice. These findings illustrate the overall effects of B[a]P or LDR independently on splenocyte cytokine secretion.

We were also interested in how these cytokine profiles changed when B[a]P treatment and LDR exposure were combined. We compared mice treated with B[a]P and LDR to mice treated only with B[a]P and found that exposure to 10 mGy but not to 100 mGy gamma rays affected cytokine secretion. Anti-inflammatory cytokines IL-2, IL-4, and IL-10 were increased significantly by 10 mGy exposure in mice treated with B[a]P compared to mice receiving B[a]P only (D2, Figure 15). This was not surprising as we had already determined that this is an overall effect of radiation exposure. However, 10 mGy exposure increased pro-inflammatory cytokines IL-1β and IL-17, augmenting the effects of B[a]P in these mice on D2 (Figure 14). Due to an insufficiency of lung cells available for culture we were unable to assess cytokine secretion in lung cell supernatant.

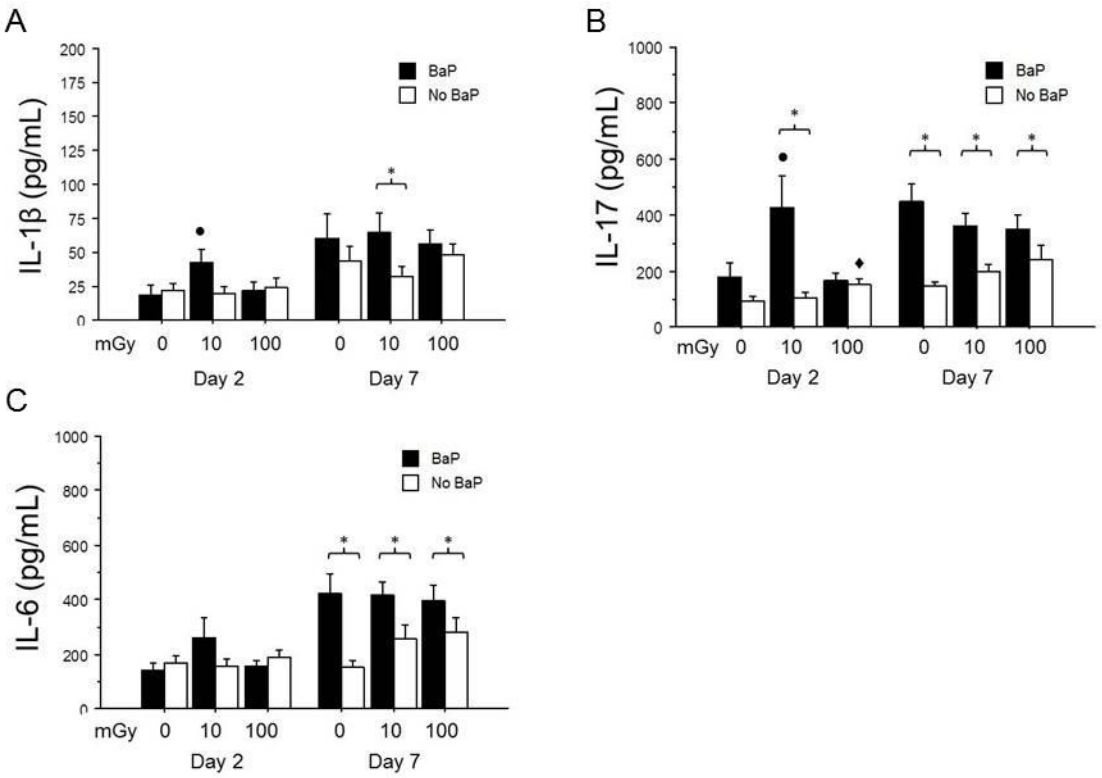

Figure 14. Spleen pro-inflammatory cytokine secretion. Secretion of IL-1β (A), IL-17 (B), and IL-6 (C) by splenocytes. Mean values ± SE obtained from two independent experiments are presented (n = 4 mice/group per experiment). * = $p < .05$ between indicated groups by ANOVA, ● = $p < .05$ compared to B[a]P, 0 mGy by unpaired t-test on that particular day, ♦ = $p < .05$ compared to No B[a]P, 0 mGy by unpaired t-test on that particular day.

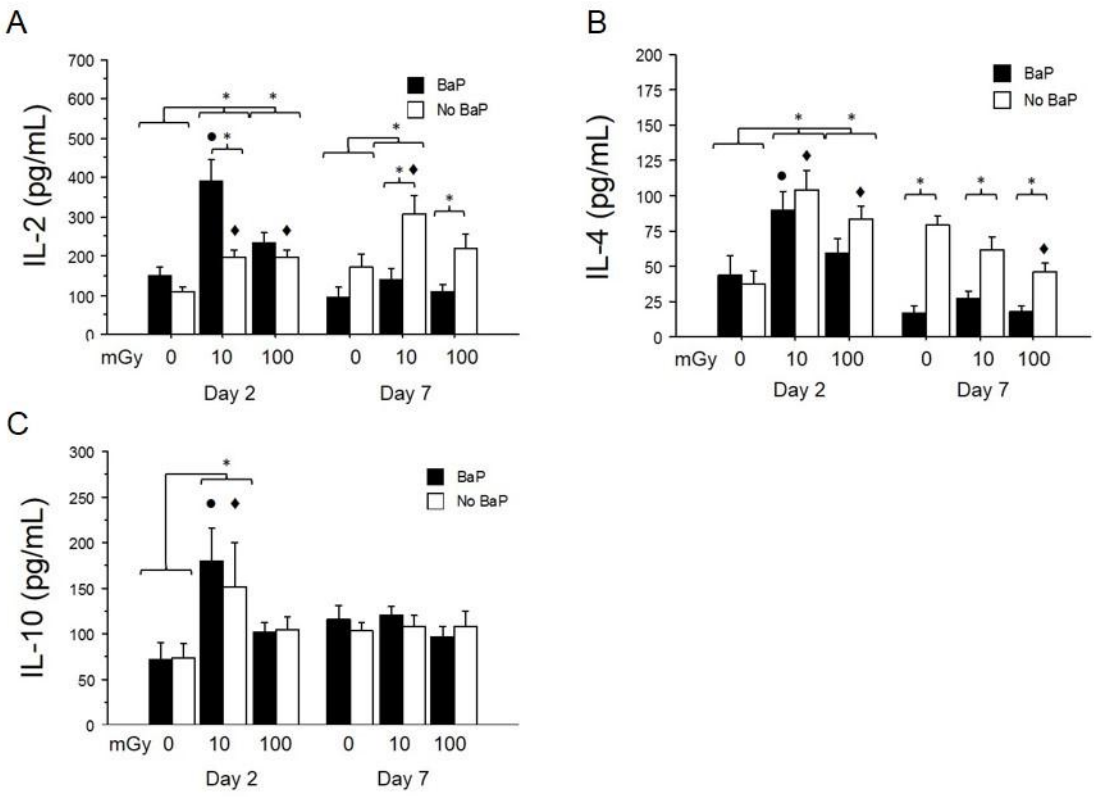

Figure 15. Spleen anti-inflammatory cytokine secretion. Secretion of IL-2 (A), IL-4 (B), and IL-10 (C) by splenocytes. Mean values ± SE obtained from two independent experiments are presented (n = 4 mice/group per experiment). * = $p < .05$ between indicated groups by ANOVA, ● = $p < .05$ compared to B[a]P, 0 mGy by unpaired t-test on that particular day, ♦ = $p < .05$ compared to No B[a]P, 0 mGy by unpaired t-test on that particular day.

Next, we examined cytokine production by individual cell types via intracellular flow cytometry (Figures 16 and 17 show the flow cytometric gating strategy associated with this analysis).

