Investigating the Immune Modulating Effects of Low Dose Ionizing Radiation as a Potential Cancer Therapy

By

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science (MSc) in Chemical Sciences

The Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Office of Graduate Studies/Bureau des études supérieures

Title of Thesis
Titre de la thèse: Investigating the Immune Modulating Effects of Low Dose Ionizing Radiation as a Potential Cancer Therapy

Name of Candidate
Nom du candidat: Valiquette, Manon

Degree

Diplôme: Master of Science

Department/Program
Département/Programme: Chemical Sciences

Date of Defence
Date de la soutenance: May 30, 2023

APPROVED/APPROUVÉ

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Abstract

Ionizing radiation is an established treatment modality for cancer. Unlike targeted high dose therapies, there is growing evidence that low dose radiation (LDR) can promote tumor reduction indirectly via stimulation of the immune system. Natural killer (NK) cells are one of the main immune cells that have been implicated in LDR induced anticancer effects. Despite this, the exact cellular and molecular mechanisms responsible for the modulation of the immune system following LDR, including the stimulation of NK cells and their cytotoxic properties, have not yet been identified. The goals of this study were twofold; to elucidate the cellular mechanisms involved in LDR immune stimulation and to investigate the dose response for NK cell cytotoxicity. Initially, a small animal model was used to comprehensively characterize the dose dependent effects on various immune cells. Mice were exposed to whole-body x-ray doses of 0.1, 0.25, 0.5 and 3 Gy and were sacrificed two days post-irradiation for isolation of their spleen, lymph nodes and blood. Flow cytometry was then used for immunophenotyping to identify potential shifts in the relative abundance of major immune cell populations. Data from this cohort of mice suggested that 2 days following an acute single exposure, LDR caused no significant changes in the numbers of the immune cell types tested, and that high dose radiation (HDR) caused a decrease in the cell populations of these cells in the irradiated mice. To further investigate the impact of LDR on the NK cells in-vitro, the NK-92 cell line was used. NK-92 cells were exposed to x-ray doses ranging from 0.1 to 1 Gy, and cell growth rates were measured post-radiation. NK cell cytotoxicity was then quantified through the co-culturing of NK-92 cells with the tumorigenic K-562 cells, and the percent cytotoxicity was measured using flow cytometry. Lastly, transcriptional analysis was performed on the main genes involved in regulating NK cytolytic activity. Overall, data showed that LDR did not cause any significant increase in the growth, cytotoxicity and gene expression of NK-92 cells. To conclude, although no evidence of immune stimulation was found

following LDR in both the mice model and NK-92 cell line, this study was successful in providing a good characterization of the baseline immune response to lower doses of radiation in healthy models.

Key Words

Cancer, immune system, ionizing radiation, low dose radiation, high dose radiation, natural killer cells, cytotoxicity, flow cytometry

Acknowledgements

My academic experience here at the Northern Ontario School of Medicine and at Laurentian University has been unforgettable thanks to many individuals who have helped me throughout my journey. Firstly, I'd like to express my sincere gratitude to Dr. Christopher Thome, my supervisor at the Northern Ontario School of Medicine for his continuous guidance, support, motivation and immense knowledge. Dr. Thome was always available to me regarding any questions and concerns I had. Moreover, I would like to thank Dr. Sujeenthar Tharmalingam for all of his help and guidance in the lab. He taught me many lab techniques I was unfamiliar with, including qPCR, and has been very insightful and helpful in making me a more productive and organized student in a laboratory setting. I would also like to acknowledge and thank my committee members Dr. Alain Simard and Dr. Vasu Appanna for their time, support and helpful advice. They have been important in ensuring my progress throughout my MSc by attending meetings and reviewing my MSc thesis. Furthermore, I would like to thank Dr. Jake Pirkkanen, Dr. Christine Lalonde and Dr. Ramya Narendrula at Laurentian University for their assistance and helpful advice as they always went above and beyond to lend a hand when I needed it. Moreover, I would like to thank my lab colleagues: Jessica Dougherty, Alex Jameus, Taylor Laframboise, Graysen Vigneux and Mike Lapointe for their constant support and all of their help throughout my master's thesis.

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APC: antigen presenting cell

BCR: B cell receptor

CSF: colony stimulating factor

CTL: Cytotoxic T lymphocyte

DC: dendritic cells

DNA: deoxyribonucleic acid

ECM: extracellular matrix

EM: electromagnetic

Gy: gray

HDR: high dose radiation

IFN-γ: Interferon gamma

LDR: low dose radiation

LD-RT: low dose radiation therapy

LET: linear energy transfer

LNT: Linear no threshold

MAPK: Mitogen activated protein kinase

mGy: milli-gray

MHC: major histocompability complex

mRNA: Messenger ribonucleic acid

mSv: millisievert

NK: Natural Killer

PAMP: Pathogen associated molecular patterns

PBMC: peripheral blood mononuclear cell

PSA: Prostate specific antigen

RT: radiation therapy

TBI: total body irradiation

TCR: T cell receptor

Th: T helper

TNF-α: tumor necrosis factor alpha

1. Introduction

1.1. Cancer

Every year, cancer is responsible for millions of deaths worldwide and, even though much progress has been achieved in medicine, there are still many issues that must be addressed in order to improve cancer therapy[1]. With over 200 known variants, cancer consists of diseases involving abnormal growth of cells with the potential of invading or spreading to healthier tissues of the body[1]. Cancer is one of the most common diseases on the face of this earth that has existed for almost the entirety of human history[1]. According to data reported by Statistics Canada, it is currently the leading cause of death in Canada, accounting for approximately 30% of all deaths, closely followed by heart disease (about 20%)[2]. Furthermore, in Canada, it is estimated that 186,400 new cancer cases and 75,700 deaths were attributable to this disease in 2021[2]. In the last decade, exceptional progress has been made in the field of oncology, specifically towards understanding cancer development and related therapies. However, with its increasing incidence, the clinical management of cancer continues to be a challenge in the 21st century[3]. Due to the wide spectra of causalities, it is challenging to identify the cause of a particular cancer regardless of its type. Indeed, even though the harmful effects of cancer have been known for a very long time, and despite the many efforts made to overcome this disease, cancer is still quite prevalent[3]. Therefore, considerable research is under way to identify the causes of the disease as well as in developing strategies for its prevention, diagnosis, treatment, and ultimately, its cure.

1.1.1. Causes

Cancer is known to arise from the transformation of normal healthy cells into tumour cells in a multi-stage process that generally progresses from a pre-cancerous lesion to a malignant tumour[4]. Although not always the case, in some instances, this can become fatal. These changes are the result of DNA mutations which can be genetically inherited or can stem from a multitude of factors

such as diet, lifestyle choices or environmental/external risk factors[5]. These include physical carcinogens (i.e. ultraviolet and ionizing radiation), chemical carcinogens (i.e. tobacco smoke and alcohol) and biological carcinogens (i.e infections from certain viruses, bacteria, or parasites)[5]. Regardless of the cause of cancer, the course of treatment will depend on numerous factors such as type and the stage of the cancer, its location, amongst others[5]. As a result, there is a wide range of treatment modalities that exists to date and that will continue to expand as more becomes known about this disease[1].

1.1.2. Types and Treatments

In the last decade, oncological research has focused not only on finding new and effective treatments for cancers, but also on alleviating critical negative side effects associated with the conventional cancer treatments that are most commonly used[6]. There are many different approaches for treating cancer, and it is not uncommon to use a combination of different therapy modalities at once[7]. Although many treatments exist, the three most common ones include: surgery, aimed at removing the tumor and sometimes nearby tissue; chemotherapy, which includes the use of cytotoxic drugs; and radiotherapy, where high energy ionizing radiation (usually x-rays) is used to destroy cancer cells[7]. The latter has gained increasing interest in the last few decades as a result of its promising benefits and success in treating cancer. In fact, 50% of cancer patients will undergo radiation therapy at one point or another during the course of their treatment regimen[8].

1.1.3. Radiotherapy

Radiotherapy or radiation therapy (RT) uses high-energy radiation, usually x-rays, to treat cancer[9]. The main goal of RT is to give a high dose of radiation to the tumor while sparing surrounding healthy cells as much as possible as this will reduce the risk of negative side effects[10]. The main goal of RT is usually to cure the cancer; however, this treatment may also be administered for

palliative care in order to help relieve symptoms[10]. In addition, RT can be given both externally and internally. External radiotherapy aims high-energy photons, or less commonly, high energy particles such as protons and neutrons, at the affected area using accelerators such as linacs[10]. Alternatively, internal radiotherapy places radioactive materials directly inside or near the tumor, which can lower the risk of damaging healthy cells nearby[11]. Although the delivery of these radiotherapies differs, both are a type of high dose ionizing radiation that aim to directly destroy cancer cells in a specific area by damaging the DNA of the cancer cells[12].

1.2. Ionizing Radiation

Radiation is the process by which energy is emitted as either particles or waves which can take the form of sound, heat, or light[12]. Large unstable atoms become more stable by emitting a quantity of energy to get rid of excess atomic energy, and this is what we call radiation[12]. This radiation can take the physical form of vibrating waves or rays, known as electromagnetic radiation, or as fast-moving particles, known as particulate radiation. Electromagnetic waves can be classified and arranged according to their various wavelengths and frequencies; this classification is known as the electromagnetic (EM) spectrum[13]. The EM spectrum is generally divided into seven regions, in order of decreasing wavelength and increasing energy and frequency[13]. The electromagnetic spectrum includes different types of energy waves. The lower energy end of the spectrum includes microwaves and radio-waves, and the higher energy end of the spectrum is made up of x-rays and gamma rays[13]. On the other hand, particulate radiation is comprised of atomic or sub-atomic particles, including alpha and beta particles and neutrons[14]. Overall, it is important to note that not all particulate radiation is considered ionizing radiation, and only x rays and gamma rays from the EM spectrum are considered ionizing. Radiation that is considered ionizing is defined as radiation with enough energy to strip electrons from atoms or molecules when it strikes or passes through a

substance[15]. The atom or molecule that loses a negatively charged electron will then become positively charged[15]. This process where an electron is either lost or gained is called ionization[15]. On the contrary, non-ionizing radiation does not have enough energy to remove electrons. The waves on the lower energy end of the electromagnetic spectrum (i.e. radio-waves and ultraviolet rays, etc.) are considered non-ionizing[16].

1.2.1. Sources of Radiation

There are natural and artificial sources of ionizing radiation that we are exposed to daily in the world around us[17]. Artificial sources of radiation include x-ray machines, radioactive isotopes used in nuclear medicine, and nuclear power plants[17]. The following are examples of natural sources of radiation: cosmic radiation, terrestrial radiation emitted by elements such as uranium and thorium present in the earth's crust, and radiation associated with radon inhalation[17]. Overall, even in small doses, humans are constantly exposed to ionizing radiation in their everyday lives, whether it comes from human-made or natural sources.

1.2.2. Radiation Doses and Units

Generally, when quantifying radiation, two measurements are commonly used. These are the "activity" and "dose" caused by the radiation[18]. The activity refers to the rate of decay of a radioisotope. On the other hand, dose measures the amount of energy that is absorbed by a material[18]. For the remainder of this thesis, radiation exposure will be referred to in terms of absorbed dose, which represents 'the energy deposited in a kilogram of substance by the radiation'[19]. This radiation dose is measured in an international (SI) unit called the gray (Gy)[19]. The important concept is that dose is a measure of how radiation interacts with substances (such as cells or DNA) and is not a description of the radiation itself[20]. This allows us to unify the measurement of different types of radiation (i.e., particles and waves) by measuring how much

energy they deposit in a material. In radiation therapy, the unit of Gy is used to measure the total amount of radiation that the patient is exposed to during their treatment regimen[21]. Adjuvant therapy doses typically range from 45 to 60 Gy for the treatment of breast, head, and neck cancers[21]. Typically, these doses are divided into multiple smaller doses, known as fractions, that are given over a period of multiple weeks[21]. Although improvements in treatment planning have increased the accuracy of beam targeting to the tumour to reduce the damage to adjacent normal tissues, the negative side effects of high dose radiation can still be quite severe. Specifically, important for my thesis, high dose radiation can lead to a supressed immune system and inhibition of antitumour immunity, and ultimately, a reduced quality of life for that patient[10].

1.2.3. Direct and Indirect Effects

When radiation encounters the human body, specifically the nucleus of the cell, severe damage to the genetic material is possible[22]. This will be dependent on many factors, the main one being the type of radiation, as this will dictate the amount of energy deposited in the tissue it encounters[22]. The energy deposited per unit distance as an ionizing particle or ray travels through a material is known as linear energy transfer (LET)[23]. Specifically, LET is used to quantify the effects of ionizing radiation on biological specimens and varies depending on the energy and charge of the kind of radiation[24]. For example, alpha particles interact very readily with the matter that they penetrate, and as a result are classified as high-LET radiation because they deposit their energy rapidly and create short and dense ionization tracks[24]. Consequently, alpha particles are known to be much more damaging for a given absorbed dose as opposed to low-LET radiation such as beta particles and gamma rays[25]. On the other hand, low-LET radiation is less likely to ionize atoms they come across, as they produce radiation tracks that are much less densely ionizing[25].

Damage to the genetic material is the primary cause of cell death induced by radiation, as this can cause a wide range of lesions in the DNA such as single strand breaks or double strand breaks[26]. Therefore, based on the energy transfer created, damaged DNA in a cell can occur through two different mechanisms: direct and indirect. The main mechanism for high-LET is through direct action. If the incident radiation itself interacts with the cell's DNA or other critical cellular components, it is referred to as a direct radiation effect[26]. On the other hand, the dominant mechanism for low-LET radiation is indirect action. For low-LET radiation, direct action represents about 20%, and indirect action is about 80%[27]. Indirect action occurs when radiation interacts with water molecules, causing reactive oxygen metabolites (atoms or molecules with unpaired electrons that are highly reactive) such as hydroxyl radicals[27]. Consequently, if there is an interaction with the genetic material as a result of exposure to ionizing radiation, the cell possesses many repair mechanisms, and so depending on the number of ionization event, exposure to radiation may not result in any increase in mutations or overall harm[27].

1.2.4. Model of Risks

In radiation protection, most biological effects from ionizing radiation are usually divided into two categories: deterministic effects and stochastic effects[28]. Deterministic effects are defined as threshold health effects that are related directly to the absorbed radiation dose, where the severity of the effect and the dose have a positive correlation[28]. In other words, the severity of these adverse health effects would increase as the dose increases. Deterministic effects are also known as immediate effects, and examples of these are: erythema (skin reddening), cataracts to lens of the eye, sterility (temporary/permanent), & epilation (loss of hair)[28]. Stochastic effects are referred to as random effects that occur solely by chance[28]. The probability of the effect occurring is assumed to be a linear function of dose without threshold. Examples of stochastic effects would be

hereditary effects and cancer development[28]. In the field of radiobiology, there are a few models in use to assess the risk of stochastic effects from radiation exposure. Currently, the linear no-threshold (LNT) model is one of the more commonly used models, particularly in the field of radiation protection[29]. The LNT model implies that there is no safe level of exposure to ionizing radiation below which there would be no risk of cancer or other biological effects[29]. However, it is quite implausible that a single radiation event can cause a tumor formation, especially since there are many ionizing events taking place in our everyday lives. For many years, the radiological risk of detrimental biological effects, including cancer, has been estimated by the LNT model[29]. Unfortunately, this has led to an increased fear of radiation and caused radiophobia for many[30]. However, in the last few decades, there is growing evidence that biological systems respond differently to lower doses of radiation, typically lower than several hundred mGy[31]. Specifically, scientific evidence based on epidemiological and pre-clinical studies have shown that, contrary to high dose exposures, radiation below certain doses could have anti-cancer properties through beneficially stimulating immune function[31]. As a result, it is believed by many that the shape of the dose response curve differs in the low dose region and that there are threshold levels for detrimental radiation effects[32]. There is also evidence that some low dose exposures can induce a hormetic response (net beneficial effect) due to the up regulation of innate repair and antioxidant capacities[33]. This model is known as the hormesis model, which postulates that lower doses of radiation can be beneficial in preventing cancer and other adverse health effects by stimulating the activation of adaptive and protective mechanisms. Consequently, the immune system has been implicated as one of the potential targets of low dose radiation (LDR). Therefore, LDR represents a potential immunotherapy for cancer and other diseases.

1.2.5. Low Dose Radiation

As mentioned above, there has been increasing evidence that radiation below certain doses could cause beneficial immunostimulatory effects. Specifically, LDR has been proven to stimulate biological immunity in-vitro as well as in-vivo[34]. This phenomenon that has been previously identified as radiation-induced hormesis, has been the subject of increased scientific interest. This stimulation of immunity involves key anti-cancer parameters, including antibody formation, natural killer cell activity and secretion of interferon and other cytokines[35]. As a whole, these parameters have allowed LDR to be examined as a treatment and management option for cancer. Therefore, low-dose radiation therapy (LD-RT) has the potential to become a viable treatment option acting as an immunotherapy, with the benefit of avoiding the negative side effects inherent to the conventional high-dose radiation therapy. LD-RT was commonly used in the 1970's and 1980's for the treatment of hematological malignancies with high success rates but has fallen out of practice in recent decades[36]. This was in part due to increased radiophobia combined with improvements in chemotherapeutics. Presently though, a lack of molecular mechanistic understanding has limited the potential of LD-RT as a plausible treatment for cancers. Therefore, a better understanding of the immune system cellular mechanisms that are involved in antitumor immunity is crucial in re-establishing LD-RT as a cancer treatment.

1.3. The Immune System

The immune system consists of various cells (e.g. macrophages, dendritic cells, lymphocytes, etc.) and molecules that tightly regulate and trigger a responses against invasive and dangerous pathogens[37]. Such pathogens may include bacteria, viruses, and toxins[37]. In addition to this role, the immune system is also very much involved in other processes including cancer prevention, homeostasis, reproduction, wound healing and metabolism amongst many others[38]. The body's

immune system will utilize two fundamental lines of defense: innate immunity and adaptive immunity.

1.3.1. Innate Immune System

The innate immune response represents the first line of defense against pathogens. This immunity is made up of elements that are already present in the body before the manifestation of an infection[38]. It is considered a less specific defense mechanism than the adaptive immune response as it recognizes conserved features of pathogens known as pathogen associated molecular patterns (PAMPs) like bacterial and fungal cell wall components (ex. gram negative cell walls)[38]. Several types of defensive barriers make up the innate immune system; these include anatomical, physiological, inflammatory as well as endocytic and phagocytic barriers[38]. The main goal of the innate immune system is to rapidly recruit immune cells in hopes of eliminating the stimuli and initiating the healing process[38]. More specifically, the immune cells that make up the innate immunity include phagocytes (macrophages, monocytes and neutrophils), natural killer (NK) cells, basophils, dendritic cells (DCs) and eosinophils[39]. All of these cells play an important role in eliminating pathogens, and some of the more common ones will be discussed in more detail. Neutrophils: in addition to their phagocytic properties, these cells contain granules and enzyme pathways that assist in the elimination of pathogenic microbes[40]. Dendritic cells also undergo phagocytosis, but mostly function as antigen presenting cells (APCs), initiating the acquired immune response and acting as important messengers between innate and adaptive immunity[41]. Specifically, activation of DC receptors can give these cells the ability to further activate or inhibit lymphocytes and to also generate both pro-inflammatory and anti-inflammatory cytokines[41]. Natural killer cells play a major role in the rejection of tumours and the destruction of cells infected by viruses. Destruction of infected cells is achieved through the release of perforins and granzymes

(proteins that cause lysis of target cells) from NK-cell granules which induce apoptosis[42]. NK cells are also an important source of another cytokine, interferon-gamma (IFN-γ), which helps to mobilize APCs and promote the development of effective anti-viral immunity[42]. Overall, one of the main roles of innate immune cells is to produce cytokines or interact with other cells directly in order to activate the adaptive immune system. Since NK cells play a vital role in immunity and participate in many antitumor responses, they are of particular interest in this study.

1.3.2. Adaptive Immune System

The adaptive immune system, unlike the innate immune system, is specific in targeting the type of pathogen and is therefore slower to respond to newly encountered pathogens. The adaptive immunity consists of an antigen-specific response through T cells and B cells and is known for its immunologic memory, allowing the system to have a faster or more efficient response in the event of a second exposure to the same or similar pathogen[43].

T cells activate cell-mediated immune responses in which they act directly against foreign antigens with the help of APCs like macrophages, DC, and B cells amongst others[44]. T cells express a variety of antigen-binding receptors called T- cell receptors (TCR) that facilitate the recognition of foreign materials in the body[44]. Originating in the bone marrow and maturing in the thymus, T cells differentiate into discrete subpopulations: CD4+ or CD8+ T cells, each having a defined repertoire of effector functions[44]. CD8+ T cells are considered as cytotoxic killer cells that eliminate substances deemed harmful by the immune system, while CD4+ T cells are the "chief conductors" of immune response regulation and function by further activating memory B cells and cytotoxic T cells[37]. CD4+ T cells can also activate eosinophils and macrophages that produce both superoxide and nitric oxide, and these cells can further collaborate within tumor site to cause its destruction[37]. While CD8+ T cells recognize tumor associated antigens presented by major

histocompatibility complex (MHC) class I molecules, CD4+ T cells recognize MHC class II-restricted epitopes[45].

B lymphocytes are a class of lymphocytes that produce antibodies, which are essential for the potentiation of the innate immune system[45]. Antibodies are key contributors to a successful immune response, as they allow the neutralization of toxins, prevent the adhesion of foreign organisms, and identify cancerous or infected cells infected for destruction by cytotoxic cells in an antibody-dependent manner[45]. Like T cells, B cells arise from hematopoietic stem cells in the bone marrow[45]. Following maturation, B cells will leave the marrow and express a unique antigen-binding receptor on their membrane. Unlike T cells, B cells can recognize antigens directly without the need for APCs, through unique antibodies expressed on their cell surface[46]. Overall, the mechanisms used by B cells and T cells are intricately regulated by the innate immunity, which is not only a pre-requisite for productive cancer immunosurveillance but also a crucial element of the anti-tumor immune response. Consequently, although different, innate, and adaptive responses operate in synergy which allows a much more efficient immune response.

1.3.3. Cytokines

Cytokines are small messenger proteins which participate in many physiological processes and play a key role in inflammation[47]. In the presence of a tumor, both the innate and adaptive immunity will release cytokines in order to mediate the activity of other immune cells and to directly affect tumor cells themselves[47]. Cytokines are a diverse group of molecules that includes more than 100 secreted factors that can be subdivided into several classes: interleukins (IL), tumor necrosis factors (TNF), interferons (IFN), transforming growth factors (TGF), colony stimulating factors (CSF) and also various chemokines[48]. Overall, two types of cytokines exist: pro-inflammatory cytokines and anti-inflammatory cytokines. Generally, both types of cytokines are

needed to mediate key immune interactions for effective antitumor activity. However, an abundance of pro-inflammatory cytokines such as interleukins (i.e. IL-1α and IL-1β) can promote various cancer processes such as invasion and tumor progression. On the other hand, amongst all cytokines, TNF-α, and IFN-γ appear to have a major role in the process of cancer elimination, as evidence shows that they are readily found in the tumor microenvironment and play a crucial role in the fight against cancer[48]. TNF-α is a potent inflammatory mediator that plays important roles in the innate immune response mainly via the production of additional cytokines, the expression of adhesion molecules and the stimulation of growth[49]. TNF-α also stimulates the proliferation of cells, has cytolytic and inhibitory effects on tumor cells and is implicated in antiviral and inflammatory activities[49]. It is mainly secreted by activated macrophages but may also be secreted by other cell types like monocytes, T cells, mast cells and NK cells[49]. Similarly, IFN-γ is also a cytotoxic cytokine expressed and released by a variety of cells including NK cells, in order to initiate apoptosis in tumor cells[50].