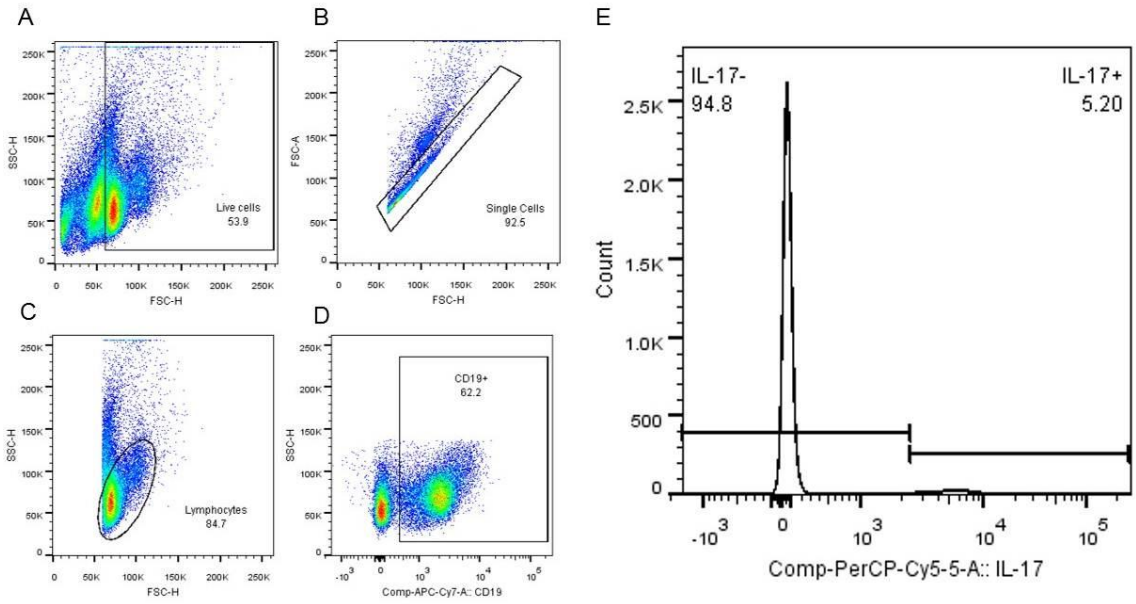

Figure 16. Example of spleen flow cytometry gating strategy for intracellular cytokine detection in B cells (CD19+). Live cells were gated to distinguish them from red blood cells and debris (Panel A). From the live cell gate, single cells were distinguished from doublets (Panel B). From the live cell singlet cells, lymphocytes were gated as side scatter low and forward scatter low to moderate (Panel C). Next, the percent of cells expressing specific cell surface proteins (i.e. CD19, Panel D) were determined from the live, singlet, lymphocytes. Gating was based on isotype control antibody staining levels (not shown) and was set such that the percentage of cells falling in the positive gate of fluoresceinated isotype control antibody stained cells was < 2%. Finally, the percent of live, singlet, lymphocyte cells (here CD19+ B cells) expressing IL-17 was determined (Panel E).

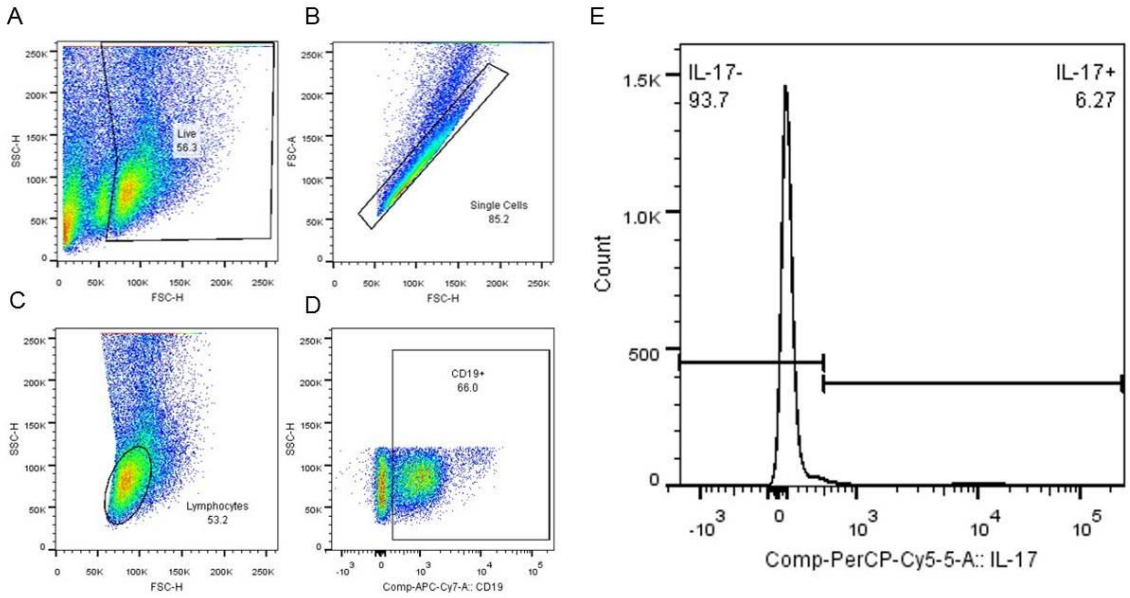

Figure 17. Example of lung flow cytometry gating strategy for intracellular cytokine detection in B cells (CD19+). Live cells were gated to distinguish them from red blood cells and debris (Panel A). From the live cell gate, single cells were distinguished from doublets (Panel B). From the live cell singlet cells, lymphocytes were gated as side scatter low and forward scatter low to moderate (Panel C). Next, the percent of cells expressing specific cell surface proteins (i.e. CD19, Panel D) were determined from the live, singlet, lymphocytes. Gating was based on isotype control antibody staining levels (not shown) and was set such that the percentage of cells falling in the positive gate of fluoresceinated isotype control antibody stained cells was < 2%. Finally, the percent of live, singlet, lymphocyte cells (here CD19+ B cells) expressing IL-17 was determined (Panel E).

We performed this experiment to address two issues; we wanted to know which cell types were responsible for cytokine secretion in the spleen but this also gave us an opportunity to look at cytokine production in the lung. Again, mice were treated as described previously (Figure 3). In the spleen B[a]P treatment increased numbers of CD8+ IL-1β+ cells (D2, not shown), Gr-1+IL-1β+ and CD19+IL-1β+ cells (D2 and D7), as well as CD8+ IL-17+ cells (D2, not shown), Gr-1+IL-17+ and CD19+IL-17+ cells (D2 and D7, Figure 18). We also

saw a significant decrease in CD4+ IL-13+ cells (D7, not shown) and a decrease of CD4+ IL-4+ cells (D7, not shown) induced by the carcinogen, although this latter finding was not significant (p = .0576). These findings are consistent with cytokine measurements from supernatants of stimulated splenocytes in response to B[a]P. We did not identify increases of any particular cell type producing IL-6 in response to B[a]P, or IL-2, IL-4, or IL-10 in response to LDR. Median fluorescence intensity (MFI) was also measured in populations responding to B[a]P treatment to determine if the carcinogen played a role in modifying a single cell's cytokine production capacity. We determined that B[a]P treatment did not affect MFI in splenocytes (Table 6). MFI was also not changed by exposure to LDR.

Table 6. Summary of B[a]P effects on cell number and MFI in spleen.