1.3.4. Anti-tumor Immunity

Cancers can generally be classified into 2 main groups: non-metastatic and metastatic cancers. The process of metastasis is when cancer cells escape from primary tumors and acquire cellular traits that allow them to travel throughout the body and colonize distant organs[51]. Both primary and metastatic cancers are complex ecosystems which are mainly composed of neoplastic cells, extracellular matrix (ECM), and "accessory" nonneoplastic cells, which include resident mesenchymal stem cells, endothelial cells, and infiltrated inflammatory immune cells[51]. In principle, tumor development can be controlled by cytotoxic innate and adaptive immune cells, and several decades of research have established that the development of cancer can be stopped or controlled through a process known as antitumour immunity[52].

Here, the immune system employs various strategies for tumour specific destruction including enhancement of immunostimulatory effects while inhibiting immunosuppressive signals. Antitumour immunity begins with immunosurveillance, a process where immune cells recognize non-self-cancer antigens which allows the immune system to attain tumour specificity[53]. The main feature of immunosurveillance lies in its diverse repertoire of antigen receptors; TCRs and BCRs. When a particular naive T cell binds to the cancer antigen of an APC, activation of the T cell will occur when the unique TCR matches the specific cancer antigen[54]. Here, activation of CD8+ cytotoxic T cells results in clonal expansion[54]. The increased number of cytotoxic T lymphocytes (CTL) specific for the target antigen binds to the cancer cell, releases cytotoxins such as perforins, granzymes, and granulysin, resulting in direct cancer cell killing[44]. Like T cells, each B cell comprises of a unique BCR and therefore binds to a specific antigen and presents it on its cell surface[44,55]. Here, CD4+ T helper 1 cells (Th1) with a matching TCR specific for the antigen presented on the B cell binds and activates the B cells[55]. This dual B cell activation transforms the B cells to plasma cells which results in production and release of antigen-specific antibodies[56]. These antibodies then bind to the antigens on the cancer cell triggering binding of NK cells to the cancer cells[57]. Here, NK cells contain the CD16 Fc receptor which recognizes the cell-bound antibodies[42]. Like CTLs, NK cells mediate lysis of the cancer cells via releasing perforins and granzymes[42]. This antibody-dependent cellular cytotoxicity (ADCC) is an important mechanism whereby the immune system destroys cancer cells[57]. CTL and NK cell mediated cancer killing are part of the immunostimulatory signature. Along with Th1 cells, other immune cells which enhance cancer killing include T17 helper cells (Th17), M1 macrophages and N1 neutrophils[58]. Conversely, the immune system also contains cell-types and molecules which result in immunosuppression, and generally work together to promote cancer by opposing the induction and proliferation of

effector T cells[58]. Cells part of this immunosuppressive phenotype include T regulatory cells, CD4+ T helper 2 cells (Th2), myeloid derived suppressor cells (MDSCs), N2 neutrophils and M2 macrophages[58]. Overall, although the main goal of anti-tumor immunity employed by the immune system is to avoid or eliminate cancer, there are unfortunately instances where the cancer will evade and metastasize to other areas of the body.

1.3.5. Cancer Evasion

Cancer cells utilize the mechanisms shared by healthy self-cells (self-tolerance) to elude defensive immune responses[59]. Moreover, these altered self-cells utilize several other mechanisms such as separation of tissue from surveillance, antigen shedding, lymphocyte killing, secretion of immunosuppressive cytokines, reduced MHC class II expression and costimulatory molecules to evade immune responses[59]. Consequently, as a result of these mechanisms, tumors cause suppression of the immune system during carcinogenesis, and therefore upregulation of immune surveillance, or the immune system itself, is crucial to fight the tumor. Therefore, LD-RT, alone or in combination with other treatments, could offer an optimal and effective therapy option to manage cancer.

1.4. Low Dose Radiation Therapy (LD-RT)

Previous studies have shown that LD-RT can successfully stimulate the immune system which ultimately induces tumor control in some cases[31]. Furthermore, research suggests that induction of innate and adaptive immune responses primarily involving NK cells, macrophages and dendritic cells increase nonspecific anti-tumor immune surveillance[60]. For instance, one study showed that 200 mGy total body irradiation in tumour bearing rats resulted in increased IFN-γ, TNF-α, and CD8+ T cells in the spleen and in the primary tumour compared to sham irradiated or locally

irradiated animals[61]. Likewise, a single dose of 100 mGy stimulated NK cell mediated cancer killing via ADCC 24–72 h post irradiation[62]. Furthermore, single exposure of 100 mGy increased macrophage activation[63]. In another similar study, LDR was shown to upregulate several other anticancer factors such as CD25 (IL-2 receptor), CD71, CD28, CD2 and CD48, and immune system stimulating signaling molecules (e.g. calcium, c-GMP and p38MAPK)[64]. In this same study, LDR also increased the IFN-γ/IL- 4 ratio of splenocytes in tumour-bearing mice, a hallmark of a shift to a T_h1 phenotype compared to T_h2[64]. Similarly, LDR promoted M1 macrophage over M2, and N1 neutrophil compared to N2. Furthermore, LDR also reduced activity of immunosuppressive cell-types and cytokines which indirectly contributed to immune system enhancement. For example, a decrease in T-regulatory cells, and a decrease in TGF-β levels have been observed with LDR treatment[65]. In addition to these promising results, LD-RT has been shown to enhance the efficacy of chemotherapeutic drugs, as well as enhance the efficacy of immunotherapy[66]. Taken together, a growing body of evidence suggests that LD-RT promotes antitumour immunity.

1.4.1. Clinical Applications

Although a considerable ongoing body of evidence supporting the potential of LD-RT exists from in-vitro and in-vivo animal studies, comparably few human clinical trials have been completed. However, in recent years LD-RT has been re-visited as potential clinical therapeutic. Since LD-RT is delivered as a whole-body or half-body treatment, the majority of these studies have involved patients with hematological malignancies[67]. The use of LD-RT in treatment of chronic lymphocytic leukemia (CLL) and Non-Hodgkin's lymphoma (NHL) was studied in the late 1960s and early 1970s, with the work of Johnson and others[36]. Over 180 patients, largely in the 1970s and 1980s, have been treated with LD-RT in randomized trials[68]. The earliest of these studies

involved 65 patients with stage III or stage IV NHL[68]. Comparison was between total body irradiation (TBI) given in 100 mGy fractions, 3–5 times per week to total doses between 1–5 Gy and multi-agent chemotherapy[68]. Patients receiving TBI (n = 32) experienced an 84% response rate, with 56% of patients achieving a complete response[68]. In the comparison with chemotherapy, there was no difference in overall survival[68].

A smaller trial randomized 39 patients with NHL to receive a total of 1.5 Gy delivered using 2 versus 3 fractions per week[69]. The majority of patients (85%) achieved a complete response with a 3-year overall survival rate of 78%[69]. Authors reported that treatments were well tolerated with patients reporting a slight decrease in appetite and mild fatigue[69]. In addition, Hoppe et al. reported a three-arm trial with 17 patients in each group comparing advanced lymphoma patients between TBI and 2 chemotherapy regimens[70]. Radiation was given in 300 mGy fractions to a total of 1.5 Gy, and patients also received a boost of 20 Gy to the primary disease site[70]. Of the TBI group, 12 achieved complete remission and after 41 months of follow up, only one patient had died of disease, fewer deaths than those seen in the two chemotherapy groups[70].

Despite considerable previous interest in LD-RT as a treatment option for lymphoma and leukemia, few reports exist regarding non-hematogenous malignancies. Half-body irradiation of an NHL patient appeared to result in control of a nasal tumor that was located outside the radiation field by stimulating the immune system[71]. Qasim reported on 30 Dutch patients with either limited or extensive small cell lung cancer[72]. In this study, patients received 100 mGy in daily treatments for two weeks for a total of 1 Gy, and were then treated with 4 Gy to the primary disease and 1 Gy to the liver[72]. Surprisingly, no patients developed brain metastases, despite an expected 3-year brain metastases rate of approximately 50%, suggesting that LD-RT was highly effective in controlling disease in chemotherapy sanctuary sites, and no treatment-related toxicity developed

in these patients[72]. Most patients experienced a mild decrease in leukocyte and platelet counts but the vast majority made a full recovery[72]. Lastly, these results are supported by a recent 2023 trial completed by I. Dayes and members of our own lab group at NOSM University looking at LDR as a treatment option for recurrent prostate cancer[73]. In this study, sixteen patients with recurrent prostate cancer received 150 mGy of nontargeted radiation twice per week, for 5 consecutive weeks[73]. It was found that LD-RT may be a potential therapy for some patients with recurrent prostate cancer by stalling rising prostate specific antigen (PSA) and that LDR is well tolerated by participants with minimal toxicities and no change in quality of life[73]. Overall, with further research and optimization, LD-RT has the potential to become an effective treatment option for managing recurrent prostate cancer as well as other forms of this malignant disease.

Of note, LD-RT is being widely used in European countries, like Germany, to treat benign diseases other than cancer, including keloids, arthritis, Grave's ophthalmopathy and meningioma[74]. Overall, LD-RT induced immune stimulation and tumor control make this treatment a viable alternative therapy to the conventional treatments for cancer. However, a comprehensive characterization of the immune response across different dose regimens, which examines all of the cell and cytokine/chemokine mediators involved in the LDR heightened anti-tumor immunity has yet to be completed. Therefore, a complete comprehensive analysis of the potential mechanisms at the root of the LDR mediated immunostimulatory response may help strengthen our understanding and acceptance of LD-RT as a cancer therapeutic.

1.5. Objectives and Hypothesis

Overall, the objective of my thesis is to evaluate the effects of LDR on the immune system and ultimately, elucidate the underlying cellular and molecular mechanisms that may be involved in antitumor immunity. It was hypothesized that LDR would stimulate the immune response while

inhibiting immunosuppressive signals. This was evaluated through two separate aims. In the first data chapter of my thesis, an in-vivo mouse model was used to identify the effects of whole-body LDR on various immune cell populations. For this aim, it was hypothesized that LDR exposures of 0.1 and 0.25 Gy would increase the number of B cells, $CD4^+$ T cells, $CD8^+$ T cells, NK cells, DC and neutrophils in the irradiated mice, and that exposures to higher doses of 0.5 and 3 Gy would cause a decrease in these cell populations. In the second chapter of my thesis, the NK-92 cell line was used to evaluate the effects of LDR in-vitro. It was hypothesized that LDR exposure of 0.1–0.25 Gy would increase cytotoxicity in these cells as well as increase expression of the main genes involved in regulating their cytolytic properties, and that higher doses of 0.5 and 1 Gy would have opposing immunosuppressive effects.

2. In-vivo Effects of LDR in Mice

2.1. Introduction

In everyday life, humans are exposed to low doses of ionizing radiation, emitted either from natural or artificial sources inherent to the environment that we live in. Therefore, studying the biological effects of low dose radiation have been the subject of acquired scientific interest. In the past several decades, many biological effects of LDR, distinguishable from those of HDR, have been reported[31]. In fact, unlike HDR that is typically immune suppressing, exposure to LDR (100 mGy or less) can conversely be immune enhancing[31]. Studies have shown that LDR helps promote cell growth and development, decrease the process of aging, enhance antioxidant capacity and the repair of DNA damage, and delay the onset of cancer[75]. One of the main features of LDR which has attracted the attention of many scientists is its ability to enhance the immune response and ultimately cause cell death in malignant cells[75]. This phenomenon, identified as radiation-induced hormesis, has made LDR a promising option for the treatment of diseases, such as cancer.

Several studies have investigated antitumor effects of LDR, specifically in delaying or eliminating cancerous cells in animals. In a study by Cheda et al. (2004), animals were subjected to whole-body irradiation, and it was demonstrated that LDR at a dose of either 0.1 or 0.2 Gy could significantly suppress pulmonary tumor metastases in BALB/c mice with syngeneic L1 sarcoma[62]. Moreover, pre-exposure to LDR has been found to reduce the incidence of lymphoma induced by high-dose fractionated total-body irradiation in C57BL/6J mice, which was accompanied by immunologic stimulation[76]. The exact mechanisms of these anti-tumour responses have yet to be identified but it is postulated that radiation induced immune stimulation plays a central role.

In its fight against cancer, the immune system utilizes a complex interplay of multiple mechanisms and strategies that have yet to be well defined and understood. Consequently, the regulatory effects of LDR on innate and adaptive immunity depends on many factors, including the status of immune

cells, the microenvironment of the immune system, and the interaction of immune cells[35]. Therefore, since LDR is being revisited as a cancer therapeutic, there has been increasing research aimed at comprehensively understanding the effects of LDR on the immune system. As mentioned above, LDR has proven to be effective in stimulating the immune response, in particular through in-vitro studies[31,62,76]. However, to date, there has also been increasing pre-clinical in-vivo studies to confirm these promising findings.

Numerous studies have shown that LDR can enhance the immune response through the augmentation of NK cells and their cytotoxic properties. In the same study conducted by Cheda *et al*. (2004), it was found that the development of tumor metastases in mice was supressed as a result of the stimulation of NK cell cytolytic activity, subsequent to exposure to LDR[62]. Specifically, a single whole-body exposure of 0.1 Gy or 0.2 Gy was delivered to BALB/c mice that were injected with L1 sarcoma cells 2 hours post irradiation[62]. A ^{51}Cr release assay, using YAC-1 tumor cells as a NK cell target, was used to measure NK cell cytotoxicity[62]. Compared to the sham-irradiated control mice, the pulmonary tumor colonies were significantly reduced in the animals exposed to LDR[62]. Similar results are also shown in an earlier study by Zhang et al. (1996) looking at NK activity in tumor-bearing C57BL/6J mice[77]. In this study, whole-body irradiations of 0.075 Gy were given to mice implanted with Lewis Lung cancer cells, and results showed that LDR caused an increase in NK cell numbers which were originally lowered due to the tumor causing immune suppression in the mice[77]. Despite many findings of LDR-induced activation of NK cells, the specific mechanisms causing these beneficial outcomes remain obscure and controversial.

Dendritic cells play an essential role as antigen presenter cells, and the immunological activity of these cells strongly depend on their state of differentiation and maturation[41]. A recent study by Shigematsu et al. (2007) reported that exposure of dendritic cells to LDR led to a greater

capacity for proliferation of T cells, and an increase in the production of IL-2, IL-12 and IFNγ[78]. Specifically, splenic dendritic cells isolated from C57BL/6 mice were exposed to various low doses of radiation from a ^{137}Cs source, and then co-cultured with T cells for 48 hours[78]. It was found that the 0.05 Gy pre-irradiated dendritic cells showed the highest proliferation of T cells[78]. So far, these studies, amongst others, confirm that LDR has the capability to stimulate innate immune cells, which in turn can lead to further activation of adaptive immune cells, ultimately leading to the enhancement of the immune response.

T lymphocytes are essential for cell-mediated immunity, and the exposure of these cells to low doses of radiation have led to an increase in the subpopulations of CD4+ and CD8+ T cells[44]. For example, a recent study by Zhou et al (2018) indicated that the adaptive immunity is also involved in the immune enhancement of mice induced by LDR, mainly through the increased proliferation and cytokine production of T cells.[34] In that study, C57BL/6 mice inoculated with Lewis Lung cancer cells were irradiated with 0.075 Gy of LDR, and 12, 16, 20 and 24 days later, the percentages of T cells in mouse spleens were measured using a splenocyte proliferation assay and flow cytometry[34]. The results of this research revealed that the percentage of CD8+ T cells in the LDR group was significantly higher than that in the sham group on days 16 and 20[34]. Coincidently, in this same study, it was found that LDR not only upregulated T cells, but also increased cytotoxicity of mouse splenocytes, and increased infiltration of T cells in the tumor tissues[34].

Lastly, besides stimulating immune cells, there is evidence that LDR can also have beneficial effects on cytokines and chemokines, which can further mediate the antitumor immunity processes employed by the immune system. Numerous studies have shown that tumor bearing mice treated with LDR not only reduced the secretion of immunosuppressive cytokines such as

IL-10, but also increased the production of immunostimulatory cytokines such as interferon (IFN)-γ, IL-2, and TNF-α[64,65].

In summary, in the past few decades, there have been many pre-clinical rodent studies suggesting that LDR stimulates the immune system by activating innate and adaptive immune cells in addition to increasing cytokine production which synergistically promotes antitumor immunity. A more comprehensive list of all the immune cell types/cytokines and their role in an enhanced immune response to LDR, can be found in Table 2.1 below.

Table 2.1. Summary of the effects of LDR on the immune system[79].

Immune system	Modification following LDR regimen	Role of immune system in LDR response
Cellular component		
Innate immunity		
NK	Increase in functionality	Lysis of tumor cells
ADCC	Increase	Lysis of tumor cells
Macrophage	Increase in functionality	Phagocytosis and antigenic presentation
Dendritic cell	Activated	Increase in T-cell proliferation and antigenic presentation
Adaptive immunity		
$CD8^+$ (CTL)	Increase in cytolysis	Lysis of tumor cells
$CD4^+$	Enhanced responsiveness	Helping other immune cells
Th1	Increase	Anti-tumor activity
Th2	No change	Proinflammatory response
T-regulatory	Decrease	Breaking of tumor tolerance during carcinogenesis and induction of anti-tumor immunity
Secretary component		
Cytokines		
IL-2	↑	T-cell proliferation
IL-12	↑	Proinflammatory response
IFN-γ	↑	Phagocytosis and antigenic presentation
TGF-β	↓	Maturation and proliferation of T and B cells
IL-10	↓	Immunactivation
TNF-α	↑	Proinflammatory response

ADCC: Antibody-dependent cell-mediated cytotoxicity; CTL: Cytotoxic T lymphocyte; LDR: Low-dose radiation; NK: Natural killer.

Overall, although research to date has shown that LDR could offer an effective treatment option for cancer through stimulation of various immune cells, a comprehensive study focusing on the exact mechanisms involved has yet to be accomplished. Therefore, the objective for this chapter of my thesis was to elucidate which cellular and molecular mechanisms are responsible for antitumor immunity. It was hypothesized that LDR would promote antitumor immunity by

stimulating the immune system while inhibiting immunosuppressive signals. Since previous studies have identified anti-cancer effects at doses less than 0.5 Gy, it was also hypothesized that LDR would be most effective at the lower doses tested (0.1 and 0.25 Gy), as opposed to the 2 highest doses that were tested (0.5 and 3 Gy). This was accomplished by exposing C57BL/6 mice to these doses of ionizing radiation and performing full immunophenotyping on the spleen, lymph nodes and blood post-irradiation.

2.2. Materials and Methods

2.2.1. Animal Housing

Forty wild-type C57BL/6 male adult mice (15-week old) were acquired from Charles River Laboratories (Wilmington, MA) and group housed (3 per cage) at the Laurentian University Animal Facility in Sudbury. The mice were maintained on a 12:12 h light/dark cycle in a pathogen-free environment with ad libitum access to food (Purina LabDiet 5001, St Louis, MO) and water. The mice were housed at the animal facility for a minimum of 1 week prior to experimental treatments to allow animals to acclimate to the facility. Therefore, treatments on the mice began at 16 weeks of age. All experimental protocols were approved by the Animal Care Committee at Laurentian University and done in accordance with the Canadian Council on Animal Care guidelines.

2.2.2. Irradiations

Mice were administered whole body x-ray exposures using an X-RAD 320 irradiation cabinet (Precision X-ray, Madison, CT) at NOSM University (NOSM U) (Figure 2.1A). Transportation and irradiation procedures followed those outlined in the “Transport and Handling of Laboratory Animals for Irradiation” Standard Operating Procedure. The mice were transported in their

housing cages by vehicle, ensuring that noises and stress to the animals were minimal. Once arrived at the facility, mice were individually placed into circular pie cages specifically designed for small animal irradiations (Figure 2.1B). Mice were not anesthetized during the transportation and irradiation.

Prior to performing irradiations on the cohorts of experimental mice, dosimetry was performed to verify the doses that would be used for irradiation. In order for doses to be measured, thermoluminescent dosimeters (TLDs) were inserted at various locations in the bodies of deceased mice. The deceased mice were then exposed to different doses of radiation. Post irradiation, TLD's were sent back to the manufacturer (Mirion Technologies Inc., Oak Ridge, TN) for the absorbed dose to be calculated. Differences between programmed and actual doses were corrected for based on dosimetry readings. Once dosimetric verification took place, animal work was able to proceed. Experimental mice were total-body irradiated with different doses of radiation. Specifically, radiation effects were evaluated across four different doses delivered as a single acute exposure of 0.1, 0.25, 0.5 and 3 Gy. The x-ray tube operating voltage was set to 320 kV with a 2 mm aluminum filter to obtain higher energy x-rays that provided a more uniform whole-body dose to the animal. Mice were placed at a distance of 60 cm from the source. Due to the wide range in total doses, two different dose rates were used to minimize the time that animals were in the pie cages. For the two lower doses of 0.1 and 0.25 Gy, the tube current was set to 0.5 mA to achieve a dose rate of 0.045 Gy/min. For the two higher dose rates of 0.5 and 3 Gy, the tube current was set to 12.5 mA to achieve a dose rate of 1.5 Gy/min. Sham irradiated control animals were also placed in the pie cage inside the irradiator, but the beam was not turned on. Post irradiation, animals were transported back to the animal facility and housed in the quarantine room until analysis 2 days later.

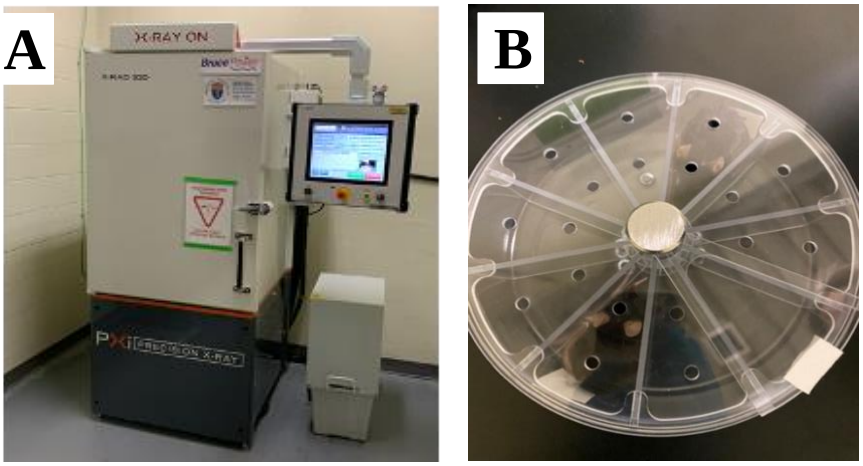

Figure 2.1. Photograph of irradiation setup at NOSM U. A) X-RAD 320 irradiation cabinet B) Circular pie cages designed for small animal irradiations.

2.2.3. Tissue Extraction and Processing

The immune response was measured 2 days post-irradiation. The spleen, lymph nodes and whole blood was collected from each mouse. Mice were placed under anesthesia using 2–3% isoflurane, (Partenaires Pharmaceutiques du Canada, Richmond Hill, ON, Canada). Once anesthetized, blood was quickly collected through cardiac puncture, followed by cervical dislocation to euthanize the mice. Dissections were then performed to remove the spleen and lymph nodes (inguinal and mediastinal).

2.2.3.1. Blood Extraction

Between 0.5 mL and 1 mL of blood was obtained through cardiac puncture using 10% 0.5 M EDTA coated syringes. Blood was then mixed with 15 µL of diluted heparin (500 USP units/ml) and equal amounts of PBS. Peripheral blood mononuclear cells (PBMCs) were then purified using Lymphoprep (STEMCELL Technologies, Vancouver, BC, Canada), where after centrifugation of the tube, the PBMC layer at the interphase could be separated from the upper plasma layer and red

blood cell (RBC) layer at the bottom of the tube. RBC lysis buffer (BioLegend, San Diego, CA, USA) was added to remove the unwanted remaining red blood cells, as per the supplier protocol. Lastly, PBMCs were washed with 5 mL of PBS and centrifuged at 400 x g for 10 min at room temperature. The total number of viable PBMCs was determined using a hemocytometer with trypan blue exclusion of dead cells.

2.2.3.2. Spleen and Lymph Node Extractions

Spleen and lymph nodes were collected and then transported to the NOSM U laboratory on ice. Samples were gently dissociated by grinding on a 70 μm BD Falcon Cell Strainer and rinsed with PBS. Single cell suspensions were then treated with 10 ml of 1X RBC lysis buffer for 10 min to remove red blood cells. The total number of viable splenocytes and lymphocytes was determined using a hemocytometer with trypan blue exclusion of dead cells.

2.2.4. Immunophenotyping by Flow Cytometry.

Single cell suspensions of splenocytes, lymphocytes and PBMCs were prepared for flow cytometry analysis at a concentration of 1 x 10^7 cells/mL. Fc receptors were blocked by 10 μg/mL of anti-mouse TruStain fcX (Biolegend, San Diego, CA, USA) for 10 minutes at room temperature. Immune cell phenotypes were analyzed by simultaneous staining for 1h at 4°C, for one or more of the following mouse cell surface antigens listed in Table 2.2 (targeted by the indicated antibody fluorescently tagged with either Alexa Fluor 647, FITC, PerCP/Cy5.5, PE, APC, APC/Cy7 or PE/Cy7). These antibodies served for the identification of $CD3^+$ T cells, differentiated as $CD4^+$ T helper cells or $CD8^+$ T cytotoxic cells, $NK1.1^+$ and $CD49b^+$ NK cells, $CD11c^+$ and HLA-DR^+ DCs, $CD86^+$ and $CD11b^+$ neutrophils, and $CD19^+$ B cells. Cell viability was assessed using 7-aminoactinomycin (7-AAD), as per the supplier protocols. The appropriate concentration of each antibody was determined through antibody titration using spleen samples from unirradiated mice.

After surface marker staining, cell suspensions were analyzed by flow cytometry using a FACSCanto II flow cytometer (Becton-Dickinson, San Jose, CA). Ultracomp compensation beads (Invitrogen, CA, USA) were used for proper compensation, as per the supplier protocol. A total of 10,000 events were run on the flow cytometry. All cytometry results were analyzed using the Kaluza Software (Beckman Coulter). First, the main cell population was identified through forward and side scatter gating (Figure 2.2A). Next, doublets were eliminated through a plot of forward scatter height vs area (Figure 2.2B). Finally, the cell population was identified by their respective surface markers (Figure 2.2C). Table 2.3 lists gating strategies used in order to identify each specific cell type through exclusion of certain negative/positive populations.

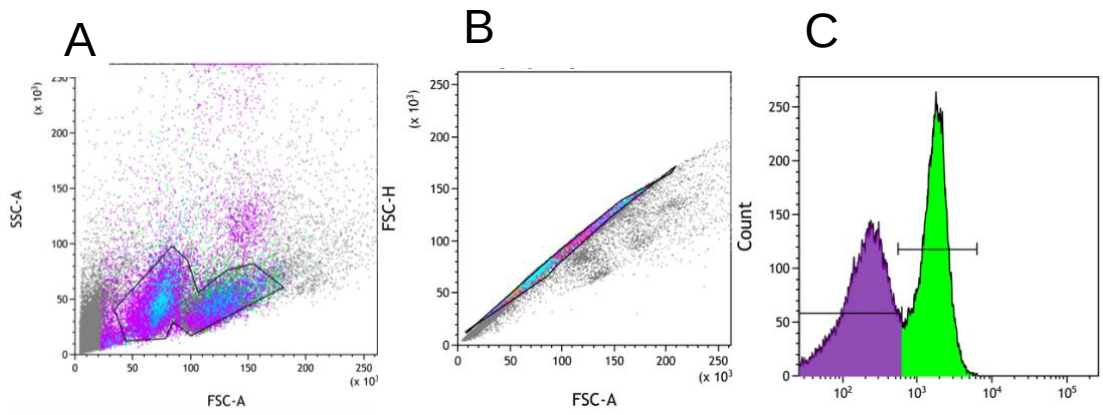

Figure 2.2. Example of gating strategies utilized to analyze and quantify specific cell populations using flow cytometry. A) Forward and Side Scatter gating is used to distinguish between cell populations based on differences in size and granularity. B) Forward scatter height vs area in order to exclude certain cell populations (e.g. debris, doublets) and C) to positively select specific populations representing immune cells of interest. The relative proportion of immune cells can then be quantified by placing gates around the distinct populations.

Table 2.2. List of antibodies used in flow cytometry analysis.

Antibody	Fluorophore	Supplier	Catalogue Number
CD19	PE-CF594	BD Biosciences	562291
NK1.1	APC	BD Biosciences	550627
CD49b	PE-Cy	BioLegend	103518
CD11c	PE	BD Biosciences	553802
CD11b	PE	BD Biosciences	557397
Ly6G	PE-Cy7	BD Biosciences	560601
CD45	APC-Cy7	BD Biosciences	557659
CD3	FITC	BD Biosciences	553061
CD8	PerCP-Cy5.5	BD Biosciences	551162
CD4	APC-Cy7	BD Biosciences	565650
HLA-DR	FITC	BD Biosciences	562009
Ly6G	PE-Cy7	BD Biosciences	560601
Live/Dead	FVS700	BD Biosciences	564997

Table 2.3. Gating strategies utilized during analysis to identify specific immune cell populations.

Organ	Cell Type	Surface Marker Gating
Spleen/Lymph	B Cell	$CD19^+$, $CD4^-$
	NK Cell	$CD4^-$, $CD19^-$, $NK1.1^+$, $CD49b^+$
	DC	$CD19^-$, $HLA\text{-}DR^+$, $CD11c^+$
	T Cell $CD4^+$	$CD3^+$, $CD4^+$, $CD8^-$
	T Cell $CD8^+$	$CD3^+$, $CD8^+$, $CD4^-$
	Neutrophils	$CD11b^+$, $Ly6G^+$
PBMCs	B Cell	$CD45^+$, $CD19^+$, $CD4^-$
	T Cell $CD4^+$	$CD45^+$, $CD3^+$, $CD4^+$, $CD8^-$
	T Cell $CD8^+$	$CD45^+$, $CD3^+$, $CD8^+$, $CD4^-$

2.2.5. Statistical Analysis

In total, 5 different treatment conditions were assessed (0, 0.1, 0.25, 0.5 and 3 Gy). A sample size of 6–8 animals per exposure was used, yielding a total of 40 mice. Each individual cell type was analyzed by a one-way ANOVA followed by Tukey's post hoc test comparing the irradiated

groups back to the sham 0 mGy group. All statistical analyses were performed with GraphPad Prism 6 software. P-values of less than 0.05 were considered statistically significant. (* = $P<0.05$, ** = $P<0.01$, **** = $P<0.001$).

2.3. Results

2.3.1. Spleen

The spleen is the largest peripheral immune organ and is also the main site at which lymphocytes respond to antigens. The spleen consists of lymphocytes, macrophages, dendritic cells, and plasma cells which all work together to destroy pathogens. The total number of splenocytes across each radiation dose tested can be found in Table 2.4 and Figure 2.3. Overall, HDR of 3 Gy caused a significant decrease in total number of splenocytes in the mice. The two major immune cell types found in the spleen were B cells and T cells. Regarding B cells specifically, Figure 2.4 shows that in the sham irradiated mice, B cell numbers ranged from $1x10^7$ to $1x10^8$. Figure 2.5 shows that B cells represent approximately 40–60% of the cells in the spleen in the 0 Gy sham irradiated mice. Regarding T cells, specifically $CD8^+$ cytotoxic T cells, Figure 2.4 shows that the cell counts in the 0 Gy sham irradiated mice ranged from $1x10^5$ to $1x10^7$, while $CD4^+$ T helper cell numbers were approximately $1x10^7$. When looking at cell percentages, $CD8^+$ cells represented 0–10% of splenocytes, while $CD4^+$ cells represented 8–18% of splenocytes in sham irradiated mice (Figure 2.5). The least common immune cell type in the spleen was NK cells. As seen in Figure 2.4, the total number of NK cells in sham irradiated mice ranged from $1x10^6$ to $1x10^7$, representing 3–4% of splenocytes (Figure 2.5). The remaining cell counts and percentages of splenocytes in the sham mice can be found in Figure 2.4 and Figure 2.5, respectively.

Table 2.4. Total number of immune cells within each organ across the different radiation doses.

Dose (Gy)	Splenocytes	Lymph cells	PBMCs
0	$7.4 \pm 0.7 \times 10^7$	$5.2 \pm 0.5 \times 10^6$	$3.7 \pm 0.8 \times 10^7$
0.1	$5.9 \pm 1.8 \times 10^7$	$5.9 \pm 0.7 \times 10^6$	$4.2 \pm 1.8 \times 10^7$
0.25	$4.1 \pm 0.6 \times 10^7$	$5.7 \pm 1.1 \times 10^6$	$3.3 \pm 0.5 \times 10^7$
0.5	$4.5 \pm 0.5 \times 10^7$	$3.6 \pm 0.4 \times 10^6$	$2.8 \pm 0.6 \times 10^7$
3	$4.2 \pm 1.2 \times 10^6$	$3.3 \pm 0.5 \times 10^6$	$9.8 \pm 1.9 \times 10^6$

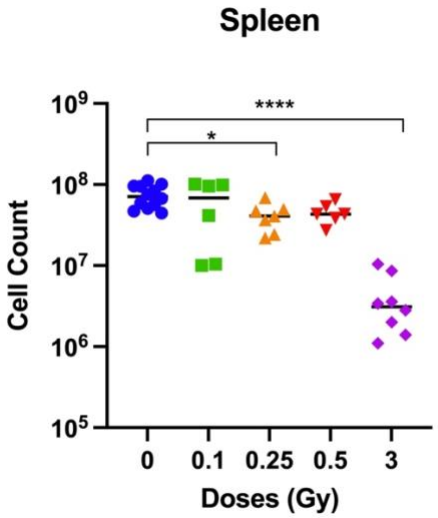

Figure 2.3. Total number of mouse spleen cells following irradiation. Cells were counted using a hemocytometer with trypan blue dye exclusion. Horizontal lines represent the average cell count for each dose (n = 6–8). Data were compared using a one-way ANOVA followed by Tukey's post-hoc test. * = $P<0.05$, **** = $P<0.001$

When the mice were exposed to 3 Gy of radiation, in most immune cell types, this high dose exposure caused a significant decrease in cell numbers. The number of B cells in the mice irradiated with 3 Gy decreased significantly ($p<0.0001$) from $1x10^7$ to $1x10^5$(Figure 2.4). For both $CD8^+$ and $CD4^+$ T cells, exposure to 3 Gy resulted in a significant decrease ($p<0.0001$) in average spleen T cell counts by approximately $1x10^7$ to $1x10^5$ and $1x10^7$ to $1x10^6$, respectively (Figure

2.4). This trend with a decrease in cell numbers after exposure to 3 Gy of radiation is also seen with NK and dendritic cells. As seen in Figure 2.4, following the 3 Gy dose of radiation, NK and dendritic cells were both significantly decreased ($p<0.0029$ and $p<0.0001$) by approximately $1x10^6$ to $1x10^5$ and $1x10^6$ to $1x10^4$, respectively. The only cell type which did not significantly decreased following 3 Gy irradiation was neutrophils (Figure 2.4). When looking at the percentage of cells (Figure 2.5), it is evident that HDR had the greatest impact on B cells. The percentage of cells in the spleen that were B cells dropped from 40–60% to less than 10%. The relative percentage of the other immune cell types remained constant or even slightly increased (Figure 2.5).

Overall, in all immune cell types examined, LDR did not cause any significant increase in cell numbers within the spleen. On a few occasions, LDR did cause a significant decrease in some of the immune cell types tested. First, mice exposed to the lowest dose of 0.1 Gy showed a significant decrease in $CD8^+$ T cells ($p<0.0367$) and B cells ($p<0.0043$) of approximately one order of magnitude (Figure 2.4). Similarly, a dose of 0.25 Gy also caused a significant ($p<0.0255$) decrease in $CD8^+$ T cells of approximately one order of magnitude (Figure 2.4). Lastly, a dose of 0.5 Gy caused a significant decrease ($p<0.0358$) in DC numbers (Figure 2.4).

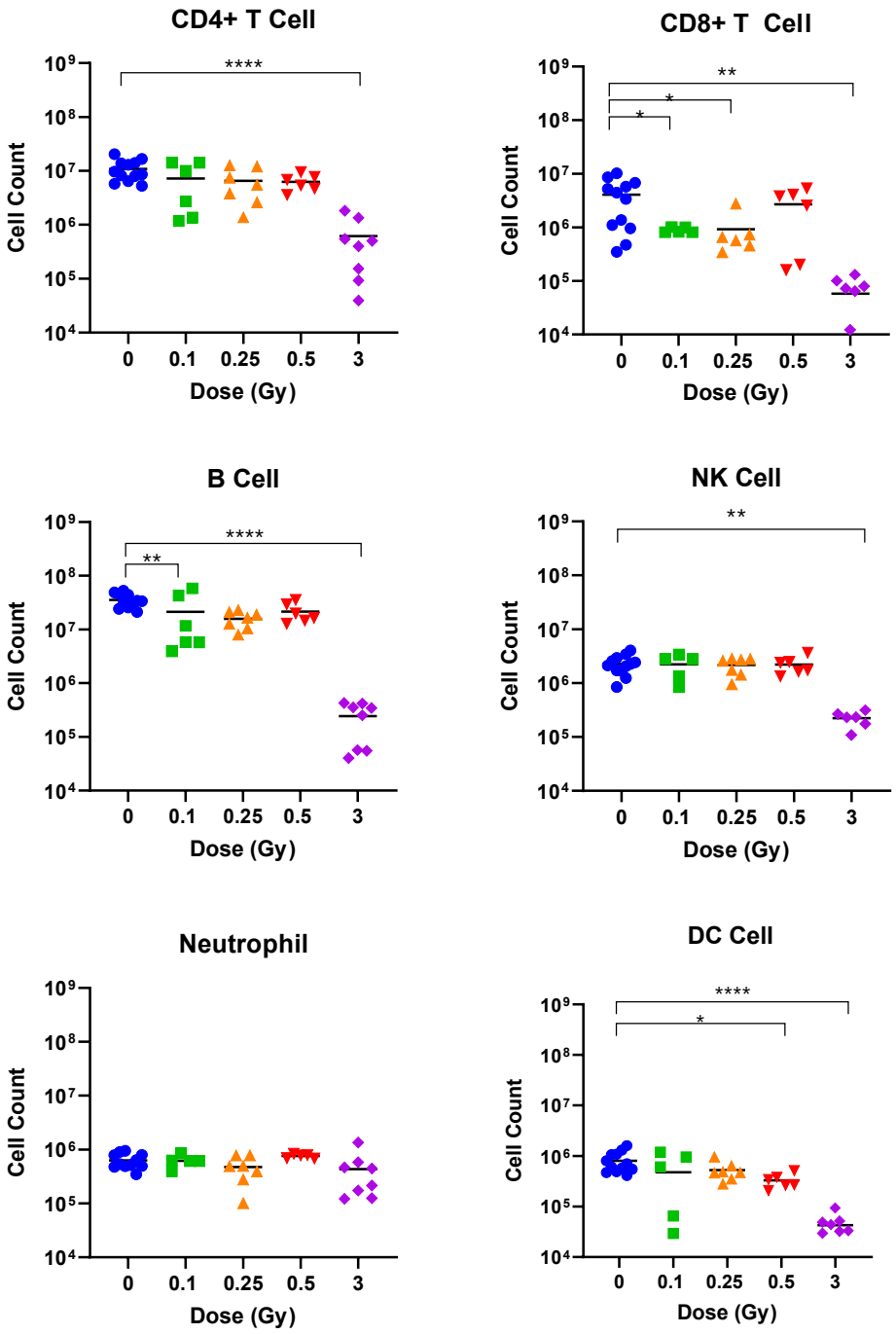

Figure 2.4. Immunophenotyping of major immune cell populations in the spleen of mice following irradiation. Cell types were identified using fluorescent-labeled monoclonal antibodies and flow cytometry. Data are presented as a total cell count for each cell type. Horizontal lines represent the average cell count (n = 6–8). Data were compared using a one-way ANOVA followed by Tukey's post-hoc test. * = P<0.05, ** = P<0.01, **** = P <0.001

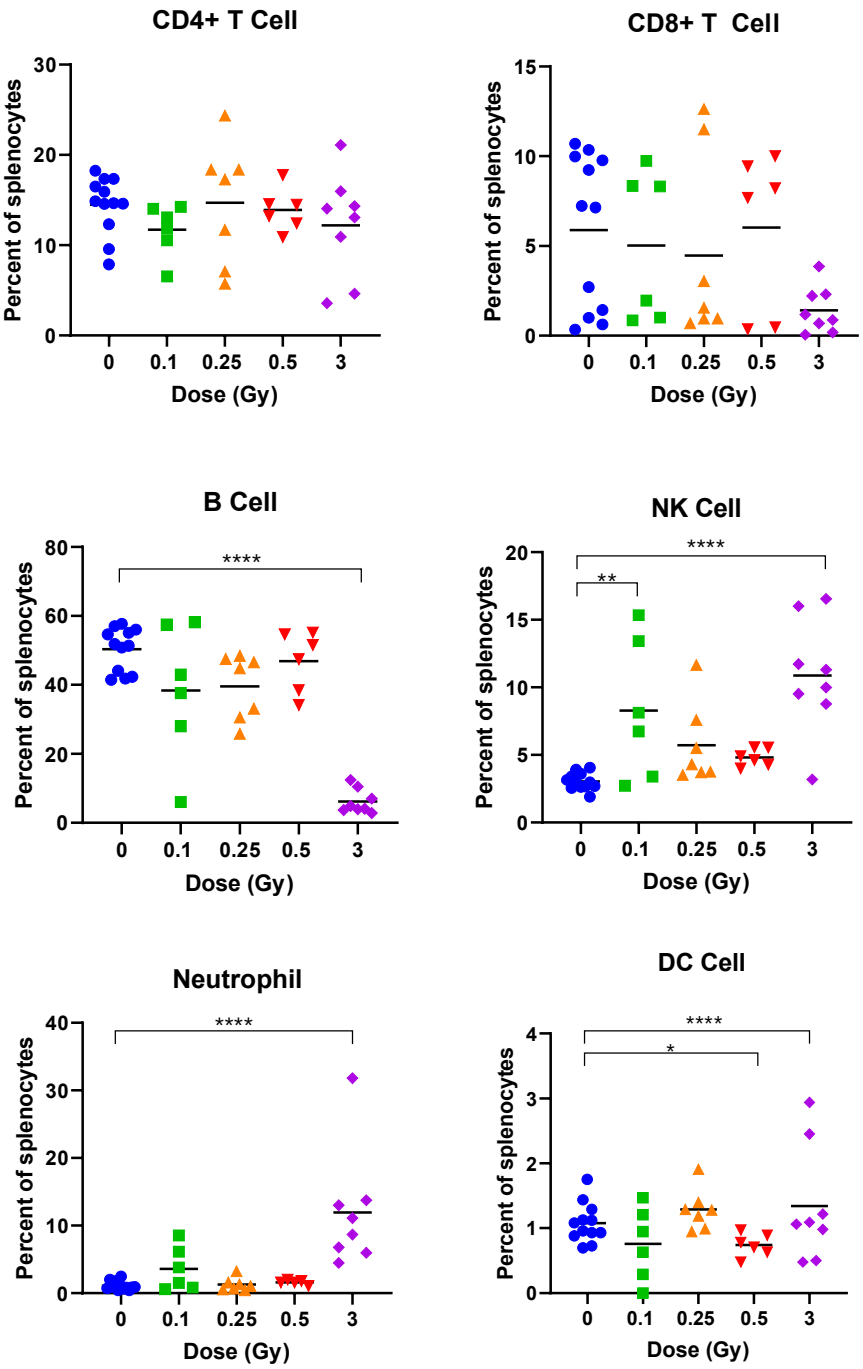

Figure 2.5. Immunophenotyping of major immune cell populations in the spleen following irradiation. Cell types were identified using fluorescent-labeled monoclonal antibodies and flow cytometry. Data are presented as a percent of the total number of splenocytes. Horizontal lines represent the average percent of splenocytes (n = 6–8). Data were compared using a one-way ANOVA followed by Tukey's post-hoc test. * = P<0.05, ** = P<0.01, **** = P <0.001

2.3.2 Lymph Nodes

After a single dose of LDR, there were no significant differences in the lymphocyte numbers in each irradiation group ranging from 0.1 Gy to 0.5 Gy compared with those in the control group, although the number of murine lymphocytes was significantly decreased in the 3 Gy irradiation group (Table 2.4 and Figure 2.6). Similar to the spleen, the two major immune cell types in the lymph nodes were B lymphocytes and T lymphocytes. Regarding the former, Figure 2.7 shows that in the sham irradiated mice, B cell numbers ranged from $1x10^5$ to $1x10^7$. Figure 2.8 shows that B cells represent approximately 10–40% of the cells in the lymph nodes in the 0 Gy sham irradiated mice. Regarding T cells, specifically CD8+ cytotoxic T cells, Figure 2.7 shows that the cell counts of these cells in 0 Gy sham irradiated mice ranged from $1x10^4$ to $1x10^6$, while T helper cell numbers were approximately $1x10^6$. When looking at cell percentages, $CD8^+$ cells represented 0–20% of lymph node cells, while $CD4^+$ cells represented 10–40% of cells in sham irradiated mice (Figure 2.8). The least common immune cell type in the lymph nodes was DC cells and NK cells. As seen in Figure 2.7, the total number of both these cells in sham irradiated mice ranged from $1x10^4$–$1x10^5$, representing less than 1% of lymph node cells. The remaining cell counts and percentages of cells in the sham mice group for the remaining immune cell types can be found in Figure 2.7 and Figure 2.8, respectively.