B[a]P effects in spleen			
Cytokine	Cell phenotype	Effect on cell number*	Effect on MFI *
IL-1β	CD8+	Increased (D2)	NC
	CD19+	Increased (D2 and D7)	NC
	Gr-1+	Increased (D2 and D7)	NC
IL-17	CD8+	Increased (D2)	NC
	CD19+	Increased (D2 and D7)	NC
	Gr-1+	Increased (D2 and D7)	NC
IL-13	CD4+	Increased (D7)	NC

* Compared to non-B[a]P treated regardless of irradiation dose.
NC = No significant change

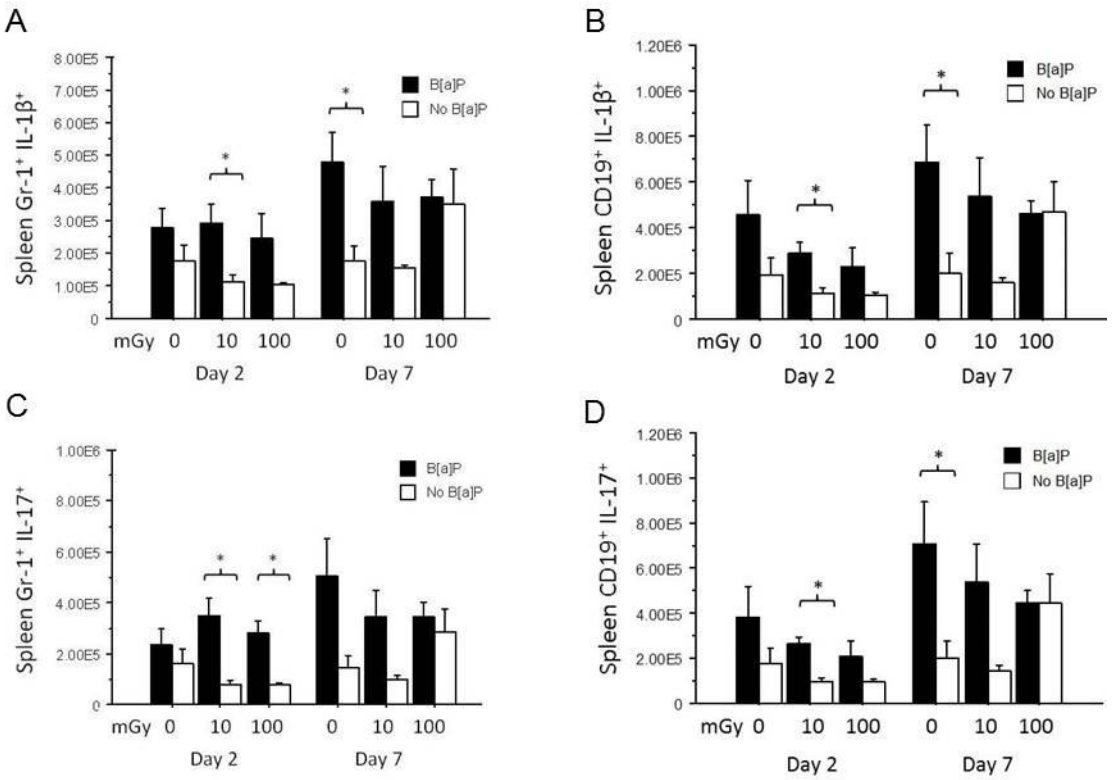

Figure 18. Spleen pro-inflammatory cytokine production. Splenic Gr-1+IL-1β+ (A), CD19+ IL-1β+ (B), Gr-1+IL-17 (C), and CD19+ IL-17+ cells (D Mean values ± SE obtained from a single experiment is presented (n = 4 mice/group).* = $p < .05$ between indicated groups by ANOVA.

In the lung we discovered that B[a]P was very stimulatory and creates a cytokine profile slightly different than we observed in the spleen. As expected, cells from mice treated with B[a]P produced pro-inflammatory cytokines (Figure 19). We observed increases in the following cell populations: CD19+ IL-1β+ and CD4+ IL-1β+ cells (D2 and D7) and CD8+ IL-1β+ (D2 and D7, not shown),; CD4+IL-6+ cells (D7); Gr-1+ IL-17+ (D2 and D7) and CD19+ IL-17+ cells (D7); CD19+ TNF-α+ (D7) and CD8+ TNF-α+ cells (D2). Interestingly, B[a]P also increased cells that produced anti-inflammatory cytokines (Figure 20). CD4+IL-

4+ and CD4+IL-13+ cells were increased by B[a]P (D2 and D7) as well as CD4+IL-10+ and CD8+IL-10+ (D2 and D7), and CD19+IL-10+ cells (D2, not shown). B[a]P not only increased the number of cytokine producing cells, but a higher MFI was also observed compared to non-B[a]P samples for several of the aforementioned (Table 7). These data indicate an elevated cytokine producing potential per cell of both inflammatory and anti-inflammatory cytokines by B[a]P in the lung.

Table 7. Summary of B[a]P effects on cell number and MFI in lung.

B[a]P effects in lung			
Cytokine	Cell phenotype	Effect on cell number*	Effect on MFI *
IL-1β	CD4+	Increased (D2 and D7)	NC
	CD8+	Increased (D2 and D7)	Increased (D7)
	CD19+	Increased (D2 and D7)	Increased (D2)
IL-6	CD4+	Increased (D7)	Increased (D7)
IL-17	CD19+	Increased (D7)	Increased (D2 and D7)
	Gr-1+	Increased (D2 and D7)	Increased (D7)
TNF-α	CD8+	Increased (D2)	NC
	CD19+	Increased (D7)	NC
IL-4	CD4+	Increased (D2 and D7)	NC
IL-10	CD4+	Increased (D2 and D7)	NC
	CD8+	Increased (D2 and D7)	Increased (D2)
	CD19+	Increased (D2)	NC
IL-13	CD4+	Increased (D2 and D7)	Increased (D2)

* Compared to non-B[a]P treated regardless of irradiation dose.
NC = No significant change

When the radiation effect was examined in cells from B[a]P treated mice, we observed an early and transient increase of CD19+TNF-α+ cells (D2) with respect to non-irradiated, B[a]P-treated controls. Otherwise, radiation overall (regardless of B[a]P), radiation alone, or in combination with B[a]P had no significant effect on cytokine production in lung cells. We also examined LDR's effect on MFI but no significant findings were detected.