When the mice were exposed to 3 Gy of radiation, in most immune cell types, this high dose radiation exposure caused a significant decrease in cell numbers. The number of B cells in the mice irradiated with 3 Gy decreased significantly ($p<0.0001$) from approximately $1x10^6$ to $1x10^4$ cells (Figure 2.7). For both $CD8^+$ and $CD4^+$ T cells, exposure to 3 Gy resulted in a significant decrease ($p <0.0486$ and $p<0.0014$) in average lymph T cell counts by 1–2 orders of magnitude each (Figure 2.7). For the remaining cell types tested, namely NK cells, DC and neutrophils, 3 Gy

of radiation did not cause any significant changes in these cells. Overall, when looking at the percentages of cells (Figure 2.8), it is evident that HDR had the greatest impact on B cells. The percentages of cells in the lymph nodes that were B cells dropped from 10–40% to less than 1–2%. The relative percentage of the other immune cell types remained constant or even slightly increased (Figure 2.8). Overall, in all immune cell types examined, LDR did not cause any significant increase in cell numbers within the lymph nodes.

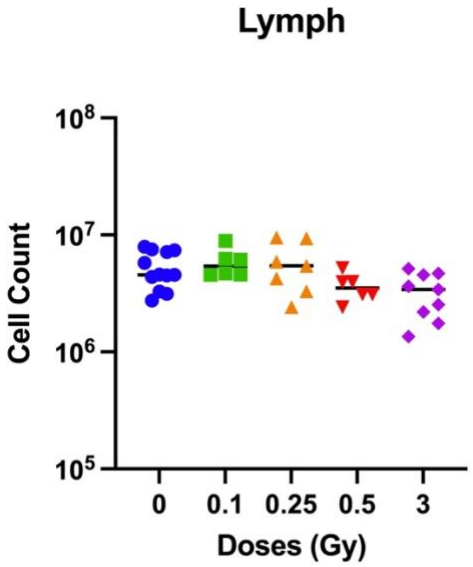

Figure 2.6. Total number of mouse lymph node cells following irradiation. Cells were counted using a hemocytometer with trypan blue dye exclusion. Horizontal lines represent the average cell count for each dose (n = 6–8). Data were compared using a one-way ANOVA followed by Tukey's post-hoc test.

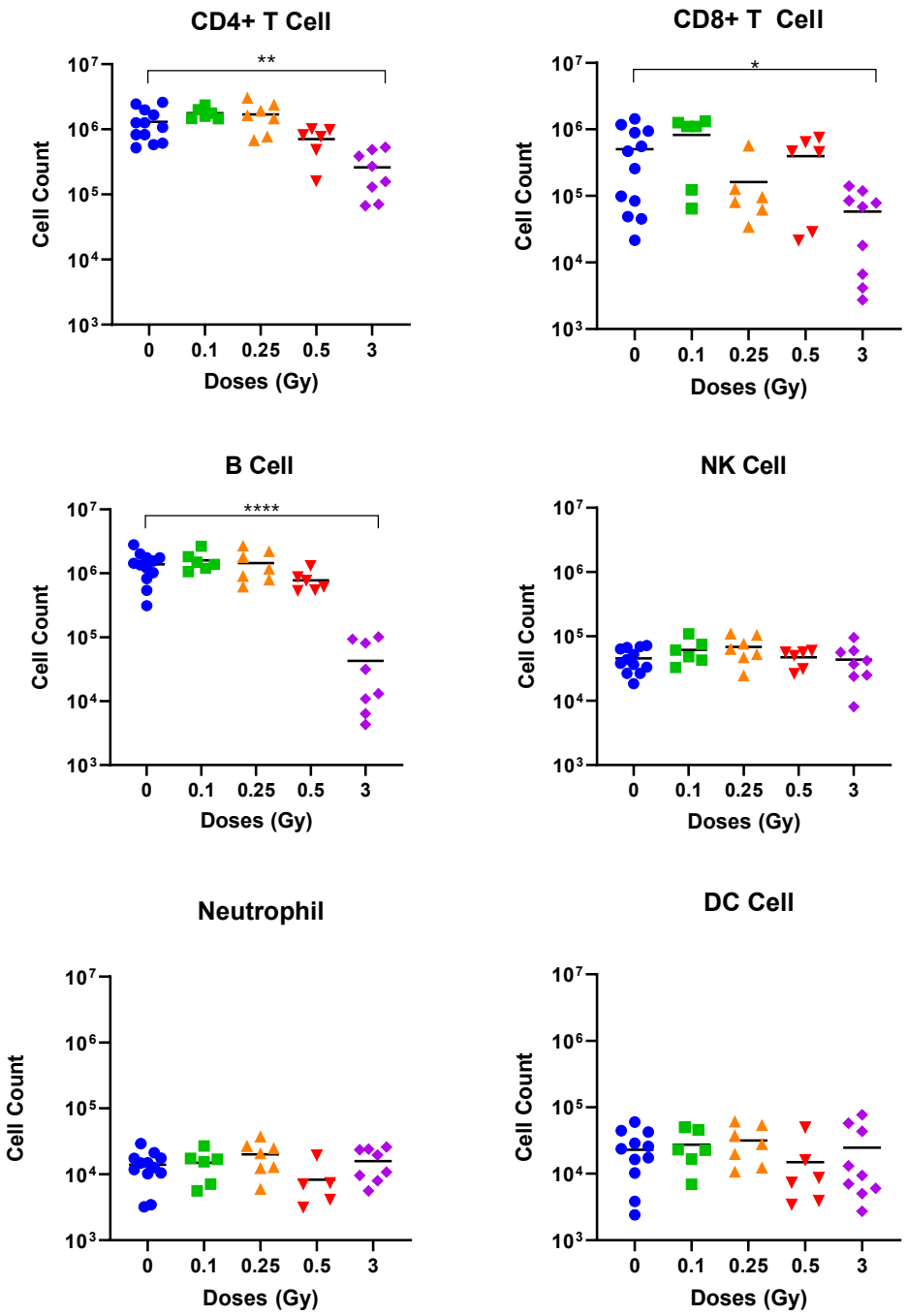

Figure 2.7. Immunophenotyping of major immune populations in lymph nodes following irradiation. Cell types were identified using fluorescent-labeled monoclonal antibodies and flow cytometry. Data are presented as a total cell count for each cell type. Horizontal lines represent the average cell count (n = 6–8). Data were compared using a one-way ANOVA followed by Tukey's post-hoc test. * = P<0.05, ** = P<0.01, **** = P <0.001

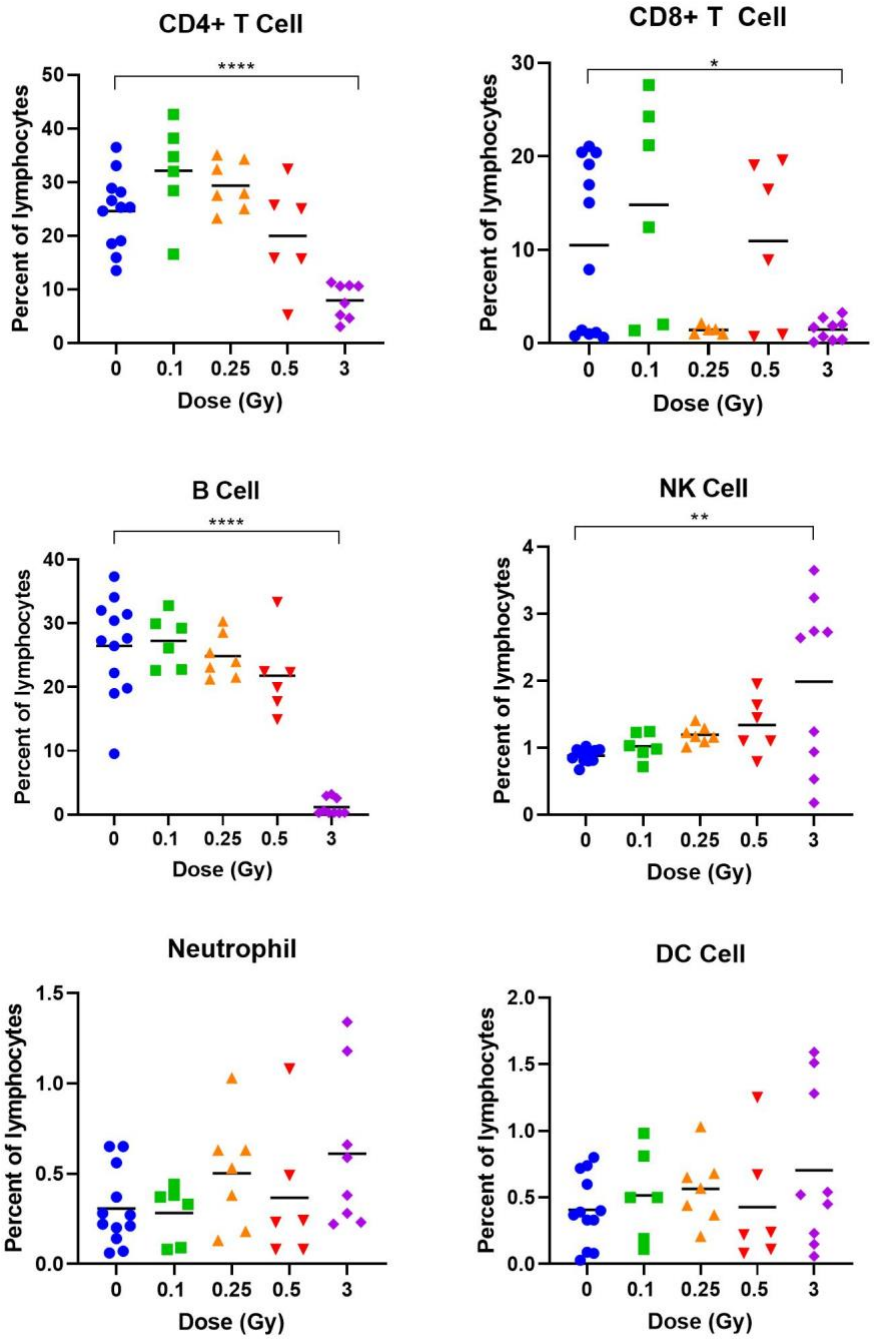

Figure 2.8. Immunophenotyping of major immune populations in lymph nodes following irradiation. Cell types were identified using fluorescent-labeled monoclonal antibodies and flow cytometry. Data are presented as a percent of the total number of lymph cells. Horizontal lines represent the average percent of lymph cells (n = 6–8). Data were compared using a one-way ANOVA followed by Tukey's post-hoc test. * = $P<0.05$, ** = $P<0.01$, **** = $P<0.001$

2.3.3 PBMCs

PBMCs include lymphocytes (T cells, B cells, and NK cells), monocytes, and dendritic cells. Although the frequency of these populations vary across individuals, lymphocytes typically account for the majority of these cell populations. The total number of lymphocytes across each radiation doses tested can be found in Table 2.4 as well as Figure 2.9. Regarding B cells specifically, Figure 2.10 shows that in the sham irradiated mice, B cell numbers ranged from $1x10^7$ to $1x10^8$. Figure 2.11 shows that B cells represent approximately 40–60% of the PBMCs in the 0 Gy sham irradiated mice. Regarding T cells, specifically $CD8^+$ cytotoxic T cells, Figure 2.10 shows that the cell counts in the 0 Gy sham irradiated mice ranged from $1x10^6$ to $1x10^7$, while $CD4^+$ T helper cell numbers were approximately in this same range. When looking at cell percentages, $CD8^+$ cells represented 5–15% of PBMCs while $CD4^+$ cells represented approximately 20% of PBMCs in sham irradiated mice (Figure 2.11).

When the mice were exposed to 3 Gy of radiation, with most immune cell types in the spleen and lymph nodes, this high dose radiation exposure caused a significant decrease in PBMC cell numbers. The number of B cells in the mice irradiated with 3 Gy decreased significantly ($p<0.0271$) from approximately $1x10^7$ to $1x10^6$ cells (Figure 2.10). For $CD8^+$ T cells, exposure to 3 Gy resulted in a significant decrease ($p <0.0006$) in cell counts by 1–2 orders of magnitude (Figure 2.10). When looking at the percentage of cells (Figure 2.11), it is evident that HDR had the greatest impact on B cells. The percentage of cells in the spleen that were B cells dropped from approximately 60% to less than 10%. Regarding the second highest dose of 0.5 Gy, the relative percentage of the $CD4^+$ cells increased significantly by approximately 20% ($p<0.0300$) (Figure 2.11).

Overall, in both the PBMC B cells and T cells, LDR did not cause any significant increase in cell numbers. In terms of relative percentages of these cells, in both B cells and $CD8^+$ T cells, LDR did cause a significant decrease after exposure to 0.1 Gy of radiation by approximately 10% in B cells and less than 5% in the $CD8^+$ T cells (Figure 2.11). In terms of the second lowest dose of 0.25 Gy, this exposure did not cause any significant changes in the numbers of the cells tested.

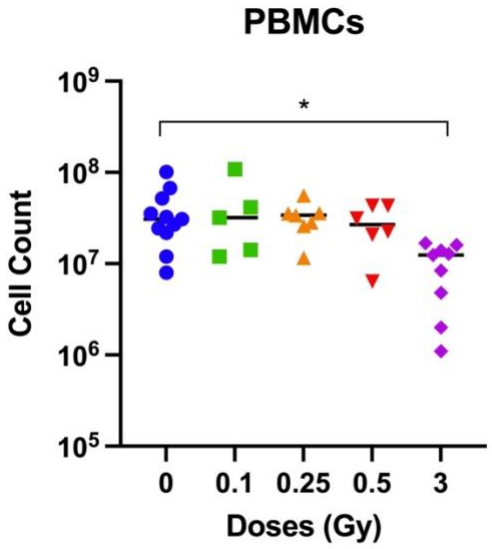

Figure 2.9. Total number of mouse PBMCs following irradiation. Cells were counted using a hemocytometer with trypan blue dye exclusion. Horizontal lines represent the average cell count for each dose (n = 6–8). Data were compared using a one-way ANOVA followed by Tukey's post-hoc test.

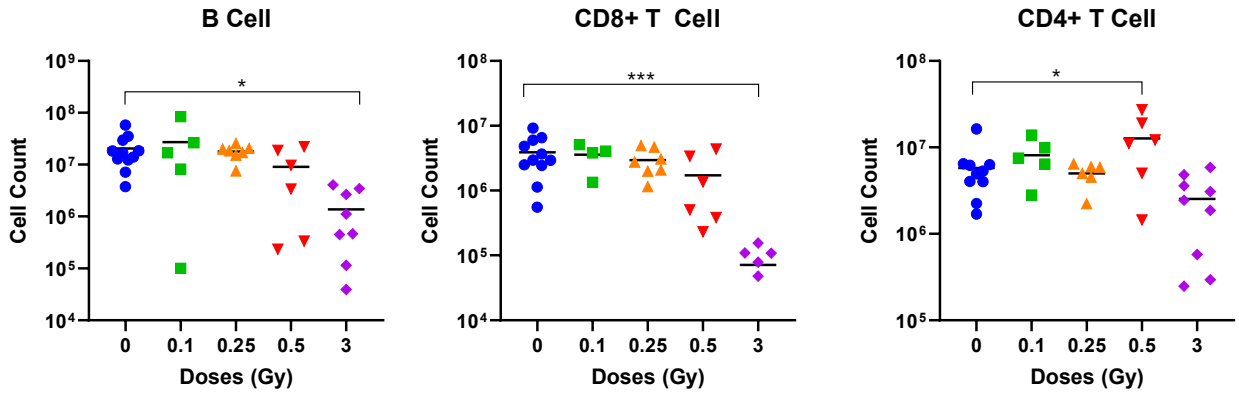

Figure 2.10. Immunophenotyping of major immune populations in PBMCs following irradiation. Cell types were identified using fluorescent-labeled monoclonal antibodies and flow cytometry. Data are presented as a total cell count for each cell type. Horizontal lines represent the average cell count (n = 6–8). Data were compared using a one-way ANOVA followed by Tukey's post-hoc test.* = P<0.05, ** = P<0.01, **** = P <0.001

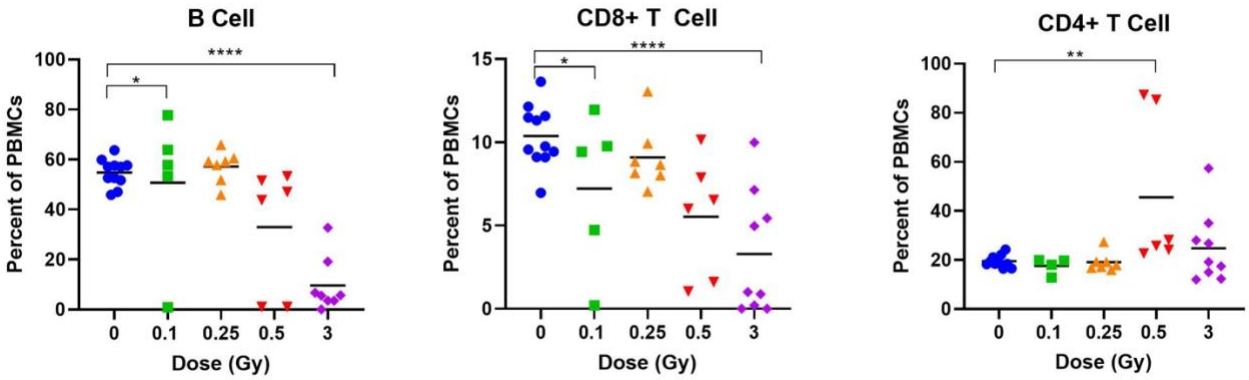

Figure 2.11. Immunophenotyping of major immune populations in PBMCs following irradiation. Cell types were identified using fluorescent-labeled monoclonal antibodies and flow cytometry. Data are presented as a percent of the total number of lymph cells. Horizontal lines represent the average percent of lymph cells (n = 6–8). Data were compared using a one-way ANOVA followed by Tukey's post-hoc test. * = P<0.05, ** = P<0.01, **** = P <0.001

2.4. Discussion

Since ionizing radiation is known to inhibit the immune system at certain elevated doses, it is crucial to identify the appropriate radiation dose regimen for optimal immune enhancement. In addition, identifying the underlying cellular and molecular mechanisms of LDR immune stimulatory effects and ultimately, cancer killing, is fundamental when revisiting LD-RT as an effective cancer therapeutic. Using a rodent model, we investigated the effects of LDR on the immune system to elucidate the underlying cellular and molecular mechanisms. Specifically, various immune cell types known to be affected/modulated by LDR were comprehensively analyzed in irradiated mice. The doses tested were single acute exposures of 0.1, 0.25, 0.5 and 3

Gy. It was hypothesized that LDR would promote antitumor immunity by stimulating the immune system, while simultaneously inhibiting immunosuppressive signals. Since previous studies have identified anti-cancer effects at doses less than 0.5 Gy, it was also hypothesized that immunostimulatory effects would be observed at the two lowest doses tested, as opposed to the highest dose of 3 Gy where immune suppressive effects would prevail. Immune cell-type identification was performed in the spleen, lymph nodes and PBMCs using flow cytometry.

Looking just at the sham irradiated (control) mice, there were differences in the percentages of immune cell types across the three organs. For example, it is known that in mice, B cells are the most abundant immune cell type in both the blood and the spleen[80]. Specifically, the total B cell percentage is most abundant in the blood (50–60%), followed by the spleen (52–56%) and then the lymph nodes (18–20%)[81]. These data are in line with our results for B cell percentages in the sham irradiated mice, where B cells constituted 57% of blood cells (Figure 2.11), 50% of splenocytes (Figure 2.5) and 25% of lymph node cells (Figure 2.8). Similarly, it has been reported that NK cells make up 3–4% of total splenocytes and less than 1% in lymph node cells in mice[82]. As seen in Figure 2.5 and 2.8 respectively, NK cells represented 4% of total splenocytes and 1% of the total lymphocytes in our study. Overall, the cell numbers obtained in our analysis are in line with the typical cell frequencies and percentages of immune cells in mice for the three organs tested. This confirms that our population of mice was healthy with a normal immune system prior to irradiation. It also confirms that our experimental protocols (i.e. dissection, surface marker staining, flow cytometry analysis) were appropriately designed.

Total cell counts of different immune cells and their relative abundance were analyzed in the spleen, lymph nodes and blood of the mice following irradiation. Overall, in most of the immune cell types tested, the highest dose of 3 Gy caused a significant decrease in cell numbers.

Since high doses of radiation, typically 1 Gy or higher, are known to cause immune suppression, these results were expected. The immune-suppressing effect of HDR has clearly been demonstrated and confirmed both experimentally and in epidemiological studies[83]. Regardless of the source, exposure to high doses of radiation, especially acute exposures, can be extremely harmful, and even fatal in some cases[83]. This is a problem for conventional radiotherapy, where despite significant advances in treatment planning, normal tissue toxicity due to HDR is a limiting factor. Immune cells are among the most radiosensitive cell types in body[84]. In a study by E. Bogandi, *et al.* (2010), looking at the effects of whole-body irradiation on the immune system of mice, authors reported similar results with HDR, where a dose of 2 Gy significantly decreased the relative numbers of all the cell populations studied[85]. In this same study, apoptosis of immune cells was also evaluated, and it was found that while LDR decreased apoptosis, irradiation with high doses (0.5 and 2 Gy) resulted in increased apoptosis in most of the cell populations studied[85]. In fact, after irradiation with 2 Gy, 23% of the total splenocytes were apoptotic, which represented a 6.6-fold increase in the frequency of apoptosis[85]. The authors reported these reductions in cell number after 1, 3 and 7 days of HDR[85]. Since our time point of 2 days falls within their first and second time points, it is of no surprise that we saw similar results in the decrease in cell numbers. Interestingly, the authors showed that B cells were significantly more sensitive to irradiation with 2 Gy than the other cell types (19.8% survival)[85]. These results are in line with our findings where HDR had the greatest impact on B cells; the percentage of B cells in the spleen dropped from 40–60% to less than 10% while the relative percentage of the other immune cell types remained fairly constant or even increased. Harrington et al. (1996) reported similar enrichment of CD4+ and NK cells after HDR, which may reflect the relatively greater radioresistance of these cells, compared to B cells[86]. Lastly, by comparing the percentage of cell populations of each cell type in the spleen

after exposure to the highest 3 Gy dose, it is possible to identify which cell type is more resistant to these higher doses of radiation. Specifically, the significant increase in the overall percentage of neutrophils, NK cells and dendritic cells (Figure 2.5) at the 3 Gy dose suggests that these cells are more radioresistant in comparison to the other immune cell types tested. On the other hand, the fact that B cells and both the CD8+ and CD4+ T cells showed a decrease or no overall change (Figure 2.5) in the percentage of splenocytes after irradiation of 3 Gy suggests that they are a more radiosensitive cell type.