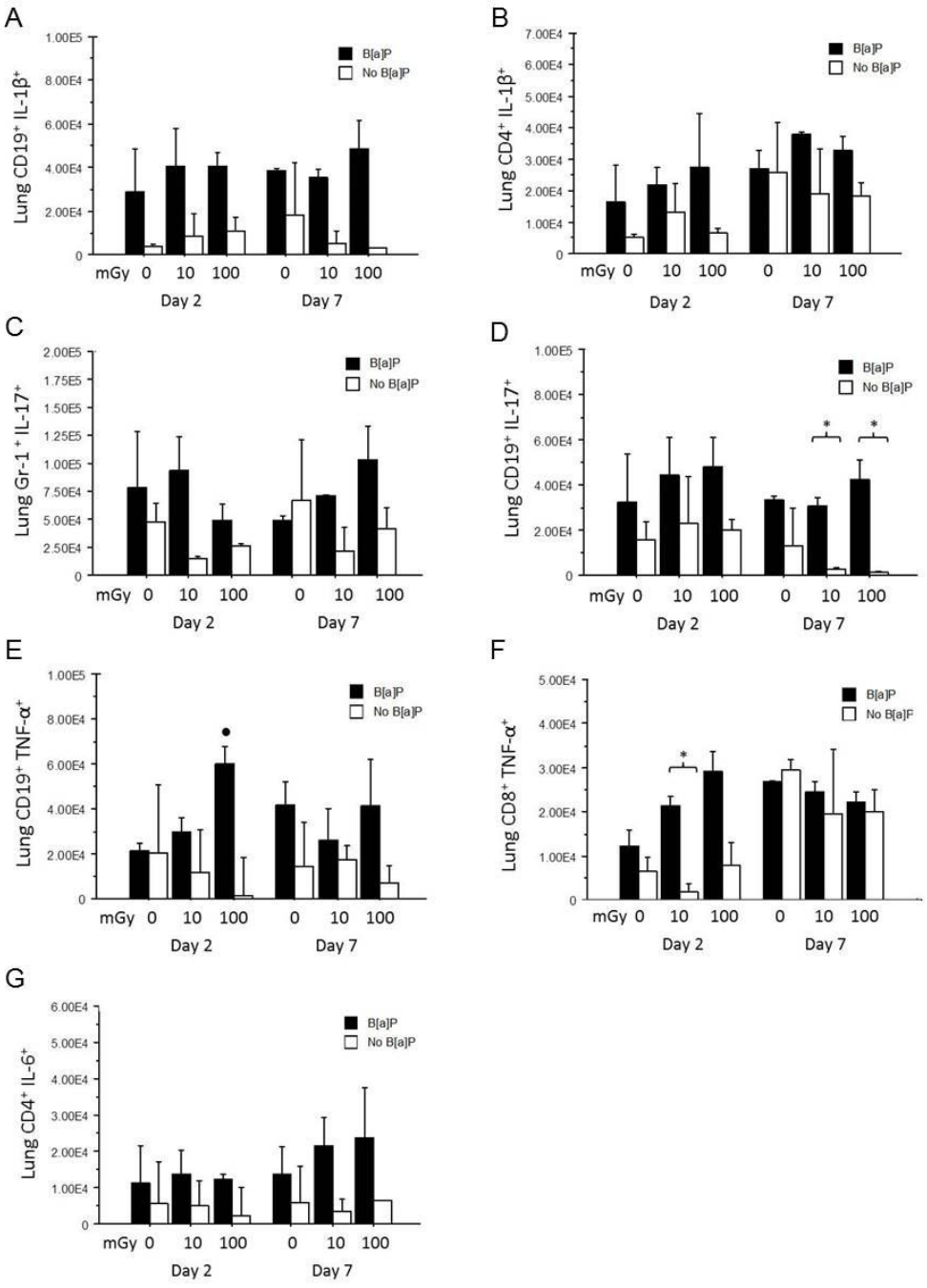

Figure 19. Lung pro-inflammatory cytokine production. Lung CD19+IL-1β+ (A), CD4+IL-1β+ (B), Gr-1+IL-17+ (C), CD19+IL-17+ (D), CD19+TNF-α+ (E), CD8+TNF-α+ (F), and CD4+IL-6+ cells (G). Mean values ± SE obtained from a single experiment is presented (pooled 2 mice/sample; n = 2/group). * = $p < .05$ between indicated groups by ANOVA, ● = $p < .05$ compared to B[a]P, 0 mGy by unpaired t-test on that particular day.

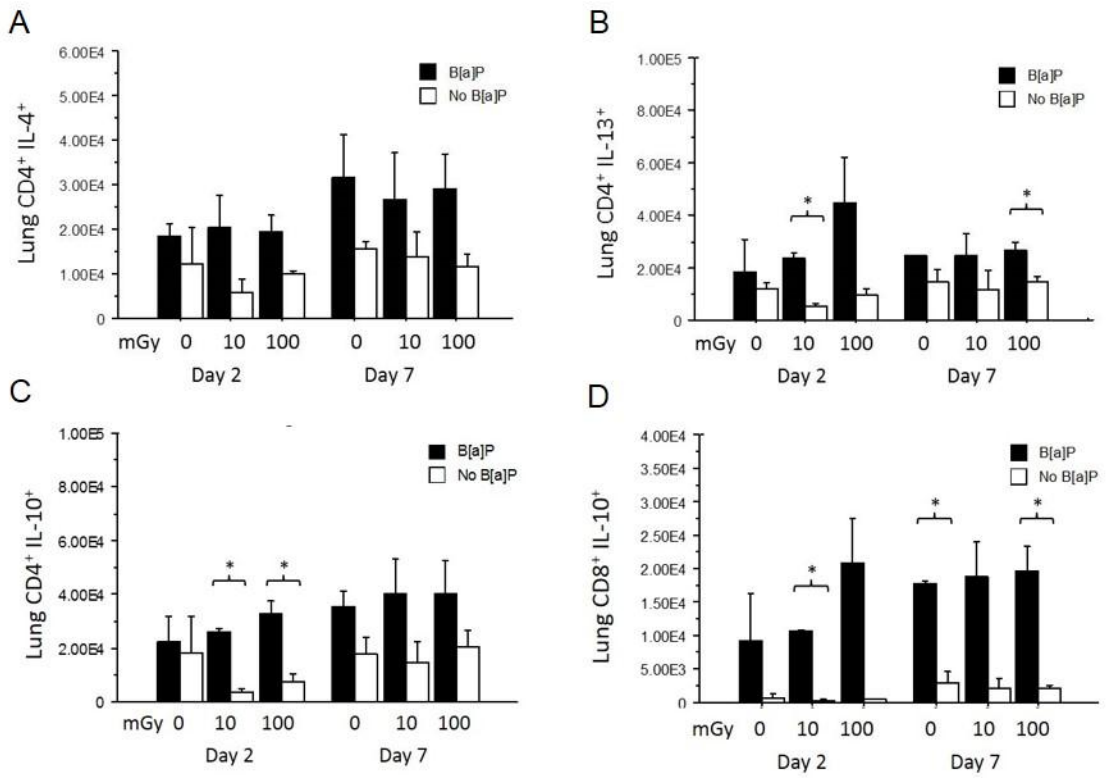

Figure 20. Lung anti-inflammatory cytokine production. Lung CD4+IL-4+ (A), CD4+IL-13+ (B), CD4+IL-10+ (C), and CD8+IL-10+ (D) cells. Mean values ± SE obtained from a single experiment is presented (pooled 2 mice/sample; n = 2/group). * = $p < .05$ between indicated groups by ANOVA.

Oxidative Stress

We investigated the effects of B[a]P and LDR on lipid peroxidation, a measure of oxidative stress, by utilizing the TBARS assay. TBARS (thiobarbituric acid reactive substances) are formed as a byproduct of lipid peroxidation, a product of oxidative stress damage. Mice were exposed to a single dose of 10 or 100 mGy gamma radiation one day prior to injection with 100 mg/kg B[a]P (Figure 4). On days two (D2) and seven (D7) post irradiation, spleen and lung tissue was harvested and lipid peroxidation was measured (Figure 21). On D7, TBARS were elevated in tissues from mice treated with

B[a]P regardless of irradiation. Western blots to detect proteins involved in the DNA damage response, γH2AX and phospho-p53 (Ser15), revealed that these proteins were not detected in spleens from B[a]P treated mice or naïve controls (Figure 22). Although radiation seemed to decrease TBARS in the lung in a dose-dependent manner (D2), this finding was not statistically significant. Radiation alone also did not affect TBARS levels overall nor did radiation reduce the elevated levels of TBARS in spleen or lung of B[a]P treated mice.

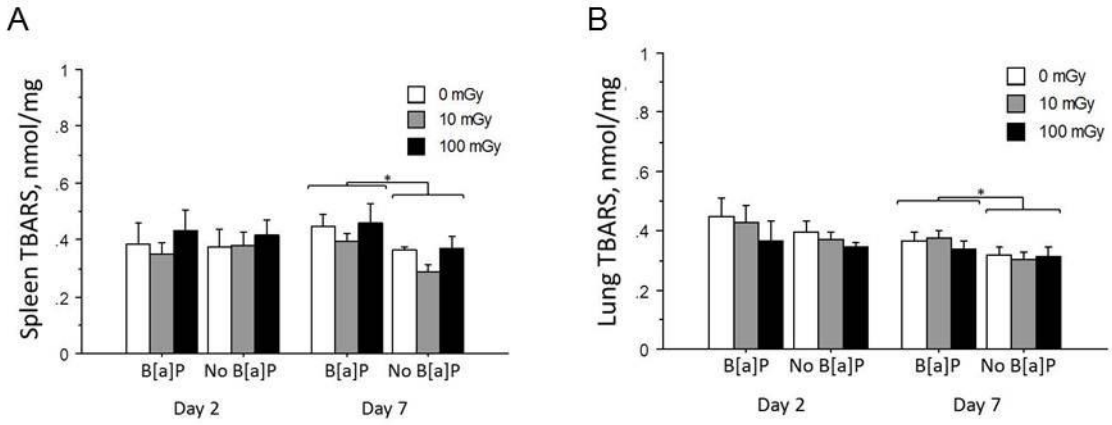

Figure 21. Lipid peroxidation in spleen (A) and lungs (B) of mice was measured using a standard TBARS assay. Mean values ± SE of nmol/mg of total protein obtained from two independent experiments are presented (n = 4 mice/experiment). * = $p < .05$ between indicated groups.

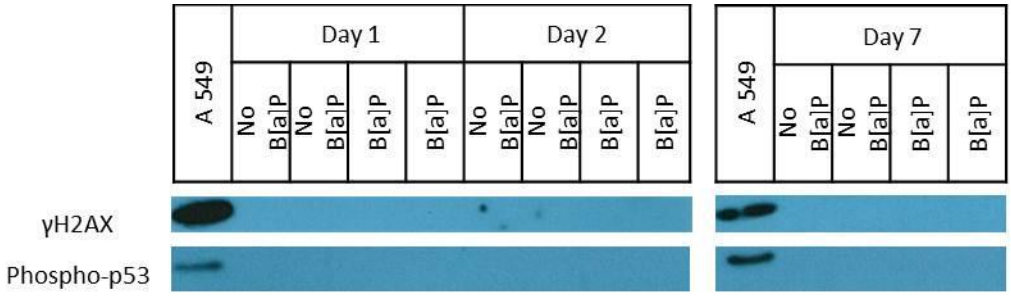

Figure 22. Western blot for γH2AX and phosphorylated p53 protein in spleen. Adriamycin-treated A549 cells are shown as a positive control.

CHAPTER 5. DISCUSSION

Summary

Is ionizing photon radiation exposure (e.g. gamma rays, X-rays) at low doses harmful? As the use of radiation increases in occupational and medical settings this is a valid question. In our studies we sought to investigate the effects of low dose gamma radiation on the development of lung cancer in an in vivo mouse model and to assess its potential as a treatment or prophylaxis against carcinogen-induced tumors.

Lung cancer is the number one cause of cancer-related death. The majority of deaths can be attributed to cigarette smoking due to the many reactive and mutagenic compounds found in cigarette smoke. We have utilized one of these compounds, benzo[a]pyrene, as a tool to induce primary lung tumors in the A/J mouse. This is an established lung cancer animal model that is useful for the study of potential lung cancer drugs and treatments. Previous human and rodent studies have shown that LDR can be effective against tumor growth and inflammatory disease (Cuttler & Pollycove 2003, Hildebrandt et al. 2003, Arenas et al. 2006, Calabrese & Calabrese 2013a, b). Thus, we hypothesized that low-dose gamma rays would suppress B[a]P-induced lung cancer when delivered as a treatment or inhibit B[a]P-induced lung cancer when delivered prophylactically. In addition, we hypothesized that the complex mechanisms involved in B[a]P-induced lung cancer would be mitigated by adaptive responses to radiation exposure.

To test our hypotheses we performed two studies, each 46 weeks in duration, to investigate carcinogenesis and tumor progression. This time point allowed us to observe lung tumors at all histological stages and to provide tumor DNA to assess Kras mutation. We also performed a series of short term studies to explore leukocyte cytotoxicity and cytokine secretion as well as oxidative stress in response to B[a]P or LDR alone, or the combination of these two treatments.

In summary, we found that bi-weekly fractionated doses of 100 mGy gamma radiation is sufficient to suppress tumor progression when administered as a treatment (one month following B[a]P) but did not prevent carcinogenesis altogether for the high level of B[a]P administered. Tumor Kras mutation frequency was the same among treatments, thus not the mechanism by which this protective phenomena occurs. LDR induced the secretion of anti-inflammatory cytokines alone yet it failed to mitigate the strong pro-inflammatory response by B[a]P. Radiation also did not reduce oxidative stress brought on by the carcinogen. Although LDR failed to mitigate the deleterious effects of B[a]P, we were able to show that radiation exposure at low doses does not alone induce lung cancer nor does it invoke inflammation or oxidative stress in our model.

Discussion of results

We showed that B[a]P readily induces lesions in our mouse model. In a cancer treatment study, 15.3 ± 2.27 (Average ± SEM, Figure 5A) lung tumors per mouse were grossly enumerated from mice treated with a single i.p. injection of

B[a]P (100 mg/kg) at the end of 46 weeks. This number was comparable to another 46 week long study performed by Anderson et al. (2008) in which 12.6 ± 1.9 lung tumors per mouse were grossly counted when B[a]P was also administered at 100 mg/kg i.p.

We also showed in our cancer treatment study that fractions of 100 mGy but not 10 mGy gamma radiation significantly reduced the number and multiplicity of adenomas in mice treated with B[a]P. As expected, B[a]P and/or its specific metabolites (e.g. BPDE, B[a]P quinones) induced lung cancer detected as hyperplastic lesions, adenomas, and carcinomas. In contrast, mice exposed to radiation alone and unexposed control mice developed no adenomas or carcinomas. Fractions of 10 mGy gamma radiation may have been too low of a dose and did not result in a reduction of B[a]P-induced lesions. When 100 mGy gamma ray fractions were given to mice injected with the carcinogen, we observed a significant reduction in the number of total adenomas and multiplicity of these lesions (2 or more) in the mouse lung. Carcinomas in mice treated with B[a]P averaged below a single lesion per mouse at the 46 week time point which made it difficult to see a further reduction or increase from radiation exposure.

In a cancer prophylaxis study we attempted to address this issue by fractionating B[a]P but increasing the final cumulative dose. By increasing the cumulative dose to 150 mg/kg we expected to induce a larger amount of lung tumors per mouse overall, thus increasing the probability of carcinomas. Surprisingly, tumor burden was not increased over what we had previously observed with 100 mg/kg B[a]P delivered as a bolus dose, however total lesions

and hyperplastic foci counted from B[a]P treated mice remained greater than those from control mice or mice exposed to radiation only (high dose, $p < .0001$; low dose, $p < .05$). Adenomas and carcinomas averaged below a single tumor per mouse in all treatment groups. Although the full doses of B[a]P were not given until 10 weeks into the study we believe that 36 weeks was still sufficient time for all B[a]P-induced tumor types to arise and develop by the end of study. Nevertheless, pre-conditioning with gamma radiation did not decrease tumor burden in either low or high dose B[a]P-treated mice nor did it suppress lung cancer progression like we observed in our cancer treatment study. Because adenoma and carcinoma counts were so low initially, a further reduction by LDR would have been hard to detect. Unfortunately we were unable to report the effects of LDR on carcinomas by the end of our studies. Since we would expect the number of carcinomas to be greater beyond 46 weeks, we cannot rule out the possible occurrence of a protective effect against this type of lesion should longer follow-up times be investigated.

In both long term studies, radiation alone did not provoke carcinogenesis as there was no significant difference between tumor numbers of control mice and mice exposed fractions of 100 mGy gamma rays. Because each dose fraction was within the low-dose range (< 200 mGy), our findings affirm the notion that a threshold for radiation related cancer risk may exist (Brenner & Raabe 2001, Ricci & Sammis, 2012). The data we have generated showed no increased lung cancers by LDR within 46 weeks however effects beyond 46 weeks were not measured. The number of subjects used in this study was

relatively small and it would be prudent to examine these effects with larger number of mice while extending the end of study beyond 46 weeks to examine long term radiation effects. This would increase the statistical power of this study as well as be important in testing if the low dose radiation effects would be the same in the long term: no increase in lung cancer risk and the protection against carcinogen induced cancers by suppression of tumor growth and progression.

Chemically induced lung tumors in mice share similar morphology and histology with human adenocarcinomas, making them a useful tool to study carcinogenesis. In humans, 30% of adenocarcinomas contain a mutation in the Kras gene; however these mutations are less common in other types of histologically-classified cancerous lesions (Wakamatsu et al. 2007). B[a]P readily creates adducts which lead to mutations in specific areas of Kras which may play a role in cancer development. Lung cancer associated mutations in the Kras gene have been documented in codons 13 and 61, but the majority of mutations have been found in codon 12 (Riely et al. 2009, Kim et al. 2013). B[a]P has been shown to induce mutations specifically in codon 12 but not in codons 13 or 61 (You et al. 1989, Gray et al. 2001). In an experiment by Gray et al. (2001), mice were injected with 100 mg/kg B[a]P and 10 Kras mutations were identified from 12 lung tumors. Ten of 12 tumors (83%) contained mutations in codon 12 whereas 0 of 12 (0%) contained mutations in codon 61. Seven of the 12 had a G → T point mutation while 3 of the 12 had a G → A point mutation (Gray et al. 2001). In our experiments we identified 5 of 9 (55%) of our tumor samples from B[a]P treated mice and 4 of 8 (50%) from B[a]P + 100 mGy treated

mice contained a mutation in Kras codon 12. This data demonstrates B[a]P mediated mutation in this specific gene region, but combined with LDR, radiation had no effect in reducing this mutation frequency. When aiming to identify a G → A mutation in Kras codon 12, we observed a 10% mutational incidence (1 out of 10 samples) in each treatment group.