In terms of the effects of LDR on the immune cells tested, our results show that LDR did not cause any significant increase in cell numbers within the three organs. Since numerous studies have reported that LDR does indeed cause immunostimulatory effects in-vitro and in-vivo, these results were unexpected and did not support our hypothesis. When testing LDR effects in immune cells at different timepoints, Bogandi., *et al*. also found that LDR did not cause an increase in cell number, and even caused a decrease in some cell types at the 3- and 7-day timepoints[85]. Although they did see an increase in some immune cell types one day after LDR, they reported that this could be due to redistribution of various lymphocyte subsets between the different compartments of the hematopoietic system due to radiation-induced stress, which occurs quickly after irradiation[85]. A plausible explanation for not seeing any increase in immune cell numbers following LDR could be that our timepoint post-irradiation was not long enough. This hypothesis is supported by results found in a recent study by X. Lui *et al*., in 2020 where the long-term effects of LDR were analyzed on the immune cells of mice[87]. Similar to our findings, the number of DC, NK, macrophages and T cells decreased compared to sham within 2 days after irradiation[87]. However, dendritic cells actually started increasing after day 7, and the proportion of macrophages increased until day 14[87]. In addition, analysis of the expression of activation markers such as CD25,

CD28 (T cells), CD69 (NK cells) and CD80/CD86 (DC) showed an increase only on days 7 and 14[87]. Results from another study by Gridley *et al*. (2009), looking at the low dose radiation effects on leukocyte distribution also support that immune effects may be longer term[88]. Specifically, a significant difference in the CD4:CD8 T cell ratio was seen on day 21 where 0.1 Gy caused an increase in the T cell ratio, however, this was not seen at the two shorter timepoints of 0 and 4 days[88]. Although the exact reason for why a longer timepoint would be needed to see a stimulation in immune cells exposed to LDR is unknown, one reason could be due to the slow adaptive immune responses. Specifically, the adaptive immunity takes longer to develop on the first exposure to a new pathogen, as specific clones of B and T cells have to become activated and expand, therefore taking a week or so before the responses are effective[43]. This same timing could apply to immune responses to ionizing radiation.

Another possible explanation for why LDR exposure did not cause any significant immune stimulation in our study could be due to the method of irradiation. In our study, a single acute dose of radiation was delivered to the mice. Conversely, there have been many studies exploring the effects of LDR that have instead used fractionated exposures, where the total dose of radiation was split up into smaller doses in multiple exposures over a given time. This was observed in one such study by Song *et al*. (2015), which focused on analyzing immune cell populations in murine splenocytes exposed to LDR[89]. In that study, female C57BL/6 mice were whole body-irradiated with a single dose or three daily fractions up to a total dose of 0.001, 0.01, or 0.1 Gy, and the spleen was harvested 2, 7 and 14 days after irradiation[89]. The authors found that although for some of the cells tested, a single acute dose and fractionated did elicit a similar pattern of change in leukocyte subpopulations, this was not the case for CD8+ T cells and NK cells[89]. These two cell types were more sensitive to fractionated exposures. The results of this study, amongst others which have

confirmed that some immune cells are more sensitive to fractionated exposures, suggests that there is likely some differences in how immune cells respond to a repeated exposure as opposed to a single acute exposure. Consequently, these previous studies support the narrative that immune cells might require a recovery period in order for immune stimulation to occur. In addition, Song *et al*. found that a single acute dose resulted in a Th1 cytokine expression profile, whereas fractionated irradiation drove a Th2 shift[89]. Since these 2 phenotypes differ in their main role and mechanisms in the immune system, this finding also suggests that the delivery method of the radiation could be responsible for the discrepancies between our results and the published literature. Lastly, Song *et al*. used very low doses of radiation, so potentially the doses used in our study might have been too high to see the immune stimulatory effects of LDR. For example, the highest dose of radiation used in their study was actually the lowest dose tested in our study. Since LDR is only recently being revisited as a new therapeutic, the exact dose for immune stimulation and cancer killing has not yet been identified. Therefore, further studies should focus on including more doses of LDR in order to better understand the LD-RT therapeutic window whereby the optimal immunostimulatory phenotype is achieved without induction of immunosuppressive effects.

A final possible explanation for why LDR exposure did not cause any significant immune stimulation in our study could be the strain or gender of mice. Specifically, the C57BL/6 strain of mice was selected because it is known to have a robust immunological response, particularly related to anticancer immunity, compared to other strains. It has also been shown to elicit an immune response following LDR exposure, and others in our lab have previously worked with C57BL/6 mice in radiobiology studies, immunology experiments and tumour implant studies. Despite this, all of the mice used were males. Although this variable is less likely than the others

mentioned above, differences in immune responses have been reported in mice based on sex. For example, it was found that adult female mice produce higher levels of T helper 1 (Th1)-type cytokines (for example, IFN-γ) than males and that regardless of age, females tend to show greater antibody responses, higher basal immunoglobulin levels and higher B cell numbers than males[90]. This stronger response in females could be due to different biologic factors such as genetic and epigenetic factors, sex hormones, and psychosocial factors[90]. Interestingly, in the same study conducted by Song et al., where it was shown that the sensitivity of the induced response varies according to the dosing method, only female C57BL/6 mice were used[89].

Most of the previous literature focused on the immune response in the spleen of irradiated mice. In our study, we provided a more comprehensive analysis as we also included immunophenotyping in the lymph nodes and circulating blood. However, we identified a very similar trend in results across all three organs. Since LDR is delivered over the entire body and previous studies have shown that LDR treatment enhances systemic antitumor immune responses, the recuring outcomes was as expected.

In conclusion, we identified an immune suppressive effect following HDR but no significant effect of LDR. As outlined above, there are multiple plausible explanations as to why a stimulation in the number of immune cells was not seen following LDR in our study. Future studies could focus on incorporating certain factors such as longer timepoints post-irradiation, fractionated exposures as well as single acute doses, and female and male mice to eliminate any sex differences that could exist. In addition, a wider range of doses could be included in order to have a better understanding of where the switch between immune stimulation and immune suppression is occurring.

3. In-vitro Effects of LDR in NK Cells

3.1. Introduction

Natural killer (NK) cells, along with B cells and T cells, are a type of lymphocyte that play an important role in the immune system. However, unlike B cells and T cells, NK cells are considered part of the innate immune system as they respond immediately to pathological invaders that the immune system encounters[37]. NK cells are large granular lymphocytes known for their cytotoxic properties[91]. In fact, NK cells are widely known for killing virally infected cells and being able to quickly detect and control cancer cells[91]. These cells represent 5–20% of all circulating lymphocytes in humans[92]. NK cells obtained their name for their ability to kill naturally. In other words, NK cells can kill tumor cells without any priming or activation unlike other immune cells such as T cells, which need activation from antigen presenting cells[92]. In general, various immune cells can identify virally infected cells in the body by detecting the major histocompatibility complex (MHC) presented on their cell surface[93]. One of the main reasons NK cells differ from other immune cells is due to their ability to recognize infected cells without the use of MHCs, leading to a very rapid immune reaction[93]. Specifically, since some cancerous cells are missing "self" markers of MHC on their surface, NK cells are the only immune cell type able to detect and destroy them[93]. To this end, NK cells can trigger target cell death by releasing cytotoxic granules containing granzymes and perforin and through death receptor-mediated pathways (e.g. FasL/Fas)[94]. In addition, NK cells are also known for their cytokine producing functions which act on other immune cells in order to obtain a stronger immune response[94].

In humans, NK cells are phenotypically identified by the expression of CD56 and the absence of CD3 and can be classically divided into two populations: $CD56^{bright}CD16^{dim}$ and $CD56^{dim}$ $CD16^{bright}$, with the former believed to be the population responsible for producing potent cytokines, and the latter responsible for the cytotoxic properties[95]. In mice, NK cells are typically

identified by lack of CD3 and the presence of NK1.1 or CD49b surface molecules[95]. Although bone marrow is the primary site of NK development, they are also found in both primary and secondary immune compartments, such as the spleen, lymph nodes, and peripheral blood[95]. NK cells are also found in mucosal tissues, including the lungs, small and large intestines, and colon[96].

Simply put, NK cells contain a repertoire of both activating and inhibiting surface receptors and the balance of these ligand/receptor interactions dictates the status of NK cell activation[97]. For instance, healthy normal cells express no or minimal levels of activating ligands, but express high levels of the MHCs that ligates to the inhibitory receptors on NK cells[97]. Therefore, the inhibitory receptors utilize the MHC molecule of healthy cells and act as a check on NK cell killing, switching 'off' the NK cell and preventing it from killing the body's own healthy cells[97]. Conversely, tumor cells have downregulated MHC expression but upregulated levels of activating ligands and thus trigger NK cell activation due to the lack of inhibitory signals and/or the presence of activating signals[98]. Consequently, activating receptors recognise the molecules expressed on the surface of cancer cells and infected cells, and 'switch on' the NK cell to initiate cytotoxic mechanisms (Figure 3.1)[98]. The main goal of the activating and inhibitory receptors is therefore to maintain a precise balance between activating costimulatory and inhibitory signals, and these interacting signals finally decide the activation and functional status of NK cells.

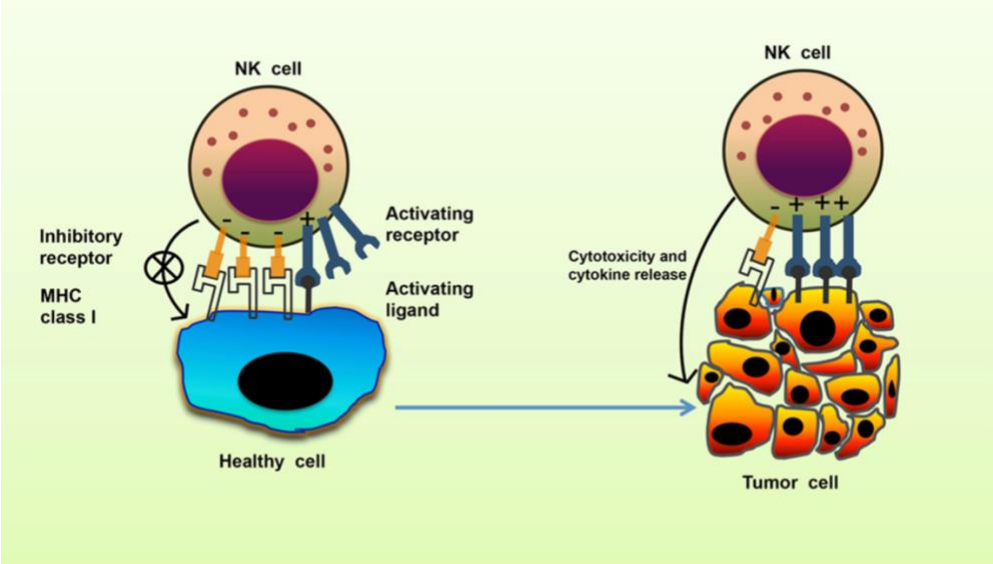

Figure 3.1. Diagram of NK cell activation. Left: NK cell activity is inhibited once in contact with a healthy cell due to MHC molecules on their cell surface and the absence of activating ligands. Right: NK cell activity is activated once in contact with a tumor cell due to the lack of MHC molecules on their cell surface and the presence of activating ligands[98].

Since there are many receptors expressed on NK cells that mediate the delivery of activating and inhibitory signals, they can be further classified by their structure, either as activating or inhibiting killer cell immunoglobulin-like receptors (KIRs) or killer cell lectin-like receptors (KLRs)[98]. Some of the most common activating receptors found on NK cells are Ly49, NCR (Natural cytotoxicity receptors) and CD16, and some common inhibiting receptors are CD94/NKG2 and ILT (immunoglobulin like receptor)[98,99]. A more comprehensive list of various activating/inhibiting receptors of NK cells is detailed in Figure 3.2.

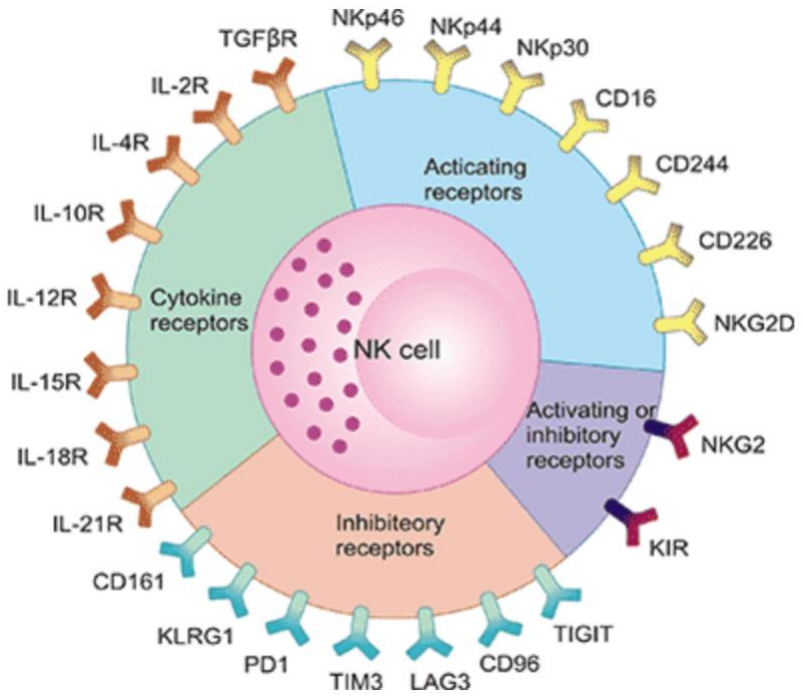

Figure 3.2. Activating and inhibitory receptors found on the cell surface of NK cells[99].

NK cells have been shown to be one of the most powerful immune effectors in tumor surveillance and control. This can be achieved by different killing strategies that the cell will choose to use. For example, NK cells can kill tumor cells indirectly by promoting dendritic and T cell interactions[98]. Although NK cells contain a wide variety of cytotoxic killing mechanisms, they typically kill target cells via 3 main pathways. These are: a) direct lysis by perforin and granzyme, b) induction of apoptosis by FasL/Fas or tumor necrosis factor (TNF)- related apoptosis-inducing ligand (TRAIL)/TRAIL receptors, and c) the release of cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) to activate systemic antitumor immunity[42,50].

In the first pathway, granule-mediated cytotoxicity is initiated by the release of lytic granules, known as perforins (pore forming proteins), directly toward a locally attached target cell[100,101]. Granzymes (cytotoxic proteins) can then enter the target cell by perforin-pores in the

plasma membrane. Once inside the cell, granzymes can then induce caspase activation, mitochondrial dysfunction, or caspase-independent apoptosis[100]. Granzymes are a family of closely related serine proteases that are expressed in cytotoxic T cells and NK cells. In humans, granzymes consists of 5 members: granzyme A, granzyme B, granzyme H, granzyme K, and granzyme M, of which granzyme B is the most characterized[101]. Together with perforin, they represent the major cytotoxic components of secretory granules of NK cells and cytotoxic T cells. Besides the release of perforins and granzymes, NK cells can also present specific ligands on their surface which will activate their respective death receptors on the surface of the target cell. There are 2 main receptor/ligand systems that can mediate apoptosis upon activation: FASL binding to FAS receptor and TRAIL binding to TRAIL-R1 and TRAIL-R2 receptors on the surface of tumor cells[98]. When the FAS and TRAIL ligands bind to their respective death receptors, activation will then induce target cell death by delivering a "death signal" to the cell[98]. This will begin with formation of a death-inducing signaling complex composed of activated death receptors, recruited FADD adaptor proteins and initiator procaspases[102]. Finally, the activation of caspase-8 and caspase-10 at the death-inducing signaling complex initiates a caspase cascade, ultimately leading to apoptosis[102].

Besides the cytotoxic action, NK cells, when activated, are capable of secreting a variety of cytokines such as TNF- α, IFN- γ, or factor granulocyte and monocyte colony stimulant (GM-CSF) which play a very important role in the proliferation, differentiation and activation of other cells and also in the regulation of the immune response[98]. These three main pathways are shown in further detail in Figure 3.3. Thus, in summary, it can be said that NK cells play a crucial role in the innate defense against viruses and tumors or in the regulation of the immune response.

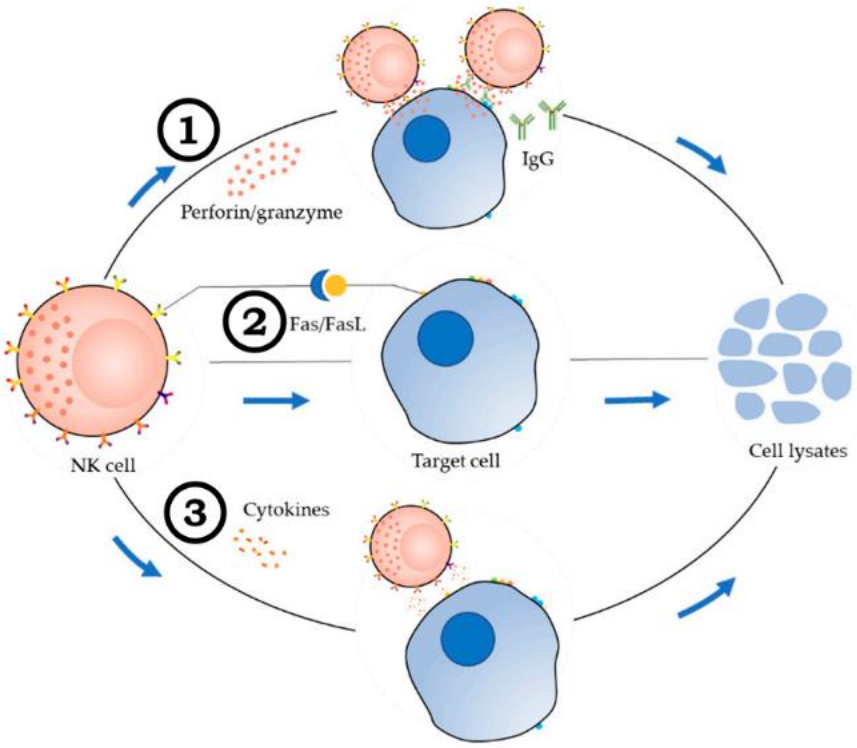

Figure 3.3. The three major cytotoxic pathways utilized by NK cells to kill a tumor. 1) The perforin/granzyme pathway 2) The Fas/TRAIL death receptor pathway 3) The cytokine/chemokine release pathway[98,102].

NK cells play a crucial role in antitumor immunity, specifically through cancer immunosurveillance and by interacting with other adoptive immune cells for an enhanced immune response[53]. In fact, numerous studies have shown that removal of NK cells can increase the incidence of cancer, suggesting that NK cells are highly involved in tumor cell elimination. Therefore, in the last decade, using NK cells for therapeutic purpose and designing NK-cell based immunotherapies has been of interest to many. To date, there are several NK cell immunotherapies that have shown promising results in reducing cancer incidence and metastasis in patients[103]. Some examples of these include adoptive cellular therapy, chimeric antigen receptor (CAR) NK cell therapy, cytokine therapy and monoclonal antibody (mAb)-based treatment[103]. In addition, with

significant advancements in cell biology technologies, many NK cell lines that can survive permanently in-vitro have been established.

Since immortal cell lines offer numerous advantages (i.e. less costly, ease of use, provide higher cell numbers, etc.) they are often used in research in place of primary cells. The NK-92 cell line has been one of the most consistent NK cell lines to show high antitumor immunity, and given their strong resemblance to NK cells, are a perfect model to study NK cells in-vitro[103,104]. This cell line was isolated from a 50-year-old male patient with rapidly progressing non-Hodgkin's lymphoma[104]. The cells require the presence of recombinant IL-2 for growth and proliferation. Furthermore, this cell line displays many characteristics of activated primary NK cells, such as the expression and activating cell surface molecules. To date, the NK-92 cell line is a critical NK cell line that has completed many preclinical and phase I and II clinical trials[104]. Although these cells are of malignant origin, many studies have shown that NK-92 cells are safe to infuse into patients if they are irradiated beforehand, which ultimately prevents in-vivo proliferation while maintaining the cell's ability to kill target cells[105]. For NK-92 cells specifically, a large body of evidence has shown that pre-irradiation with 10 Gy of radiation stops the cells from proliferating uncontrollably, however functional cytotoxicity of these cells are still maintained[105]. In this chapter of my thesis, the NK-92 cell line was used to study the effects of LDR on NK cell cytotoxicity.

As mentioned previously, in the last few decades, LD-RT has been revisited and has been found to be a promising cancer therapeutic through its ability to stimulate and enhance the immune response. Since NK cells are one of the main cell types involved in the immune system's defence against cancer, it is of no surprise that NK cells are one of the cell types that have been implicated in LDR induced anticancer effects. In fact, numerous studies have shown that LDR could increase the cytotoxicity of NK cells. For example, in mice exposed to a single acute whole-body dose of

100–500 mGy, spleen isolated NK cells showed increased cytotoxicity in-culture compared to sham irradiated controls when measured using the ^{51}Cr release assay[106]. In addition, Shin *et al.* showed that irradiation of mice with low dose x-rays (0.1 Gy) significantly stimulated NK cell-mediated tumor cell lysis[107]. Despite these promising findings, the exact mechanism by which LDR can stimulate NK cytotoxicity has not been identified.

Overall, since the NK-92 cell line mirrors most of the characteristics of human primary NK cells, it is receiving much attention in immunotherapies to treat a range of malignancies. It would then be of great interest to test the effects of LDR on the NK-92 cell line. Therefore, the objective of this chapter of my thesis was to elucidate potential mechanisms of LDR induced cytotoxicity in NK-92 cells. It was hypothesized that radiation exposure below 0.5 Gy would increase the cytotoxicity in NK cells, mainly through the enhancement of both the ligand mediated and perforin/granzyme mediated apoptotic signals and through the release of cytotoxic cytokines. This was tested through in-vitro irradiation of NK-92 cells, following which cell growth, cell cytotoxicity and gene expression was measured.

3.2. Material and Methods

3.2.1. Cell Lines and Culture

Experiments were conducted using two different cell lines; NK-92 (CRL-2407) and K562 (CCL-243). The NK-92 cell line is a human cytotoxic cell line composed of allogeneic, activated, interleukin-2 (IL-2) dependent-natural killer cells derived from a 50-year-old male patient with rapidly progressive non-Hodgkin's lymphoma. The K562 cell line is a tumorigenic cell line that are lymphoblast cells isolated from the bone marrow of a 53-year-old chronic myelogenous leukemia patient. Both cell lines were purchased from American Type Culture Collection (ATCC). NK-92 cells were cultured in alpha Minimum Essential Media with L-glutamine and sodium

pyruvate, no ribonucleosides or deoxyribonucleosides (Gibco, Waltham MA), with 0.2 mM inositol (Thermo Fisher Scientific, Waltham MA), 0.2 mM 2-mercaptoethanol (Thermo Fisher Scientific, Waltham MA), 0.02 mM folic acid (Thermo Fisher Scientific, Waltham MA), 12.5% horse serum (Gibco, Waltham MA), 12.5% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch,GA), and 1000 U/ml recombinant interleukin 2 (IL-2) (Miltenyi Biotech, CA) at 5% CO_2 and 37°C. K562 cells (ATCC CCL-243) were cultured in Dulbecco's modified Eagle Medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning Life Sciences) and with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (pen-strep) solution (MilliporeSigma, Burlington MA) at 5% CO_2 and 37°C.