In our studies we have shown that the LDR does not reduce the occurrence of B[a]P mediated Kras mutation nor does it increase these mutations found in mouse lung tumors. This data is supported by a rodent study in which mice irradiated with ≥ 1 Gy (1000 mGy) demonstrated Kras mutations in codons 12, 13, and 61, however no mutations in Kras were detected at the 0.25 Gy (250 mGy) dose (Nishimura et al. 1999). Thus, Kras mutation in response to radiation exposure may occur at higher doses but not within the low dose range. For a more thorough investigation of Kras mutation, we would need to sequence Kras codon 12 which is known to be a mutational hotspot in lung cancer as well as with B[a]P exposure.

In a series of short term mechanistic studies we showed that the B[a]P is cytotoxic to lymphocytes in lung and spleen tissues within the first week post-exposure. Preliminary data from splenocytes suggested that neither B[a]P nor LDR induced apoptosis by 4 and 24 hours post-exposure, respectively. In the lung, B[a]P decreased T cells, B cells, and DCs, but in the spleen only CD4+ T cells were reduced. Regardless, the total cell number in each tissue was unaffected. These deficits were likely made up over time by the recruitment of monocytes and neutrophils into the tissue. These cell types are recruited to sites

of inflammation through the up-regulation of soluble factors such as cytokines and chemokines, often to where tissue has been damaged. In response to radiation alone, a transient reduction in total lung cells were observed. We hypothesize that this is due to the elimination of radio-sensitive cell types (T cells and DCs) and not antigen mediated cell mobilization to different tissues. In addition, LDR failed to provide protection against B[a]P-mediated cytotoxicity for most leukocytes with the exception of CD8+ T cells and CD19+ cells in the lung, the loss of which was mitigated by delivery of the 10 mGy dose.

Since we observed effects on cell number by each treatment, we also wanted to know if there were effects on cell function that could help explain our findings on tumorigenesis. Previous studies have shown that cytokines can modulate the balance between tumor progression and immunity against tumors (Carmi et al. 2011). Podechard et al. (2008), showed that B[a]P leads to lung inflammation through the up-regulation of pro-inflammatory cytokines (e.g. IL-8) and neutrophilia, while Anandakumar et al. (2012) showed increased protein expression of IL-6, TNF-α, COX-2, and NF-κB in murine blood. Other studies have shown that low-dose radiation can dampen the inflammatory response in both animal and human populations (Arenas et al. 2012, Sanders 2012, Kataoka 2013). With this in mind we hypothesized that LDR would mitigate B[a]P-induced inflammation by reducing pro-inflammatory cytokines.

In support of our hypothesis, B[a]P stimulated the secretion of pro-inflammatory cytokines from splenocytes as well as mediated the production of these cytokines by leukocytes in both spleen and lung tissues. At 24 hours post-

injection, we observed that B[a]P stimulated splenocyte secretion of IL-1β and IL-17, likely produced by CD19+, Gr-1+, and CD8+ cells. IL-6 secretion was also elevated but we did not identify the cellular source responsible for producing this cytokine. As a secondary lymphoid organ, the spleen allows us insight as to what is occurring systemically in the immune system so we anticipated a similar response in the lung which is the primary organ B[a]P affects. In the lung, IL-1β, IL-17, and TNF-α were produced early by T cells, B cells and Gr-1+ cells and remained increased by six days post B[a]P injection. In addition to these, IL-6 was induced at the later time point in CD4+T cells. We also observed an increased cytokine production capacity per cell when lung cells were examined but failed to observe this in splenocytes which reflects the potential differences in metabolism of this carcinogen in the lung compared to other tissues.

Cytokines like IL-1β and IL-17 are important in recruiting neutrophils and monocytes to sites of inflammation through the up-regulation of chemokines and adhesion factors (Carmi et al. 2011). In a separate study, our colleagues determined that BPDE stimulates the secretion of the chemokines CXCL1 and CXCL5 from human lung fibroblasts and induces IL-6 secretion via NF-κB activation (Chen et al. 2012). This combination of pro-inflammatory cytokines and chemokines are conducive for the recruitment of neutrophils (Sims & Smith 2010, Liu et al. 2011). Therefore, we believe that production of pro-inflammatory cytokines is related to the cytotoxicity of lymphocytes and subsequent recruitment of inflammatory cells caused by B[a]P.

Pro-inflammatory cytokines can be produced by many different cells types to drive inflammation in response to tissue damage (Murugaiyan & Saha 2009, Sims & Smith 2010). Recent studies have shown that TNF-α, along with IL-1β, have the ability to upregulate iNOS (inducible nitric oxide synthase) and COX-2, and enhances the stability of B[a]P-induced DNA adducts leading to its carcinogenic potential (Umannova et al. 2011). TNF-α can also perpetuate inflammation through increased NF-κB signaling, IL-1β and IL-6 expression.

Transcription factors such as NF- κB, or STATs, are critical in the transcription of inflammatory cytokines and pathway activation/inhibition is one way these cytokines can be regulated. In other instances, regulation of these pathways and subsequent inflammatory processes may be caused by epigenetic modifications such as histone methylation or acetylation (Lotem & Sachs 2002, Bayarsaihan, 2011). Ligand/receptor binding is another mechanism by which these cytokines may be regulated. For instance, B[a]P has been identified as a ligand for the aryl hydrocarbon receptor (AhR), a known regulator of both innate and adaptive immune processes, and can regulate IL-6 and TNF-α expression via the AhR pathway (Hur et al. 2013). Interestingly, these are cytokines we observed increased in our mouse model with B[a]P treatment.

The types of cytokines induced by B[a]P in our studies, are associated with increased ROS/RNS production which can potentiate a state of inflammation favoring tumor development and progression (Azad et al. 2008). A strong correlation exists between increased oxidative stress and lung cancer risk in humans (Ray et al. 2012). Sustained oxidative stress can further induce DNA

damage and elevate the probability of cancer inducing mutations. ROS/RNS can also lead to further production of pro-inflammatory cytokines, continuing the cycle of inflammation. For instance, nitric oxide (NO) can induce COX-2 to amplify the production of IL-1β and TNF-α (Umannova et al. 2011). Additionally, oxygen radicals such as superoxide ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) can upregulate adhesion molecules to enhance neutrophil recruitment in areas of inflammation (Fraticelli et al. 1996). Recruited phagocytes are considered a primary source of ROS/RNS production and can perpetuate an inflammatory response potentially leading to a state of oxidative stress (Martin et al. 1998).

In our model, B[a]P significantly elevated lipid peroxidation (oxidation of lipids by ROS) by D7 in both spleen and lung tissue which corresponded with elevations in inflammatory cytokine production and neutrophil recruitment into tissues. Components of lipid peroxidation can also lead to subsequent DNA damage however we did not find evidence of elevated γH2AX or phospho-p53(Ser15) in spleens from B[a]P treated mice. Therefore we do not believe that increased ROS/RNS induced significant DNA damage in our study, at least at the time points we investigated.

In addition to the inflammatory response we expected, B[a]P suppressed anti-inflammatory cytokines production by splenocytes. IL-4 and IL-13, capable of polarizing T cells toward a T-helper type 2 (Th2) response, were decreased in spleen cell supernatants. This was corroborated by a reduction in the number of IL-4 and IL-13 producing splenic CD4+ T cells. In excess, T-helper type 1 (Th1) cytokines (e.g. IFN-γ) can counteract a Th2 response including the production of

IL-4 and IL-13. Elevated levels of Th1 cytokines were not detected in supernatants, thus we do not believe this effect is due to Th1 polarization of cells (not shown). However in the lung we observed quite a different response to B[a]P. Cytokine secretion by lung cells was not measured, but we were surprised to see increases in anti-inflammatory cytokine producing CD4+ and CD8+ T cells and CD19+ cells after B[a]P treatment. These data suggest tissue specific immune responses. We hypothesize that increased anti-inflammatory cytokine secretion in response to B[a]P could be linked to COX-2, an enzyme generally involved in inflammation. As part of its inflammatory response B[a]P and BPDE can induce the expression of COX-2 (O'Byrne & Dalgleish 2001, Dreij et al. 2010, Umannova et al. 2011, Anandakumar et al. 2012). However, it has been shown that Th2 cytokines are also produced in COX-2 expressing environments. Current literature has shown that induction of IL-4 and IL-10 by CD4+ T cells acts to suppress further inflammation by down-regulating COX-2 and other pro-inflammatory cytokines (O'Byrne & Dalgleish 2001, Brown & DuBois 2004). Although COX-2 was not measured in our study, we postulate that the anti-inflammatory cytokine production is related to COX-2 induction in the lung.

Chen et al. (2012) showed that exposing BPDE-treated fibroblasts with low-dose gamma rays inhibits IL-6 secretion via suppressing NF-κB signaling. They also observed a decrease in STAT3 activation of BPDE-treated human bronchial epithelial cells when incubated with conditioned media from irradiated fibroblasts (Chen et al. 2012). These results lead us to believe that LDR can

induce the secretion of soluble factors involved in cell signaling which can regulate inflammation by B[a]P and its metabolites.

Exposure to LDR increased overall secretion of anti-inflammatory cytokines in the spleen but most effects were short lived and were only observed on D2 post-radiation. The 10 mGy dose increased IL-4, IL-10 and IL-2 whereas the 100 mGy dose increased secretion of only IL-4 and IL-10. IL-4 is produced mainly by T cells and can facilitate a Th2 type immune response. Th2 cytokines such as IL-4 and IL-13 play an important role in B cell activation and antibody production. This response may be beneficial in mounting an antibody response against tumor antigens (i.e. MUC-1 in lung cancer) but this function would need to be tested in our model (Reuschenbach et al. 2009). It is interesting that both doses enhanced the secretion of the same cytokines, however neither dose of radiation inhibited pro-inflammatory cytokine secretion in mice treated with B[a]P. Intracellular evidence of anti-inflammatory cytokine production was also not detected in lung T cells, although cytokine secretion was not measured.

Our findings are different than those of other groups who have reported increased secretion of Th1 but not Th2 cytokines subsequent to LDR-mediated immune stimulation. Th1 cytokines such as IFN-γ and IL-12 along with inflammatory cytokines have been implicated in the increased NK cell cytotoxicity and macrophage activation against implanted (i.e. foreign) tumor cells in rodents induced by LDR treatment (Liu 2003a,b, 2007, Nowosielska et al. 2011). These variations in findings are possibly due to the method of cancer induction: foreign tumor cell implantation versus chemical carcinogenesis of primary tumors, and/or

to immunological differences which are inherent to different mouse strains. For example, a commonly used strain, C57BL/6 elicits a predominately Th1 immune response whereas the A/J mouse strain used in our model elicits a predominately Th2 immune response (Sellers et al. 2012). Still, we chose to utilize the A/J strain because it is commonly used in toxicological studies and its natural susceptibility to carcinogen induced lung tumors (Belinsky et al. 1992, Malkinson 1992, Wakamatsu et al. 2007). It is prudent to take these factors into account when designing and interpreting these type of studies.

Low-dose radiation itself did not induce lipid peroxidation in our model. But neither did it protect against nor relieve carcinogen-induced oxidative stress. Because there was no change in lipid peroxidation induced by LDR overall, we did not investigate its association with γH2AX or phospho-p53 (Ser15) changes in mice who received radiation only. While ROS/RNS still may play a role in B[a]P-induced carcinogenesis we do not believe that relief of oxidative stress is a mechanism by which LDR may be suppressing tumor progression. Therefore, our data did not support our hypothesis that LDR would dampen the inflammatory response induced by B[a]P.

Other mechanisms or cell populations may be responsible for the suppression of lung cancer in response to LDR. In our cancer treatment model, irradiations were given during the development of hyperplastic foci. Therefore, LDR may have acted directly on growing hyperplasias to suppress their growth into adenomas. LDR may have exerted its effects through suppressing cell division, or by inducing cell senescence or apoptosis (Sun & Yang 2010). To test

these parameters, a commonly used method such as immunohistochemical (IHC) staining of tissues would be suitable. Proliferation markers such as nuclear antigen (PCNA) or Ki-67 can be used to examine tumor cell proliferation between treatment groups: B[a]P versus B[a]P + LDR treated mice. Increased expression of PCNA is associated with proliferating cells in the G1 and S phase and is reduced in G2 and M phase. Heightened levels of PCNA staining has been observed in lung tumors from mice treated with B[a]P (Anadakumar et al. 2009, Kamaraj et al. 2009). Increased apoptosis of tumor cells may be another testable mechanism of LDR suppression of lung tumor progression. Again, sections of tumors can be assayed using IHC staining for markers of apoptosis (i.e. TUNEL). To investigate tumor cell senescence, β-galactosidase expression can be measured in tumors and compared amongst treatments. This method can be used in our mouse model for B[a]P-induced lung tumors however it is anticipated that multiple time points would need to be studied which would also increase the number of mice needed on study.

Another model in which radiation effects on lung tumors can be performed is using a xenograft model in which lung tumors would be implanted into an immunocompromised mouse host. Xenograft mouse models are helpful in investigating the effects of drugs and therapies on cancers (Richmond & Su 2008). Because established tumor cells are used (primary or cell lines), this cuts back on the time needed for neoplastic transformation to occur in normal cells and may provide some convenience. For lung cancer, various methods of implantation ranging from tail vein or subcutaneous injection to intrathoracic

injection have been used (Cui et al. 2006, Harris et al. 2011). The tail vein method has been used to study radiation effects with some success on tumor cell metastases to the lung (Nowosielka, 2010). However, the intrathoracic injection method may provide a slight advantage in that this does not rely on metastasis to the lung but would place the tumor cells directly in the tissue. Although this may be an alternative to our A/J model, because immunocompromised mice are used, this would not be a good model to thoroughly study any potential immune effects that LDR may have on tumorigenesis. In addition, the use of foreign cells in an immunocompetent strain could also be an issue since they be more easily recognized by the immune system.

In response to LDR, soluble factors such as TGF-β, are also known to play a role against tumor progression and can be secreted by immune and non-immune cells surrounding the tumor (i.e. fibroblasts, endothelial cells). This cytokine has been implicated in ROS mediated tumor cell apoptosis and has been found upregulated in mice resistant to cancer (Bauer 2007, Zhang et al. 2015). Although TGF- β was not examined in our current study, excision of lung tumors and cells in their microenvironment from fresh tissue followed by placement in culture and measurements of TGF- β in supernatants by ELISA could be accomplished in future studies. Because it is unknown whether radiation may be having an effect directly or indirectly on the lung tumors, modulations in tumor cell processes as well as changes in the tumor microenvironment by LDR should be examined in the future.

Limitations

In our studies it is important to acknowledge and understand the limitations that potentially impacted our outcomes. One of the challenges we encountered in our studies was the constraint of time. Naturally, primary tumors in mice take a considerable amount of time to develop, especially carcinomas for which development can extend beyond the 46 weeks we allotted for our long term studies. We attempted to address this issue by fractionating B[a]P to increase tumor induction and subsequent adenoma and carcinoma growth without extending our study timeline. However, in our cancer prophylaxis study, fractionated B[a]P treatment failed to induce a large number of lung tumors, particularly adenomas and carcinomas. For example, 25 mg/kg B[a]P given six times bi-weekly (150 mg/kg cumulative dose) resulted in an average of 5.7 histoproliferative lesions per mouse and 0.9 adenomas/mouse. Hecht et al. (1994) used fractionated doses of B[a]P weekly for eight weeks resulting in an average of 7.2 adenomas per mouse at the end of 26 weeks. Considering the longer intervals between B[a]P treatments in our study, we hypothesize that the low tumor burden observed from our experiment is a result of B[a]P being efficiently metabolized and cleared between injections before reaching a cumulative dose for optimal carcinogenicity. For future studies, to observe more advanced tumors with fractionated B[a]P treatments, i.p. injection of B[a]P within shorter time intervals may be prudent. It may also be beneficial to use a larger number of animals and to extend the study timeline out further than 46 weeks to

observe higher numbers of carcinomas as well as radiation effects on tumors at longer time points.