3.2.2. Irradiations

Irradiations were performed on an X-RAD 320 irradiation cabinet (Precision X-ray, Madison, CT) at NOSM University operated at 320 kV and 12.5 mAs with a 2 mm Al filter. Doses for all experiments were delivered as single acute exposures. A range of doses were used depending on the endpoint. For growth analysis, cells were exposed to doses of 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 Gy. For the cytotoxicity assay, cells were exposed to doses of 0.1 and 0.25 Gy. For RT-qPCR, cells were exposed to doses of 0.1, 0.25, 0.5 and 1 Gy.

3.2.3. Cell Growth Curve

A growth curve was carried out for the NK-92 cell line in order to evaluate the growth characteristics and population doubling time of this cell line post radiation exposure. On day 0, cells were seeded into separate flasks before immediately being irradiated at the doses mentioned above. Specifically**,** NK-92 cells were seeded at a density of 300,000 cells/ml on Day 0 in T25 flasks. Total viable cells were counted using a hemocytometer with trypan blue dye exclusion (SIGMA-Aldrich) at the same time each day for 7 days. To calculate a cell doubling time, an

exponential curve was fit to the log phase of the growth curve (days 1-4) using GraphPad Prism Software. Growth curves were performed in triplicate.

3.2.4. Cytotoxicity Assay

A cytotoxicity assay was performed to quantify the ability of NK-92 cells (effector cell) to recognize and kill the tumorigenic K562 cells (target cell). First, K562 cells were labeled with 2 μM carboxyfluorescein succinimidyl ester (CFSE) for 20 minutes to enable the discrimination of K562 cells from NK-92 cells during flow cytometry analysis. Then, NK-92 cells were co-cultured with CFSE labeled K562 target cells in a 96 well plate. Each well contained a volume of 200 μL. A total of 40,000 K562 target cells were added to each well. The number of NK-92 cells added to each well depending on the effector to target (E:T) ratio. A negative control well with K562 cells alone was also used. After co-culture for 4 hours at 37°C and 5% CO_2, the cell mixture was stained with propidium iodide (PI) (Beckman Coulter, Milan, Italy) at a concentration of 50 ug/ml for 20 min in the dark in order to differentiate live vs dead cells. After staining, cells were analyzed on a SONY Flow Cytometer (SA3800 Software). The fluorescence intensity was measured for both CFSE and PI. Data were analyzed using a dotplot of CFSE vs PI to which quadrants were applied. NK cytotoxicity (%) was calculated based on the ratio of dead K562 cells (positive for both CFSE and PI) to total K562 cells (all CFSE positive cells).

Prior to irradiations, different E:T ratios were tested in the optimization process in order to find the most optimal ratio for the experiment. This would be determined by the ratio which would yield 40–60% K562 cell killing. The ratios tested were 25:1, 12:1, 6:1, 3:1 and 1:1 NK-92:K562 cells. As seen in Figure 3.4, the 6:1 and 3:1 ratios were the most optimal ratios to observe NK-92 cytotoxicity since they resulted in 40–60% K562 cell killing. Therefore, these ratio were used for radiation experiments.

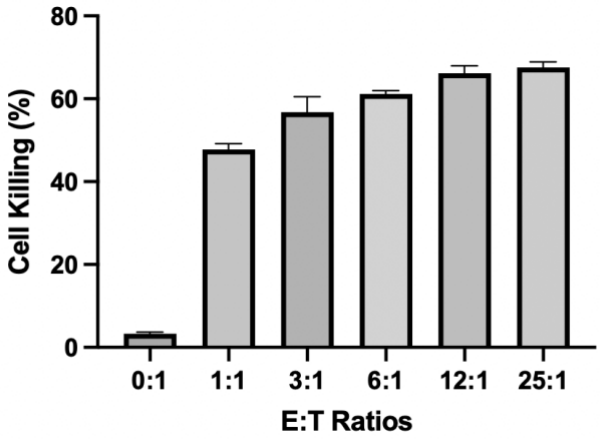

Figure 3.4. Effector to target (E:T) cell ratio optimization for cytotoxicity assay. Ratios tested ranged from 1:1 to 25:1 NK-92:K562 cells. Data represent the average of 3 independent replicates ± SEM. The 3:1 and 6:1 ratios were chosen as the most optimal since they resulted in a 40–60% K562 cell killing.

3.2.5. RT-qPCR

The mRNA levels in irradiated NK-92 cells were quantified for nineteen genes involved in NK cytotoxicity. Cells were seeded on Day 0 at a density of 200,000 cells/ml and immediately irradiated with their respective doses. After radiation exposure, either 24 or 48 hours later, total RNA was isolated from NK-92 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The concentration of total RNA was then determined with the qPCR Thermo Cycler NanoQuant instrument (MJ Mini 48-Well Personal Thermal Cycler, BioRad). Next, 2 µg of RNA was reversely transcribed into cDNAs following the Super Script III First-Strand kit's protocols (Invitrogen, Carlsbad, CA, USA). The reverse transcription condition was 65ºC for 5 min, 50ºC for 50 min, 85ºC for 5 min. qPCR was performed with SYBR green premix (Takara Biomedical Technology, Beijing). The qPCR program was 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s, 60ºC for 1 min. qPCR was performed on a QuantStudio 5 Dx Real-Time PCR machine (Thermo Scientific, Massachusetts) with gene specific primers (Table

3.1). The comparative threshold cycle (CT) method was used with β-actin as an internal control. Lastly, the mRNA expression levels of target genes were calculated by $2^{\triangle\triangle Ct}$ method and normalized to a non-irradiated NK-92 group. Primers were designed based on previously published sequences for these target genes in NK-92 cells.

Table 3.1. qPCR primers used to evaluate the genes involved in NK-92 cytotoxicity post radiation exposure**.**

Gene	Forward Primer	Reverse Primer
Perforin/Granzyme Pathway		
PRF1	GAGCCTCGGTGAAGAGAGGA	GGCACTTGGGCTCTGGAAT
GZMA	AGAGACTCGTGCAATGGAGAT	CCAAAGGAAGTGACCCCTCG
GZMB	CCAGGGCAGATGCAGACTTTTC	GTCGTCTCGTATCAGGAAGCC
FAS/TRAIL Ligand Mediated Pathway		
FASLG	CTTGGTAGGATTGGGCCTGG	CTGGCTGGTAGACTCTCGGA
TNFSF12	TGGGAGGAAGCCAGAATCAAC	CTCATCAAAGTGCACCTGACAGT
Cytokine/Chemokine Pathway		
IFNG	GAGTGTGGAGACCATCAAGGA	TGGACATTCAAGTCAGTTACCGAA
TNF	TGCACTTTGGAGTGATCGGC	GCTTGAGGGTTTGCTACAACA
Activating/Inhibiting Receptors		
NRC3	CTTCGCCAACTGGGACATCT	GTACGAATCTCAGGGGGCTG
CD69	ACGCAGGTAGAGAGGAACAC	ACCCTGTAACGTTGAACCAGT
KLRC1	ACTAACCTGGCCTCTCCACT	TTTGGGTTTGGGGGCAGATT
KLRD1	AGCCAGCATTTACTCCAGGAC	TGCCGACTTTCGTTCCAAGT
KLRK1	TCCCTCTCTGAGCAGGAATC	CCACGAATCCACCCCATCAA
KIR3DL1	ACCATGTTGCTCATGGTCGT	CTTGTCCTGACCACCCACG
LILBR1	CTCCACATCTGCAGGCCC	CCACCAAGATGCCGATCACA
FCGR3A	CTGGCATGCGGACTGAAGAT	TCCTTCTCGAGCACCCTGTA
TNFSF9	TTCCTCACGCTCCGTTTCTC	TGTACTGGTCTCATAAATGGTTGT
TNFSF10	GTCAGCTCGTTAGAAAGACTCCA	TGCTCAGGAATGAATGCCCA
NRC1	CCAGTGAAGCTCCTGGTCAC	CCCAGAGGGCATGGTCTTTC

3.2.6. Statistical Analysis

Statistical analysis was completed using GraphPad PRISM 6 software. Cell doubling times were compared using a one-way ANOVA with radiation dose as the independent variable. Cytotoxicity data and gene expression data were also compared using a one-way ANOVA comparing each dose back to the control group of 0 Gy. P values less than 0.05 were considered statistically significant.

3.3. Results

3.3.1. Cell Growth

NK-92 cell growth and doubling times were quantified post irradiation. Cell counts were performed up until day 7. However, data is only shown up to day 4 as this was where growth plateaued, and cell death began to occur. Specifically, for the lower doses ranging from 0.01 to 1 Gy, cells showed exponential growth up until day 4 (Figure 3.5A). On the other hand, in the higher doses of 5 and 10 Gy, no cell growth was observed, and cells had reached 100% mortality by the 4^{th} day post-irradiation. Based off the exponential fit of the growth curve, the doubling time of the cells was calculated for doses up to 1 Gy (Figure 3.5B). The doubling times ranged from 1.3 to 1.6 days, but overall there was no significant changes in the irradiated cells compared to the sham control group of 0 Gy.

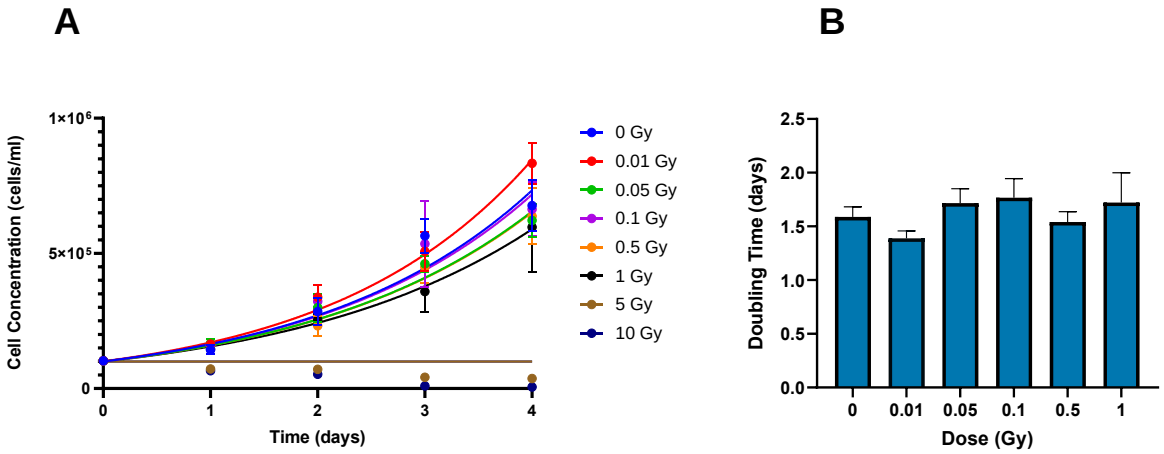

Figure 3.5. Growth curve (A) and doubling time (B) analysis for irradiated NK-92 cells. Data represent the average of three independent replicates ± SEM. Doubling times were compared using a one-way ANOVA.

3.3.2. Cytotoxicity

The cytolytic capacity of the irradiated NK-92 cells was analyzed in a killing assay using K562 cells as target cells. At 24 hours post irradiation, LDR at a ratio of 3:1 and 6:1 did not cause any significant effects on NK-92 cell cytotoxicity, as seen in Figure 3.6A ($p = 0.1014$, $p = 0.5711$). Figure 3.6B shows these same non-significant results at a timepoint of 48 hours post irradiation ($p = 0.9268$, $p = 0.7303$). In Figure 3.6A, compared to the control NK-92 cells, LDR exposure of 0.1 and 0.25 Gy did cause a slight increase in cell cytotoxicity at both ratios, however, this was not significant.

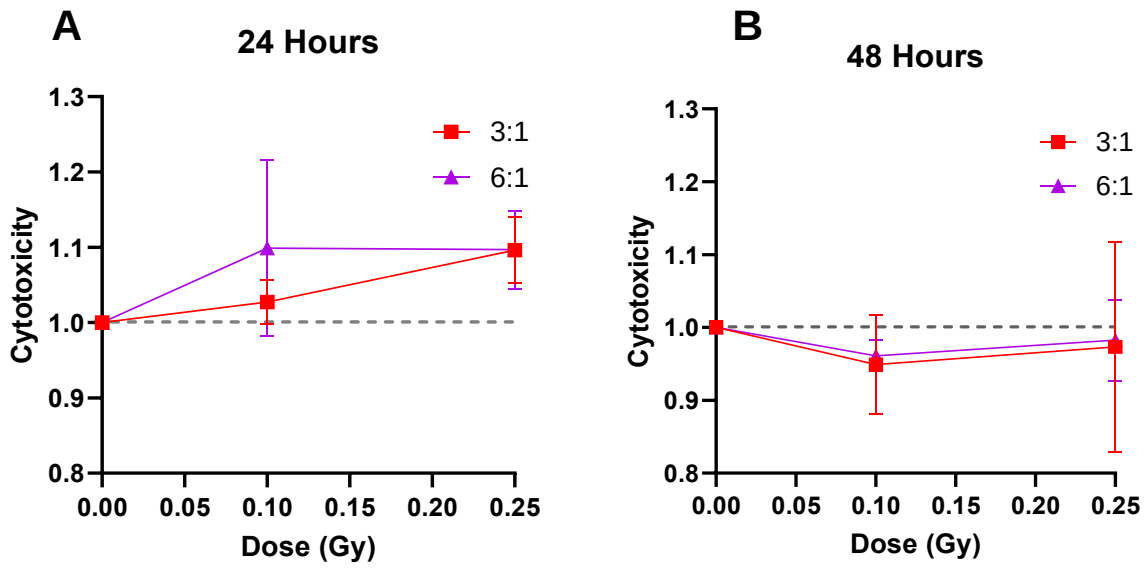

Figure 3.6. Cytotoxicity of NK-92 cells at (A) 24- and (B) 48-hours post irradiation. Cytotoxicity was measured using a 3:1 and 6:1 ratio of NK-92:K562. Data are presented relative to control cytotoxicity and represent the average of three independent replicates ± SEM. Data were compared using a one-way ANOVA.

3.3.3. RT-qPCR

Gene expression levels were quantified post irradiation in NK-92 cells using RT-qPCR. Overall, LDR exposure did not cause any significant changes in the main genes related to NK-92 cytotoxicity (Figure 3.7, Table 3.2). Regarding the Granzyme B gene which is involved the NK cell perforin/granzyme pathway, at 48 hours post irradiation the 0.1 Gy exposure was trending towards an increase in expression, although results were non-significant (Figure 3.7B). For the *FASL/TRAIL* pathway, it did appear that both the *FAS* and *TRAIL* genes followed a similar trend to that of Granzyme B. Specifically, although non-significant, mRNA levels of *FAS* were slightly increased with 0.1 Gy of radiation at the 48-hour timepoint. For *TRAIL*, mRNA levels were slightly increased in response to 0.25 Gy of radiation at the 48h timepoint and at the 0.1 Gy dose, for both the 24h and 48h timepoints (Figure 3.7C,D). Lastly, when looking at the third pathway of NK cell

cytotoxicity, the cytokine/chemokine pathway, the TNF and IFNG genes also show a similar trend. For example, for both *TNF* and *IFNG*, at 48 hours post irradiation with 0.1 Gy, gene expression levels were slightly increased compared to the sham group. In addition, for the *IFNG*, 0.25 Gy at the 24-hour timepoint also caused an increase in mRNA levels, although nonsignificant. All other genes related to NK-92 activating and inhibiting receptors show a similar trend (Table 3.2). Despite these slight increases, in all 19 genes tested, LDR did not have a significant effect on the transcriptional levels of the main genes regulating cytotoxicity of NK-92 cells.

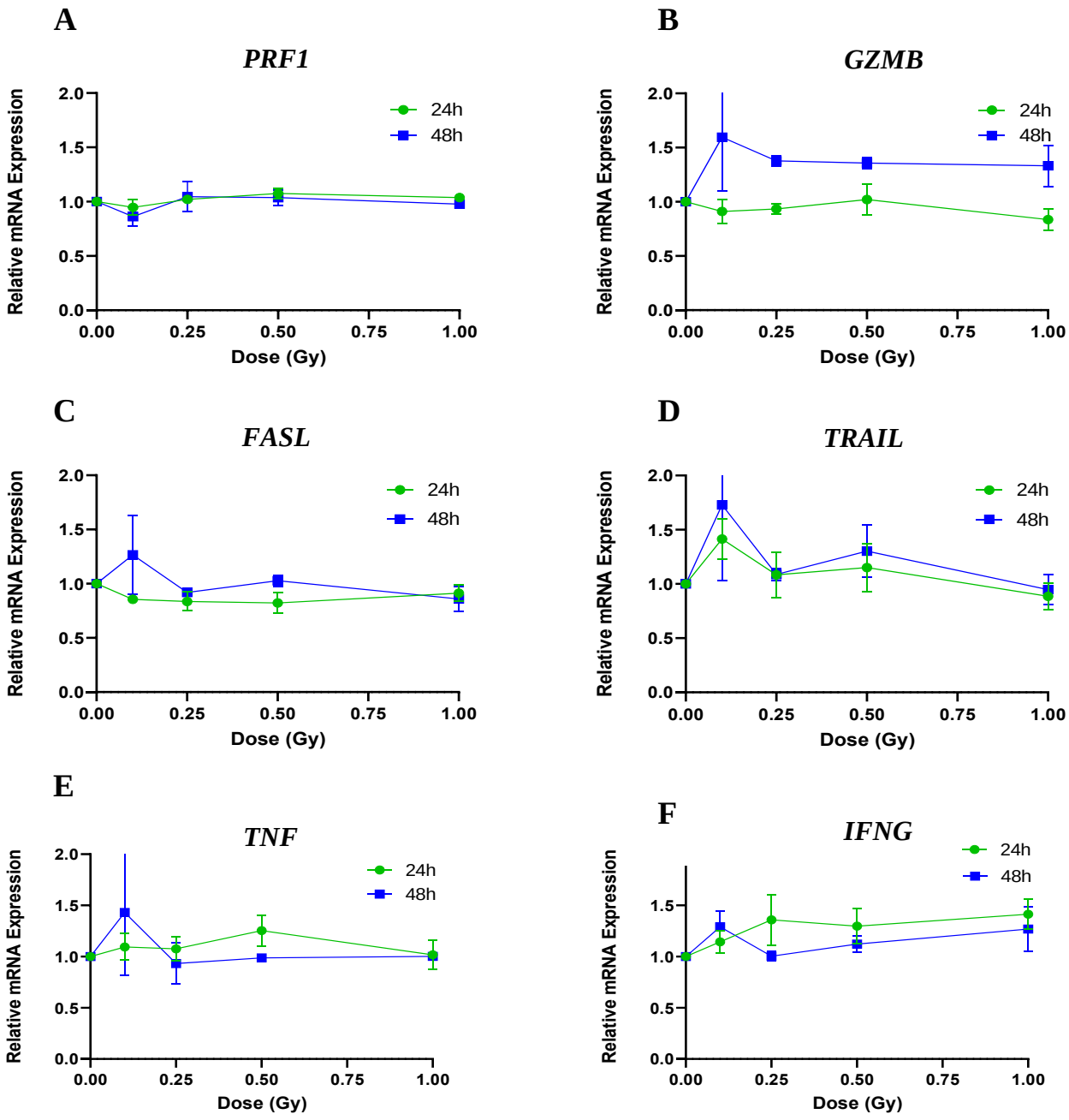

Figure 3.7. Transcriptional levels of six main genes involved in NK cell cytotoxicity. Relative mRNA expression was quantified using RT-qPCR 24- and 48-hours post irradiation. Data are presented relative to sham irradiated (control) mRNA expression at that respective timepoint and represent the average of three independent replicates ± SEM. Data were compared using a one-way ANOVA. *PRF1* = Perforin, *GZMB* = Granzyme B, *FasL* = Fas Ligand, *TNF* = Tumor Necrosis Factor, *TRAIL* = TNF-Related Apoptosis-Inducing Ligand, *IFNG* = Interferon Gamma.

Table 3.2. Transcriptional levels of genes involved in NK cell cytotoxicity. Relative mRNA expression was quantified using RT-qPCR 24- and 48-hours post irradiation. Data are presented relative to sham irradiated (control) mRNA expression and represent the average of three independent replicates ± SEM. Data were compared using a one-way ANOVA.

	Relative Gene Expression (mean ± SEM)							
	24 Hours				48 Hours			
Gene	0.1 Gy	0.25 Gy	0.5 Gy	1 Gy	0.1 Gy	0.25 Gy	0.5 Gy	1 Gy
BIRC3	1.08±0.13	1.02±0.23	1.04±0.12	0.83±0.07	1.39±0.28	1.15±0.28	1.61±0.33	1.48±0.23
NRC3	1.00±0.09	1.00±0.14	1.09±0.20	1.12±0.07	1.08±0.24	1.08±0.24	1.06±0.18	0.90±0.24
CD69	0.66±0.06	0.77±0.13	0.86±0.16	0.97±0.13	1.05±0.14	0.94±0.13	0.89±0.19	1.33±0.24
KLRC1	0.91±0.06	1.04±0.10	0.97±0.12	1.07±0.06	1.06±0.21	0.86±0.23	0.90±0.19	0.89±0.23
KLRD1	0.89±0.13	1.10±0.13	0.94±0.07	0.91±0.05	0.91±0.05	1.04±0.06	1.15±0.09	1.18±0.11
KLRK1	1.02±0.12	1.02±0.16	1.02±0.15	0.84±0.08	1.10±0.27	0.96±0.16	1.20±0.21	1.06±0.21
KIR3DL1	1.10±0.19	0.90±0.18	1.02±0.20	0.87±0.08	1.03±0.05	1.04±0.11	1.84±0.57	1.48±0.31
LILBR1	1.12±0.34	1.12±0.29	1.14±0.06	0.98±0.16	1.04±0.36	1.07±0.13	0.84±0.05	1.15±0.30
FCGR3A	1.25±0.10	1.00±0.07	1.09±0.09	0.88±0.06	0.84±0.12	1.23±0.11	1.42±0.08	1.61±0.04
TNFSF9	0.70±0.19	0.87±0.21	0.77±0.10	0.90±0.16	0.71±0.16	0.95±0.28	1.12±0.28	1.70±0.44
TNFSF12	0.97±0.07	1.05±0.14	1.03±0.07	0.86±0.07	0.75±0.10	0.76±0.11	0.88±0.12	0.93±0.25
NRC1	0.93±0.09	0.92±0.15	0.94±0.07	0.75±0.09	1.05±0.23	0.79±0.07	0.75±0.11	0.79±0.13
GZMA	1.06±0.19	1.20±0.22	1.36±0.27	1.21±0.18	0.90±0.24	0.83±0.08	0.83±0.12	1.13±0.25

3.4. Discussion

NK cells are one of the main cell types involved in the immune system's defense against cancer. They are highly cytotoxic anti-tumor and anti-infection immune effectors, and therefore are promising candidates for cancer immunotherapy[104]. However, since there exist significant challenges with the use of blood-derived NK cells for therapeutic purposes, investigators have been trying to generate stable NK cell lines. To date, the NK-92 cell line is the only FDA-approved cell line to be used in clinical trials and many immunotherapies have in fact already been conducted with these cells, and overall demonstrated the lack of infusion-associated toxicities in cancer patients[99]. Therefore, NK-92 cells are an extremely attractive candidate for cancer therapies.