Aside from pulmonary tumors, we did observe a number of non-pulmonary proliferative lesions in our cancer treatment study which may have contributed to the early deaths of 12 mice. Non-pulmonary lesions were also found in mice at the conclusion of the 46 week study. These could be classified as gastric squamous cell hyperplasia or carcinoma, fibrosarcoma, or other sarcoma. The occurrence of non-pulmonary lesions did not come as a surprise as B[a]P can induce these types of lesions when delivered i.p. or subcutaneously (Hecht et al. 1994, Culp et al. 1998, Balansky et al. 2006). At the end of 46 weeks, non-pulmonary lesions consisted mostly of abdominal squamous cell hyperplasia and carcinoma. B[a]P treated mice exhibited 3 lesions compared to 8 lesions in mice which had also received fractions of 100 mGy. When statistically analyzed using Fisher's Exact Test, this increase in non-pulmonary lesions was not statistically significant ($p = 0.085$). It is possible that radiation may have enhanced the incidence and progression of B[a]P-induced proliferative lesions in mice however this did not impact survival.

Finally, we found it impractical to utilize a long term study design to identify testable mechanisms. Therefore we adopted a short one week study design in which we could test the effects of B[a]P, LDR, or the combination in parallel with our long term studies. This method provided a means to quickly generate data but it is not clear if our findings were a result of an acute response or the initiation of a chronic response. Although we did not observe many radio-

adaptive effects to a single dose of 100 mGy in our short term studies, we did not measure these effects after exposure to multiple fractionated doses of 100 mGy. It is possible that results would differ under these circumstances. To further investigate mechanistic findings, these could be tested utilizing our cancer treatment study design; measured after each treatment and after multiple treatments. It would be very interesting to see if each treatment induced a transient and acute response or if these responses compounded into a cumulative effect at the end of the treatment regimen.

Implications for future research

Our research contributes to the existing knowledge of the effects of Benzo[a]pyrene and presented a potential application for the use of low dose gamma rays or a similar type of radiation (e.g. X-rays).

In our study we further characterized the effects of this carcinogen in the A/J mouse model of lung cancer currently being used in cancer research. In the A/J mouse we showed the histological profile of tumors expected within a year of a single high dose (100 mg/kg) or bi-weekly, fractionated doses (150 mg/kg or 75 mg/kg total dose) of injected B[a]P. Immediate cytokine responses by leukocytes in addition to lipid peroxidation in target tissues were also described. This data may be important when considering the use of this model to test lung cancer drugs and treatments in the future.

We were unable to identify the specific mechanism behind our observations, but have shown that fractionated exposures to 100 mGy can be an

effective treatment against B[a]P-induced tumor progression. This was shown in a carcinogen-induced animal model without the use of injected tumor cells which allows for the natural interactions of the body's cells to occur throughout the carcinogenic process. Although this effect was small, it was significant and it provides a basis for further research of low-dose radiation as a feasible therapy against lung cancer. Therapeutic and/or preventative potential uses of LDR exposure are currently being studied for arthritis, diabetes, and various cancers (Sakamoto et al. 1997, Wang et al. 2008. Calabrese & Calabrese 2013a,b.). LDR therapy alone may not effectively cure cancer, but may be an effective co-treatment in addition to those currently employed.

Importantly, we showed that radiation alone does not increase lung cancer incidence or tumorigenesis in mice. Although more work needs to be performed in the arena of low dose radiation research, we feel that this finding adds to the bodies of evidence that brings into question the use of the LNT model for all types and doses of radiation (Jaworowski 1997, Aurengo et al. 2005, Tubiana et al. 2009, Averbeck 2009). The LNT model assumes that the smallest amount of radiation increases cancer risk proportionally. Even though there is a lack of empirical evidence suggesting that radiation at low doses cause cancer, this assumption has caused concern among the general population and has raised fear of radiation exposure at any dose. It has been reported that some individuals are choosing to forego potentially lifesaving medical tests (i.e. X-rays, CT scans) due to radiation fears (Scott et al. 2008). Radiation doses from the average chest X-ray or CT scan fall well within the low dose range, yet we have

shown no increase in lung cancer risk at these doses. Our studies support current data that radiation, especially at low doses and dose rates, may not be as harmful as some people have been led to think (Aurengo et al. 2005, Luckey 2006). Nonetheless, one of the main challenges is translating our findings in a mouse model to the reality of human cancer risk. It is important to acknowledge that genetic variation, lifestyle, and environmental factors may confound attempts at distinguishing an individual's risk for cancer due to radiation exposure alone.

Conclusions

The data presented here has shown that LDR has the potential to be utilized as a treatment for lung cancer. Low-dose gamma radiation alone does not increase lung cancer risk and suppresses tumor growth in mice treated with a carcinogen to exhibit primary tumors. These findings support our hypothesis that exposure to low dose gamma rays would suppress the progression of lung cancer. However our hypothesis that LDR suppresses lung cancer progression through reduction in inflammatory processes or Kras mutation was not supported in our study. Neither did we find that LDR was effective against preventing carcinogenesis. The mechanistic studies we performed were limited in that we only assessed effects out to a single week post-exposure, however when taken in the context of our cancer treatment study, radiation effects have the potential to be manifested differently. At this point we are unable to tell if effects by radiation occur only acutely or if these become prolonged or even compounded after multiple exposures. This is a question we must address in the future.

Because we were unable to resolve the specific radio-adaptive response mechanisms at this time, we believe that additional studies are needed to clarify the mechanisms behind our finding that LDR potentially protects against cancer progression. It is also possible that immune modulation and changes in oxidative status cannot explain our findings. Alternatively, direct effects on tumor cells and other factors in the tumor environment have yet to be studied using our model. Studies extending beyond 46 weeks would also be needed to examine the long term effects of fractionated radiation exposure. Nevertheless, we believe that our findings are a valuable contribution to the field of radiation research.

Although the effects by radiation in our studies were small, the evidence we have presented supports the existence of a threshold or hormetic model for biological effects but does not support the no-threshold model. Much of the literature supporting these alternative models has been derived from in vitro and animal studies or from modelling of epidemiological data in areas of high natural background. Therefore the challenge lies in providing empirical evidence in humans that support this data. Although we know that cancer risk increases at high radiation doses, it is currently unknown what the exact implication of genetic and epigenetic variation has on an individual's biological response to radiation even at low doses.

In this body of work, we have also further described the carcinogenic potential Benzo[a]pyrene and its associated inflammatory processes in our mouse model. We have described the consequences of a single high dose and fractionated bi-weekly doses of B[a]P that occur within a year of administration.

Both of these treatment schedules were effective at inducing primary lung tumors of all histological stages: hyperplastic foci, adenomas, and carcinomas. Shortly following B[a]P injection, we were able to show the carcinogen's immediate impact on cells of the immune system. It has already been established that B[a]P promotes inflammation and we have confirmed this by showing that both lung and splenic leukocytes contribute to this phenotype by producing pro-inflammatory cytokines which correlate with increased oxidative stress and the recruitment of inflammatory cell types in these tissues. Interestingly, we have also shown that there are tissue specific differences regarding anti-inflammatory cytokine production. This information may be of value when utilizing B[a]P and the A/J mouse model of lung cancer to investigate potential treatment targets.

Radiation sources surround us so we are constantly being exposed to small doses daily throughout life. In addition to this daily exposure, the use of radiation in industry and medicine is increasing. Radiation can be a powerful tool and its biological effects are dependent on dose, dose rate, and type. As a result, its use is tightly regulated. In our studies we have examined the biological effects of low-dose gamma radiation in a carcinogen-induced mouse model of lung cancer. Our findings are consistent with the view that low-dose ionizing photon radiation (e.g. gamma radiation and X-rays) is unlikely to cause lung cancer and may provide health benefits. This work supports the continued research of LDR in animal models to identify radio-adaptive mechanisms in anticipation of human treatment in the future.

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