As mentioned above, studies have suggested that LDR could increase the cytotoxicity of NK cells. Since clinical studies have established the safety of administering NK-92 cells as allogeneic cell therapy in hematologic and solid cancers, merging LDR with NK-92 cells would be of benefit. Using the NK-92 cell line, we hypothesized that LDR would lead to effective expansion and a greater activity enhancement of NK cells and their cytotoxic properties. However, our results showed that LDR did not significantly impact NK-92 cell growth, cytotoxicity or the transcriptional levels of the cell's main genes regulating cytotoxicity. Since previous studies have shown that LDR can stimulate NK cell cytotoxicity, these results were unexpected. For example, a recent study by Yang *et al*. looked at the effects of LDR on the cytotoxic properties of primary NK cells isolated from mice[108]. NK cells were irradiated in-vitro and the K562 tumorigenic cell line was used as a target, similar to our experiments[108]. They found that 0.75 Gy of radiation significantly increased the cytotoxicity of NK cells 24 hours post exposure[108].

It is possible that the NK-92 cell line is not a good model to observe the stimulatory effects of LDR. The NK-92 cell line is a human immortalized cell line, and thus may not adequately represent in-vivo primary cells. The NK-92 cell line was chosen since in-vitro models are an easy and cost-effective method that is useful to examine specific signaling pathways and regulatory mechanisms within the cell. Evidence has shown that the NK-92 cells have much broader and greater cytotoxicity than primary NK cells[104]. Since the exact mechanisms of primary NK cell cytotoxicity are unknown, it is possible that there are slight differences between the killing strategies used by primary NK cells and NK-92 cells. For example, although the two cell types share many of the same cell surface receptors, there are a few differences between their activating and inhibitory receptors. In addition, in the presence of a tumor, it could be that these two cell types release different cytokines and chemokines, or a different quantity of perforin and granzymes

proteins, and collectively, these minor differences may have been sufficient to not see any effects from LDR.

Another reason for the lack of a response to LDR in NK-92 cells could be the environment in which they were irradiated. In a study by Cheda *et al.*, where immunological changes in response to LDR were analyzed in mice, it was found that 0.1 Gy of X-irradiation significantly stimulated NK cell-mediated tumor cell lysis[62]. However, it is important to note that these NK cells were irradiated in-vivo in mice before being isolated and purified for analysis. On the contrary, in our study, NK-92 cells were irradiated in-vitro where they were isolated from the rest of the immune system. Since the immune system utilizes a complex interplay of multiple mechanisms in the face of a pathogen or cancer, it is highly likely that NK cells utilize other cells and mediators in order to be activated and to initiate cytotoxic responses against tumor cells. For example, it is well known that NK cells can kill tumor cells indirectly by promoting dendritic and T cell interactions[42]. In addition, NK cell function is regulated by a variety of cytokines and in turn, act synergistically with other cytokines to become activated and elicit more robust cytotoxic responses. When NK cells are irradiated in-vivo, the entire immune system of that mice is also exposed to radiation. It is therefore possible that the strong cytotoxic properties of NK cells are dependent on other immune cells and mediators which are also stimulated by LDR. This hypothesis is supported by results from a study in 2012 by Sonn, *et al*[106]. The authors found that when purified NK cells received 0.2 Gy in-vitro, no significant difference in cell viability was observed[106]. In addition, no functional changes were detected in LDR-exposed NK cells, demonstrating that LDR alone was insufficient to generate changes at the cellular level[106]. In a review paper from J. Chen *et al,* it was shown that other immune cells such as T regulatory cells, DCs and tumor associated neutrophils, are highly likely to induce NK cell activation when exposed to LDR[109]. Therefore, when NK cells

are radiated in-vitro, they are missing the systemic immune response which may influence the cell's choice or method of killing strategy.

As mentioned, there have been studies documenting the clinical success and safety of NK-92 cells in immunotherapies in solid tumors as well as hematological malignancies[104,105]. This was one of the main reasons for selecting the NK-92 cell line in our study. In addition, the cost of preparation and administration of NK-92 cells are significantly less than that of primary NK cells, and it is fairly easy to grow NK-92 cells in high numbers. Therefore, since a large body of evidence suggests that LDR can increase the cytotoxicity of NK cells, if LDR would have significantly increased the cytotoxicity of NK-92 cells, this could have made for a promising new cancer therapeutic in the field of immuno-oncology. Specifically, LDR could have been administered to the NK-92 cells in-vitro before being implanted into patients in order to see more of a robust cytotoxic effect against the tumor cells.

Ionizing radiation is currently used during NK-92 cell therapies, however, not as a immunostimulant. Clinical trials using NK-92 have irradiated the cells with a dose of 10 Gy prior to infusion[105]. This HDR is used in order to prevent over-proliferation of this cell line within the patient. A dose of 10 Gy prevents in-vivo proliferation but still maintains the cell's ability to kill target cancer cells and produce immune active cytokines[110]. A study conducted by I. Montagner et al., investigating the effects of engineered NK-92 cells as an off the shelf cell therapy for prostate cancer, used a 10 Gy irradiation of the effector population prior to infusion as a safety measure to prevent permanent engraftment[111]. Results showed that NK cells pre-irradiated with 10 Gy did not replicate any further and over time gradually declined, with living cells no longer detectable after 5–7 days[111]. In addition, it was reported that the NK cells retained a high level of target specific killing activity, and in fact, had a higher cytotoxicity than sham irradiated cells[111]. These findings

support the results from our growth curve assay, where in higher doses of 5 and 10 Gy, no cell growth was observed, and cells had reached 100% mortality by the 4th day post-irradiation. Therefore, although the NK-92 cells did not experience growth during the 7 days following radiation exposure, it is possible that the cells were still alive and capable of cytolytic activity. However, since the objective of this research was to investigate immunostimulatory effects of LDR, we did not test the cytotoxicity of the 5 or 10 Gy irradiated cells.

This study focused on the direct effects of LDR on NK-92 cells since it is believed that LD-RT functions primarily through stimulating immune cells. However, we cannot rule out the possibility that LDR could also be acting directly on the tumor, making it more sensitive to destruction by the host's immune system. To test this, the K562 tumorigenic cell line could also have been exposed to LDR instead of, or in addition to NK-92 cells. To date, with in-vivo studies on the effects of NK cytotoxicity on tumor cells, the exact mechanisms for NK cell-mediated tumor cell lysis are yet to be identified. Specifically, since both cell types are being exposed to LDR in an in-vivo model, it is unclear as to if the antitumor effects caused by the radiation originate from the stimulated NK cells or from the tumor cells or both. For example, one hypothesis would be that LDR cause the overexpression of certain markers on the surface of the tumor cells, making them more identifiable or vulnerable to the immune system. Kaushik *et al.*, claimed that LDR decreased tumor progression via the inhibition of the JAK1/STAT3 pathway in breast cancer cells[112]. Firstly, authors assessed LDR effects on a breast cancer cell line in-vitro and reported that LDR decreased the migration and invasion of the cancer cells and downregulated the expression of critical markers associated with tumor cell progression (vimentin and SNAI2)[112]. Furthermore, to confirm whether LDR can decrease breast metastasis in-vivo, the metastatic MDA-MB231 LM2 breast cancer cells were transplanted into mice[112]. Compared to controls, lung metastasis was

markedly decreased in LDR-treated tumors[112]. Collectively, these results suggest that LDR can have a direct impact on the cancer cells. Therefore, an important future experiment would be to expose the cancerous K562 cell line to LDR prior to co-culture with NK cells in order to have a better understanding of whether LDR immune enhancing and anticancer effects are acting on cells of the immune system or on cancer cells themselves.

In order to obtain a more in depth understanding as to which cytotoxic mechanism NK cells utilize in response to LDR, gene expression levels of the NK-92 cells were quantified post irradiation using RT-qPCR. As seen in Figure 3.7 and Table 3.2, LDR exposure did not induce any significant changes in the main genes related to NK-92 cytotoxicity. The genes selected in this panel represent the major genes involved in the three main NK cell cytotoxicity pathways. Specifically, *GZMA*, *GZMB* and *PRF1* represent the lytic compounds involved in the perforin/granzyme pathway of NK cells. The *FAS* and *TNFSF12* genes represent the FAS and TRAIL ligands from the death receptor-mediated pathway of NK cells. Lastly, *IFNG* and *TNF* genes were selected to represent the cytokines used in the cytokine/chemokine pathway of NK cells. Since previous studies reported that NK cells use these three pathways in order to eliminate tumor cells, the non-significant results fail to support our hypothesis. Results from a study by Yang *et al.,* showed that expression levels of IFN-γ and TNF-α in supernatants of NK cells were visibly increased after an LDR exposure of 0.75 Gy[108]. In addition, Yoon *et al*. found that LDR not only increased the expression of chemokines in tumor cells, but more importantly, increased the expression of CXCL16 (ligand of CXCR6) in NK cells[113]. The authors reported that LDR enhanced the migration of NK cells to tumor sites which led to more robust and effective tumor control[113]. The reasons detailed above as to why we failed to see an increase in NK-92 cytotoxicity with LDR could also explain why we did not see any gene expression changes.

In conclusion, we did not identify any immunostimulatory effects in NK-92 cells based on cytotoxicity and gene expression 24 and 48 hrs post LDR (0.1 and 0.25 Gy) exposure. Future studies are needed to further elucidate the mechanisms of LDR induced NK cell cytotoxicity. In addition, studies focusing on the effects of LDR on NK-92 cells could include a wider range of doses and timepoints and should also consider exposing the cancerous target cells to LDR prior to co-culture with NK cells. Overall, although our results were insignificant, evidence has shown that LDR does cause an increase in NK cell cytotoxicity, in-vitro and in-vivo, proving to be an attractive candidate for numerous clinical applications.

4. Discussion and Conclusions

4.1. Discussion

The goal of this thesis was to investigate whether LDR could have stimulatory effects on the immune system, and to elucidate which cellular and molecular mechanisms are involved in the reported LDR antitumor immunity processes. This was achieved through 2 different aims, using both an animal model and an NK-92 cell model. In both of these models, results showed that LDR did not stimulate the immune system, as no significant increase of immune cells was observed. The results found were opposing to our hypothesis, where it was expected that LDR would increase the number of immune cells in the irradiated mice and increase the cytotoxicity of NK cells irradiated in-vitro. These results were surprising considering the number of experimental studies which have confirmed the modulatory effects of LDR on innate and adaptive immunities, including regulating the status of immune cells, the microenvironment of the immune system, and the interaction of immune cells[35]. On the other hand, in both models, HDR of 3 Gy caused a significant decrease in all immune cell types tested. Since high doses of radiation are associated with immune suppression, these results were not unexpected.

As mentioned above, there are several possible hypotheses for why LDR failed to cause immunostimulatory effects both in the C57BL/6 mice and the NK-92 cell line. For example, whole-body irradiation at a dose of 0.02–0.25 Gy has been reported by numerous studies to inhibit the growth and metastasis of tumors[31,76]. It is possible that the doses used in this study, in combination with other factors such as the timing and delivery of radiation, as well as the sex and the strain of the mice, were responsible for the insignificant effects of LDR. In addition, it is possible that the expected stimulation of immune cells in the mice would have been more prominent in a cancer model. Immune stimulatory effects caused by LDR might only be initiated in a stressed or diseased state, so future studies could consider implanting tumors into the mouse

before LDR exposure. Specifically, innate immunity is typically initiated once it encounters a foreign invader in the body. Therefore, LDR may not have resulted in an increased immune response since the baseline immune system of a healthy mouse is different than that of a cancerous one, where the immune cells and antitumor mechanisms of the body are already partially activated. This theory is supported clinically, since in all of the human studies which have successfully identified the immune modulatory effects of LDR, radiation was administered to cancer patients.

In regard to the second aim with the NK-92 cell line, the fact that LDR did not cause a significant impact on the growth, cytotoxicity and transcriptional levels of the cells is likely due to using an immortalized cell line in place of fresh primary NK cells. Similar to the findings in the first data chapter of my thesis, a decrease in growth was seen with higher doses of radiation. In the mice, the highest dose of 3 Gy caused a significant drop in circulating NK cells. On the other hand, in the in-vitro model, the highest doses of 5 and 10 Gy caused the suppression of NK-92 cell growth. Overall, these findings suggest that NK cells are sensitive to higher doses of radiation, of at least 3 Gy. In addition, it has been reported that NK cells are one of the immune cell types that are the most sensitive to fractionated exposures[114]. Therefore, future studies should consider adding a different radiation delivery method by including fractionated exposures. This would have the added benefit of being more easily translated into clinical applications, since in human studies, fractionated exposures are most commonly used on cancers[115]. For instance, a common dose regimen used in humans to treat cancer through LD-RT has been fractionated exposures of 100–150 mGy multiple times per week for a few weeks[115].

Overall, although no significant LDR effects on the immune system were found in my study, this does not mean it will not be of therapeutic benefit. It would be of interest for future studies to focus on identifying more clearly the cellular and molecular mechanisms behind LDR

and its anticancer effects. For example, in order to eliminate any radiophobia and progress towards using LDR as a cancer treatment in humans, assays such as full immunophenotyping should be conducted in humans. More importantly, well-designed clinical trials should be conducted in order to study the safe and effective dose, dose rate, time interval between fractions and so on. Once we have a better understanding, LD-RT could be more clinically accepted and there will be less concern about LDR causing any potential late carcinogenic risks. Currently, in European countries, LD-RT is practised for the treatment of a variety of inflammatory and painful joint diseases[74]. In fact, total doses of LD-RT comprise 5–10% of those given to tumor patients[74].

Since radiosensitivity varies considerably among individuals, the radiation dose or radiation frequency required for inducing anti-tumor effects likely also varies[112]. Most immune cells studied in my thesis are known to be very sensitive to radiation. The bone marrow is the site of production of immune cells, and it is in this location that they are more sensitive to radiation as they are in the process of cellular division[43]. Specifically, studies have shown that immature undifferentiated hematopoietic cells are more sensitive to radiation compared to differentiated cells[116]. Therefore, another plausible explanation as to why no significant effects were seen from LDR in my study is that most of the cell types studied were terminally differentiated, therefore rendering them more resistant to radiation.

It is known from previous studies that high doses of ionizing radiation, on the order of multiple Gy, causes deleterious biological effects in humans, including an overall immune suppression, and ultimately an increased risk of cancer induction. This was supported by the results of my thesis. On the other hand, the biological effects caused by low doses of radiation are much less clear and have been investigated for more than a century[31]. Therefore, establishing the dose response relationship for low dose biological effects is one of the key topics in radiobiological

research. To date, there exists several radiation risk models. The first model is the LNT model, which assumes that even very low doses of ionizing radiation could have adverse effects on human health[29]. The second model is the hormesis model, which states that adaptive and protective mechanisms can in fact be induced by low doses of radiation and therefore be beneficial in preventing cancer and other adverse health effects[33]. The third model is the threshold model, which suggests that below certain exposure levels, radiation is harmless and causes no observable risk[117]. Data from previous studies on LD-RT support the hormesis model, showing that low doses of radiation can stimulate the immune system and have anticancer effects[33]. Therefore, it was hypothesized in this thesis that LDR would stimulate the immune cells in mice and NK cells in-vitro. However, the data obtained from both data chapters of my thesis were more in support of the threshold model, since at the lowest doses tested, there was no significant immune effects. Specifically, with the immune cells that were studied in the mice with a 48-hour timepoint, and the NK cells from my in-vitro model with a 24- and 48-hour timepoint, results show that there is in fact a threshold effect. This threshold appears to be between 0.5 and 3 Gy. In the in-vitro chapter of my thesis, a decrease in growth of NK-92 cells was observed at 5 and 10 Gy, but not at 1 Gy, suggesting that the threshold effect was somewhere between 1 and 5 Gy. On the other hand, in the in-vivo chapter of my thesis, a decrease in immune cell counts, including NK cells, was seen at the 0.5 and 3 Gy doses, suggesting that the threshold effect was somewhere around 0.5 Gy. Although these model systems are both very different, one being in-vivo and the other in-vitro, they both suggest a similar threshold range for immune suppressive effects.

Finally, the results obtained in my study may help to alleviate any concerns that may exist regarding exposure to LDR. The use of LD-RT as a potential cancer therapeutic has fallen out of practice in the last decade due to increased radiophobia[30]. In addition, although the radiation doses

used in medical diagnostic procedures such as computed tomography (CT) are very low, many people are still concerned about adverse health effects from these exposures. Studies have suggested that radiation exposures less than 100 mGy are too low to detect any statistically significant cancer excess in the presence of naturally occurring malignancies[118]. Based on the data from my thesis, LDR does not appear to have any detrimental effects on the immune system.

4.2. Conclusion

Overall, there is a lack of consistent evidence regarding the effects of LDR on the various cell types of the immune system. What remains unclear is the circumstances under which LDR can modulate the number/activity of certain immune cell types, and how these LDR-induced effects can potentially be used in the prevention and treatment of disease. Taken together, this thesis provides insight into the biological effects caused by both LDR and HDR on the immune system. Specifically, this study reports that HDR, ranging from 0.5 to 3 Gy, causes a suppression in most immune cell types, in particular NK cells. In addition, contrary to our initial hypothesis, these findings demonstrate that across the biological models and radiation doses used, LDR does not have a stimulatory effect on immune cells and does not cause an increase in the cytotoxicity of NK cells. Overall, much work is still required regarding using LDR as an alternative cancer therapeutic. For example, further studies should focus on studying LDR in cancer models and testing fractionated exposures in addition to single acute doses. Once the ideal dose regimen has been identified for immune stimulation, follow up studies can further investigate the mechanisms of tumour cell killing. To conclude, these advances could help contribute to better understanding LDR and its potential as an anti-cancer therapy as well as the treatment of non-cancer disease.

5. References

1. Pucci, C., Martinelli, C., & Ciofani, G. (2019). Innovative approaches for cancer treatment: Current perspectives and new challenges. *Ecancermedicalscience*, *13*, 961.
2. *Cancer statistics at a glance | Canadian Cancer Society*. (n.d.). Retrieved April 23, 2023, from https://cancer.ca/en/research/cancer-statistics/cancer-statistics-at-a-glance
3. Moreno-Smith, M., Lutgendorf, S. K., & Sood, A. K. (2010). Impact of stress on cancer metastasis. *Future Oncology (London, England)*, *6*(12), 1863–1881.
4. Cooper, G. M. (2000). The Development and Causes of Cancer. *The Cell: A Molecular Approach. 2nd Edition*.
5. Alegría-Torres, J. A., Baccarelli, A., & Bollati, V. (2011). Epigenetics and lifestyle. *Epigenomics*, *3*(3), 267–277.
6. Mokhtari, R. B., Homayouni, T. S., Baluch, N., Morgatskaya, E., Kumar, S., Das, B., & Yeger, H. (2017). Combination therapy in combating cancer. *Oncotarget*, *8*(23), 38022–38043.
7. *Cancer treatments: MedlinePlus Medical Encyclopedia*. (n.d.). Retrieved April 23, 2023, from https://medlineplus.gov/ency/patientinstructions/000901.htm
8. Morris, Z. S., & Harari, P. M. (2014). Interaction of Radiation Therapy With Molecular Targeted Agents. *Journal of Clinical Oncology*, *32*(26), 2886–2893.
9. *Radiation Therapy for Cancer—NCI* (nciglobal,ncienterprise). (2015, April 29). [CgvArticle]. https://www.cancer.gov/about-cancer/treatment/types/radiation-therapy
10. Moding, E. J., Kastan, M. B., & Kirsch, D. G. (2013). Strategies for optimizing the response of cancer and normal tissues to radiation. *Nature Reviews. Drug Discovery*, *12*(7), 526–542.
11. *Internal radiation therapy | Canadian Cancer Society*. (n.d.). Retrieved April 23, 2023, from https://cancer.ca/en/treatments/treatment-types/radiation-therapy/internal-radiation-therapy

12. *Radiation Basics*. (n.d.). NRC Web. Retrieved April 23, 2023, from https://www.nrc.gov/about-nrc/radiation/health-effects/radiation-basics.html
13. Hu, C., Zuo, H., & Li, Y. (2021). Effects of Radiofrequency Electromagnetic Radiation on Neurotransmitters in the Brain. *Frontiers in Public Health*, *9*, 691880.
14. Lee, B. Q., Kibédi, T., Stuchbery, A. E., & Robertson, K. A. (2012). Atomic Radiations in the Decay of Medical Radioisotopes: A Physics Perspective. *Computational and Mathematical Methods in Medicine*, *2012*, 651475.
15. Reisz, J. A., Bansal, N., Qian, J., Zhao, W., & Furdui, C. M. (2014). Effects of Ionizing Radiation on Biological Molecules—Mechanisms of Damage and Emerging Methods of Detection. *Antioxidants & Redox Signaling*, *21*(2), 260–292.
16. Tuieng, R. J., Cartmell, S. H., Kirwan, C. C., & Sherratt, M. J. (2021). The Effects of Ionising and Non-Ionising Electromagnetic Radiation on Extracellular Matrix Proteins. *Cells*, *10*(11), 3041.
17. Shahbazi-Gahrouei, D., Gholami, M., & Setayandeh, S. (2013). A review on natural background radiation. *Advanced Biomedical Research*, *2*, 65.
18. *Radiation Dose Assessment—Analysis of Cancer Risks in Populations Near Nuclear Facilities—NCBI Bookshelf*. (n.d.). Retrieved April 23, 2023, from https://www.ncbi.nlm.nih.gov/books/NBK201992/
19. Materials, N. R. C. (US) C. on E. of E. G. for E. to N. O. R. (1999). Radiation Quantities and Units, Definitions, Acronyms. In *Evaluation of Guidelines for Exposures to Technologically Enhanced Naturally Occurring Radioactive Materials*. National Academies Press (US).
20. Brooks, A. L., Hoel, D. G., & Preston, R. J. (2016). The role of dose rate in radiation cancer risk: Evaluating the effect of dose rate at the molecular, cellular and tissue levels using key events in

critical pathways following exposure to low LET radiation. *International Journal of Radiation Biology*, *92*(8), 405–426.

21. Gharzai, L. A., Green, M. D., Griffith, K. A., Else, T., Mayo, C. S., Hesseltine, E., Spratt, D. E., Ben-Josef, E., Sabolch, A., Miller, B. S., Worden, F., Giordano, T. J., Hammer, G. D., & Jolly, S. (2019). Adjuvant Radiation Improves Recurrence-Free Survival and Overall Survival in Adrenocortical Carcinoma. *The Journal of Clinical Endocrinology and Metabolism*, *104*(9), 3743–3750.
22. Verschaeve L. (2005). Genetic effects of radiofrequency radiation (RFR). *Toxicology and applied pharmacology*, *207*(2 Suppl), 336–341.
23. Park, S. H., & Kang, J. O. (2011). Basics of particle therapy I: Physics. *Radiation Oncology Journal*, *29*(3), 135–146.
24. Rana, S., Kumar, R., Sultana, S., & Sharma, R. K. (2010). Radiation-induced biomarkers for the detection and assessment of absorbed radiation doses. *Journal of pharmacy & bioallied sciences*, *2*(3), 189–196.
25. Templin, T., Sharma, P., Guida, P., & Grabham, P. (2016). Short-Term Effects of Low-LET Radiation on the Endothelial Barrier: Uncoupling of PECAM-1 and the Production of Endothelial Microparticles. *Radiation research*, *186*(6), 602–613.
26. Borrego-Soto, G., Ortiz-López, R., & Rojas-Martínez, A. (2015). Ionizing radiation-induced DNA injury and damage detection in patients with breast cancer. *Genetics and molecular biology*, *38*(4), 420–432.
27. Desouky, O., Ding, N., & Zhou, G. (2015). Targeted and non-targeted effects of ionizing radiation. *Journal of Radiation Research and Applied Sciences*, *8*(2), 247–254.

28. Hamada, N., & Fujimichi, Y. (2014). Classification of radiation effects for dose limitation purposes: history, current situation and future prospects. *Journal of radiation research*, *55*(4), 629–640.
29. Cardarelli, J. J., 2nd, & Ulsh, B. A. (2018). It Is Time to Move Beyond the Linear No-Threshold Theory for Low-Dose Radiation Protection. *Dose-response : a publication of International Hormesis Society*, *16*(3), 1559325818779651.
30. Sacks, B., & Siegel, J. A. (2017). Preserving the Anti-Scientific Linear No-Threshold Myth: Authority, Agnosticism, Transparency, and the Standard of Care. *Dose-response : a publication of International Hormesis Society*, *15*(3), 1559325817717839.
31. Nowosielska, E. M., Wrembel-Wargocka, J., Cheda, A., Lisiak, E., & Janiak, M. K. (2006). Enhanced Cytotoxic Activity of Macrophages and Suppressed Tumor Metastases in Mice Irradiated with Low Doses of X- rays. *Journal of Radiation Research*, *47*(3/4), 229–236.
32. Scott, B. R., & Tharmalingam, S. (2019). The LNT model for cancer induction is not supported by radiobiological data. *Chemico-biological interactions*, *301*, 34–53.
33. Lau, Y. S., Chew, M. T., Alqahtani, A., Jones, B., Hill, M. A., Nisbet, A., & Bradley, D. A. (2021). Low Dose Ionising Radiation-Induced Hormesis: Therapeutic Implications to Human Health. *Applied Sciences*, *11*(19), 19.
34. Zhou, L., Zhang, X., Li, H., Niu, C., Yu, D., Yang, G., Liang, X., Wen, X., Li, M., & Cui, J. (2018). Validating the pivotal role of the immune system in low-dose radiation-induced tumor inhibition in Lewis lung cancer-bearing mice. *Cancer medicine*, *7*(4), 1338–1348.
35. Cui, J., Yang, G., Pan, Z., Zhao, Y., Liang, X., Li, W., & Cai, L. (2017). Hormetic Response to Low-Dose Radiation: Focus on the Immune System and Its Clinical Implications. *International journal of molecular sciences*, *18*(2), 280.

36. Jameus, A., Kennedy, A. E., & Thome, C. (2021). Hematological Changes Following Low Dose Radiation Therapy and Comparison to Current Standard of Care Cancer Treatments. *Dose-response : a publication of International Hormesis Society*, *19*(4), 15593258211056196.
37. Chaplin D. D. (2010). Overview of the immune response. *The Journal of allergy and clinical immunology*, *125*(2 Suppl 2), S3–S23.
38. Ganeshan, K., & Chawla, A. (2014). Metabolic regulation of immune responses. *Annual review of immunology*, *32*, 609–634.
39. Marshall, J. S., Warrington, R., Watson, W., & Kim, H. L. (2018). An introduction to immunology and immunopathology. *Allergy, Asthma & Clinical Immunology*, *14*(2), 49.
40. Rosales C. (2018). Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types?. *Frontiers in physiology*, *9*, 113.
41. Hirayama, D., Iida, T., & Nakase, H. (2017). The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. *International journal of molecular sciences*, *19*(1), 92.
42. Sharma, P., Kumar, P., & Sharma, R. (2017). Natural Killer Cells - Their Role in Tumour Immunosurveillance. *Journal of clinical and diagnostic research : JCDR*, *11*(8), BE01–BE05.
43. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). The Adaptive Immune System. *Molecular Biology of the Cell. 4th Edition*.
44. Charles A Janeway, J., Travers, P., Walport, M., & Shlomchik, M. J. (2001). T Cell-Mediated Immunity. *Immunobiology: The Immune System in Health and Disease. 5th Edition*.
45. Waldman, A.D., Fritz, J.M. & Lenardo, M.J. (2020). A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nat Rev Immunol* 20, 651–668.
46. Gaudino SJ and Kumar P (2019) Cross-Talk Between Antigen Presenting Cells and T Cells Impacts Intestinal Homeostasis, Bacterial Infections, and Tumorigenesis. Front. Immunol. 10:360.

47. Zhang, J. M., & An, J. (2007). Cytokines, inflammation, and pain. *International anesthesiology clinics*, *45*(2), 27–37.

48. Borish, L. C., & Steinke, J. W. (2003). 2. Cytokines and chemokines. *Journal of Allergy and Clinical Immunology*, *111*(2), S460–S475.

49. Jang, D. I., Lee, A. H., Shin, H. Y., Song, H. R., Park, J. H., Kang, T. B., Lee, S. R., & Yang, S. H. (2021). The Role of Tumor Necrosis Factor Alpha (TNF-α) in Autoimmune Disease and Current TNF-α Inhibitors in Therapeutics. *International journal of molecular sciences*, *22*(5), 2719.

50. Jorgovanovic, D., Song, M., Wang, L., & Zhang, Y. (2020). Roles of IFN-γ in tumor progression and regression: a review. *Biomarker research*, *8*, 49.

51. *How cancer can spread | Cancer Research UK*. (n.d.). Retrieved April 23, 2023, from https://www.cancerresearchuk.org/about-cancer/what-is-cancer/how-cancer-can-spread

52. Gonzalez, H., Hagerling, C., & Werb, Z. (2018). Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes & development*, *32*(19-20), 1267–1284.

53. Vesely, M. D., & Schreiber, R. D. (2013). Cancer immunoediting: antigens, mechanisms, and implications to cancer immunotherapy. *Annals of the New York Academy of Sciences*, *1284*(1), 1–5.

54. Pan, M., & Li, B. (2022). T cell receptor convergence is an indicator of antigen-specific T cell response in cancer immunotherapies. *eLife*, *11*, e81952.

55. Cano, R. L. E., & Lopera, H. D. E. (2013). Introduction to T and B lymphocytes. In *Autoimmunity: From Bench to Bedside [Internet]*. El Rosario University Press. https://www.ncbi.nlm.nih.gov/books/NBK459471/

56. Catalán, D., Mansilla, M. A., Ferrier, A., Soto, L., Oleinika, K., Aguillón, J. C., & Aravena, O. (2021). Immunosuppressive Mechanisms of Regulatory B Cells. *Frontiers in Immunology*, *12*.

57. Hu, W., Wang, G., Huang, D., Sui, M., & Xu, Y. (2019). Cancer Immunotherapy Based on Natural Killer Cells: Current Progress and New Opportunities. *Frontiers in immunology*, *10*, 1205.

58. Grivennikov, S. I., Greten, F. R., & Karin, M. (2010). Immunity, inflammation, and cancer. *Cell*, *140*(6), 883–899.

59. Rabinovich, G. A., Gabrilovich, D., & Sotomayor, E. M. (2007). Immunosuppressive strategies that are mediated by tumor cells. *Annual review of immunology*, *25*, 267–296.

60. Paust, S., Senman, B., & von Andrian, U. H. (2010). Adaptive immune responses mediated by natural killer cells. *Immunological reviews*, *235*(1), 286–296.

61. Hosoi, Y., & Sakamoto, K. (1993). Suppressive effect of low dose total body irradiation on lung metastasis: dose dependency and effective period. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology*, *26*(2), 177–179.

62. Cheda, A., Wrembel-Wargocka, J., Lisiak, E., Nowosielska, E. M., Marciniak, M., & Janiak, M. K. (2004). Single low doses of X rays inhibit the development of experimental tumor metastases and trigger the activities of NK cells in mice. *Radiation research*, *161*(3), 335–340.

63. Nowosielska, E. M., Wrembel-Wargocka, J., Cheda, A., Lisiak, E., & Janiak, M. K. (2006). Enhanced cytotoxic activity of macrophages and suppressed tumor metastases in mice irradiated with low doses of X- rays. *Journal of radiation research*, *47*(3-4), 229–236.

64. Liu S. Z. (2003). Nonlinear dose-response relationship in the immune system following exposure to ionizing radiation: mechanisms and implications. *Nonlinearity in biology, toxicology, medicine*, *1*(1), 71–92.

65. Fourquet, A., Teillaud, J. L., Lando, D., & Fridman, W. H. (1993). Effects of low dose total body irradiation (LDTBI) and recombinant human interleukin-2 in mice. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology*, *26*(3), 219–225.

66. Liu, Y. P., Zheng, C. C., Huang, Y. N., He, M. L., Xu, W. W., & Li, B. (2021). Molecular mechanisms of chemo- and radiotherapy resistance and the potential implications for cancer treatment. *MedComm*, *2*(3), 315–340.

67. Johnson, R. E. (1972). Remission induction and remission duration with primary radiotherapy in advanced lymphosarcoma. *Cancer*, *29*(6), 1473–1476.

68. Canellos, G. P., DeVita, V. T., Young, R. C., Chabner, B. A., Schein, P. S., & Johnson, R. E. (1975). Therapy of advanced lymphocytic lymphoma a preliminary report of a randomized trial between combination chemotherapy (CVP) and intensive radiotherapy. *The British journal of cancer. Supplement*, *2*, 474–480.

69. Choi, N. C., Timothy, A. R., Kaufman, S. D., Carey, R. W., & Aisenberg, A. C. (1979). Low dose fractionated whole body irradiation in the treatment of advanced non-Hodgkin's lymphoma. *Cancer*, *43*(5), 1636–1642.

70. Hoppe, R. T., Kushlan, P., Kaplan, H. S., Rosenberg, S. A., & Brown, B. W. (1981). The treatment of advanced stage favorable histology non-Hodgkin's lymphoma: a preliminary report of a randomized trial comparing single agent chemotherapy, combination chemotherapy, and whole body irradiation. *Blood*, *58*(3), 592–598.

71. *Non-Hodgkin Lymphoma Treatment (PDQ®)—PDQ Cancer Information Summaries—NCBI Bookshelf*. (n.d.). Retrieved April 23, 2023, from https://www.ncbi.nlm.nih.gov/books/NBK66057/

72. Qasim M. M. (1979). Total body irradiation as a primary therapy in non-Hodgkin lymphoma. *Clinical radiology*, *30*(3), .

73. Low-Dose Hemibody Radiation, a Treatment Option for Recurrent Prostate Cancer: A Phase 2 Single-Arm Trial. *Advances in Radiation Oncology*. 8:101066-101066. 2023

74. Donaubauer, A.-J., Zhou, J.-G., Ott, O. J., Putz, F., Fietkau, R., Keilholz, L., Gaipl, U. S., Frey, B., & Weissmann, T. (2020). Low Dose Radiation Therapy, Particularly with 0.5 Gy, Improves Pain in Degenerative Joint Disease of the Fingers: Results of a Retrospective Analysis. *International Journal of Molecular Sciences*, *21*(16), 16.

75. Lumniczky, K., Impens, N., Armengol, G., Candéias, S., Georgakilas, A. G., Hornhardt, S., Martin, O. A., Rödel, F., & Schaue, D. (2021). Low dose ionizing radiation effects on the immune system. *Environment international*, *149*, 106212.

76. Yang, G., Li, W., Jiang, H., Liang, X., Zhao, Y., Yu, D., Zhou, L., Wang, G., Tian, H., Han, F., Cai, L., & Cui, J. (2016). Low-dose radiation may be a novel approach to enhance the effectiveness of cancer therapeutics. *International journal of cancer*, *139*(10), 2157–2168.

77. Zhang Y., Liu S.Z., (1996). Effect of Low Dose Radiation on Immune Functions of Tumor-Bearing Mice". Chin J Radiol Health 5:235–237.

78. Shigematsu, A., Adachi, Y., Koike-Kiriyama, N., Suzuki, Y., Iwasaki, M., Koike, Y., Nakano, K., Mukaide, H., Imamura, M., & Ikehara, S. (2007). Effects of low-dose irradiation on enhancement of immunity by dendritic cells. *Journal of radiation research*, *48*(1), 51–55.

79. Farooque, A., Mathur, R., Verma, A., Kaul, V., Bhatt, A. N., Adhikari, J. S., Afrin, F., Singh, S., & Dwarakanath, B. S. (2011). Low-dose radiation therapy of cancer: role of immune enhancement. *Expert review of anticancer therapy*, *11*(5), 791–802.

80. Hensel, J. A., Khattar, V., Ashton, R., & Ponnazhagan, S. (2019). Characterization of immune cell subtypes in three commonly used mouse strains reveals gender and strain-specific variations. *Laboratory investigation; a journal of technical methods and pathology*, *99*(1), 93–106.

81. *Peripheral Blood | Whole blood | Handbook | Miltenyi Biotec | Canada*. (n.d.). Retrieved April 23, 2023, from https://www.miltenyibiotec.com/CA-en/resources/macs-handbook/human-cells-and-organs/human-cell-sources/blood-human.html?countryRedirected=1#gref
82. *NK cells (mouse) | Mouse cell types | Mouse cells and organs | MACS Handbook | Resources | Miltenyi Biotec | Canada*. (n.d.). Retrieved April 23, 2023, from https://www.miltenyibiotec.com/CA-en/resources/macs-handbook/mouse-cells-and-organs/mouse-cell-types/nk-cells-mouse.html?countryRedirected=1#gref
83. Kutanzi, K. R., Lumen, A., Koturbash, I., & Miousse, I. R. (2016). Pediatric Exposures to Ionizing Radiation: Carcinogenic Considerations. *International journal of environmental research and public health*, *13*(11), 1057.
84. Heylmann, D., Ponath, V., Kindler, T., & Kaina, B. (2021). Comparison of DNA repair and radiosensitivity of different blood cell populations. *Scientific reports*, *11*(1), 2478.
85. Bogdándi, E. N., Balogh, A., Felgyinszki, N., Szatmári, T., Persa, E., Hildebrandt, G., Sáfrány, G., & Lumniczky, K. (2010). Effects of low-dose radiation on the immune system of mice after total-body irradiation. *Radiation research*, *174*(4), 480–489.
86. Harrington, N. P., Chambers, K. A., Ross, W. M., & Filion, L. G. (1997). Radiation damage and immune suppression in splenic mononuclear cell populations. *Clinical and Experimental Immunology*, *107*(2), 417–424.
87. Liu, X., Liu, Z., Wang, D., Han, Y., Hu, S., Xie, Y., Liu, Y., Zhu, M., Guan, H., Gu, Y., & Zhou, P. K. (2020). Effects of low dose radiation on immune cells subsets and cytokines in mice. *Toxicology research*, *9*(3), 249–262.

88. Gridley, D. S., Rizvi, A., Luo-Owen, X., Makinde, A. Y., & Pecaut, M. J. (2009). Low Dose, Low Dose Rate Photon Radiation Modifies Leukocyte Distribution and Gene Expression in CD4+ T Cells. *Journal of Radiation Research*, *50*(2), 139–150.

89. Song, K. H., Kim, M. H., Kang, S. M., Jung, S. Y., Ahn, J., Woo, H. J., Nam, S. Y., Hwang, S. G., Ryu, S. Y., & Song, J. Y. (2015). Analysis of immune cell populations and cytokine profiles in murine splenocytes exposed to whole-body low-dose irradiation. *International journal of radiation biology*, *91*(10), 795–803.

90. Klein, S. L., & Flanagan, K. L. (2016). Sex differences in immune responses. *Nature reviews. Immunology*, *16*(10), 626–638.

91. Rahman, M., & Bordoni, B. (2023). Histology, Natural Killer Cells. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK565844/

92. Perera Molligoda Arachchige A. S. (2021). Human NK cells: From development to effector functions. *Innate immunity*, *27*(3), 212–229.

93. He, Y., & Tian, Z. (2017). NK cell education via nonclassical MHC and non-MHC ligands. *Cellular & molecular immunology*, *14*(4), 321–330.

94. Prager, I., & Watzl, C. (2019). Mechanisms of natural killer cell-mediated cellular cytotoxicity. *Journal of leukocyte biology*, *105*(6), 1319–1329.

95. Walzer, T., Bléry, M., Chaix, J., Fuseri, N., Chasson, L., Robbins, S. H., Jaeger, S., André, P., Gauthier, L., Daniel, L., Chemin, K., Morel, Y., Dalod, M., Imbert, J., Pierres, M., Moretta, A., Romagné, F., & Vivier, E. (2007). Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(9), 3384–3389.

96. Fuchs, A., & Colonna, M. (2011). Natural killer (NK) and NK-like cells at mucosal epithelia: Mediators of anti-microbial defense and maintenance of tissue integrity. *European journal of microbiology & immunology*, *1*(4), 257–266.

97. Sivori, S., Vacca, P., Del Zotto, G., Munari, E., Mingari, M. C., & Moretta, L. (2019). Human NK cells: surface receptors, inhibitory checkpoints, and translational applications. *Cellular & molecular immunology*, *16*(5), 430–441.

98. Paul, S., & Lal, G. (2017). The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. *Frontiers in Immunology*, *8*.

99. Zhang, J., Zheng, H., & Diao, Y. (2019). Natural Killer Cells and Current Applications of Chimeric Antigen Receptor-Modified NK-92 Cells in Tumor Immunotherapy. *International Journal of Molecular Sciences*, *20*(2), 2.

100. Prager, I., Liesche, C., van Ooijen, H., Urlaub, D., Verron, Q., Sandström, N., Fasbender, F., Claus, M., Eils, R., Beaudouin, J., Önfelt, B., & Watzl, C. (2019). NK cells switch from granzyme B to death receptor-mediated cytotoxicity during serial killing. *The Journal of experimental medicine*, *216*(9), 2113–2127.

101. Trapani J. A. (2001). Granzymes: a family of lymphocyte granule serine proteases. *Genome biology*, *2*(12), REVIEWS3014.

102. Sordo-Bahamonde, C., Lorenzo-Herrero, S., Payer, Á. R., Gonzalez, S., & López-Soto, A. (2020). Mechanisms of Apoptosis Resistance to NK Cell-Mediated Cytotoxicity in Cancer. *International journal of molecular sciences*, *21*(10), 3726.

103. Chu, J., Gao, F., Yan, M., Zhao, S., Yan, Z., Shi, B., & Liu, Y. (2022). Natural killer cells: A promising immunotherapy for cancer. *Journal of Translational Medicine*, *20*(1), 240.

104. Klingemann, H. (2023). The NK-92 cell line—30 years later: Its impact on natural killer cell research and treatment of cancer. *Cytotherapy*, *25*(5), 451–457.

105. Liu, Q., Xu, Y., Mou, J., Tang, K., Fu, X., Li, Y., Xing, Y., Rao, Q., Xing, H., Tian, Z., Wang, M., & Wang, J. (2020). Irradiated chimeric antigen receptor engineered NK-92MI cells show effective cytotoxicity against CD19+ malignancy in a mouse model. *Cytotherapy*, *22*(10), 552–562.

106. Sonn, C. H., Choi, J. R., Kim, T.-J., Yu, Y.-B., Kim, K., Shin, S. C., Park, G.-H., Shirakawa, T., Kim, H. S., & Lee, K.-M. (2012). Augmentation of natural cytotoxicity by chronic low-dose ionizing radiation in murine natural killer cells primed by IL-2. *Journal of Radiation Research*, *53*(6), 823–829.

107. Shin, S. C., Lee, K. M., Kang, Y. M., Kim, K., Kim, C. S., Yang, K. H., Jin, Y. W., Kim, C. S., & Kim, H. S. (2010). Alteration of cytokine profiles in mice exposed to chronic low-dose ionizing radiation. *Biochemical and biophysical research communications*, *397*(4), 644–649.

108. Yang, G., Kong, Q., Wang, G., Jin, H., Zhou, L., Yu, D., Niu, C., Han, W., Li, W., & Cui, J. (2014). Low-dose ionizing radiation induces direct activation of natural killer cells and provides a novel approach for adoptive cellular immunotherapy. *Cancer biotherapy & radiopharmaceuticals*, *29*(10), 428–434.

109. Chen, J., Liu, X., Zeng, Z., Li, J., Luo, Y., Sun, W., Gong, Y., Zhang, J., Wu, Q., & Xie, C. (2020). Immunomodulation of NK Cells by Ionizing Radiation. *Frontiers in oncology*, *10*, 874.

110. Walcher, L., Kistenmacher, A. K., Sommer, C., Böhlen, S., Ziemann, C., Dehmel, S., Braun, A., Tretbar, U. S., Klöß, S., Schambach, A., Morgan, M., Löffler, D., Kämpf, C., Blumert, C., Reiche, K., Beckmann, J., König, U., Standfest, B., Thoma, M., Makert, G. R., … Fricke, S. (2021). Low Energy Electron Irradiation Is a Potent Alternative to Gamma Irradiation for the Inactivation of (CAR-)NK-92 Cells in ATMP Manufacturing. *Frontiers in immunology*, *12*, 684052.

111. Montagner, I. M., Penna, A., Fracasso, G., Carpanese, D., Dalla Pietà, A., Barbieri, V., Zuccolotto, G., & Rosato, A. (2020). Anti-PSMA CAR-engineered NK-92 Cells: An Off-the-shelf Cell Therapy for Prostate Cancer. *Cells*, *9*(6), 1382.

112. Kaushik, N., Kim, M. J., Kim, R. K., Kumar Kaushik, N., Seong, K. M., Nam, S. Y., & Lee, S. J. (2017). Low-dose radiation decreases tumor progression via the inhibition of the JAK1/STAT3 signaling axis in breast cancer cell lines. *Scientific reports*, *7*, 43361.

113. Yoon, M. S., Pham, C. T., Phan, M. T., Shin, D. J., Jang, Y. Y., Park, M. H., Kim, S. K., Kim, S., & Cho, D. (2016). Irradiation of breast cancer cells enhances CXCL16 ligand expression and induces the migration of natural killer cells expressing the CXCR6 receptor. *Cytotherapy*, *18*(12), 1532–1542.

114. Diegeler, S., & Hellweg, C. E. (2017). Intercellular Communication of Tumor Cells and Immune Cells after Exposure to Different Ionizing Radiation Qualities. *Frontiers in Immunology*, *8*.

115. Tsai, C. J., Chang, K. W., Yang, B. H., Wu, P. H., Lin, K. H., Wong, C. Y. O., Lee, H. L., & Huang, W. S. (2022). Very-Low-Dose Radiation and Clinical Molecular Nuclear Medicine. *Life (Basel, Switzerland)*, *12*(6), 912.

116. *Radiosensitivity of Organs and Tissues [MOE]*. (n.d.). Retrieved April 24, 2023, from https://www.env.go.jp/en/chemi/rhm/basic-info/2018/03-02-07.html

117. Safety Commission, C. N. (2014, February 3). *Linear-Non-Threshold Model*. http://nuclearsafety.gc.ca/eng/resources/health/linear-non-threshold-model/index.cfm

118. Dahal, S., & Budoff, M. J. (2019). Low-dose ionizing radiation and cancer risk: not so easy to tell. *Quantitative imaging in medicine and surgery*, *9*(12), 2023–2026.