IMMUNE SYSTEM MODULATION BY LOW DOSE IONIZING RADIATION

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Abstract

The historical narrative and our understanding about the low dose effects of radiation on the immune system has changed drastically from the beginning of the 20th century to now. A paradigm shift from the DNA target hit model to the one that also considers non-targeted effects (NTE) has attracted a lot of interest recently. Investigations to delineate mechanisms of NTE in the biological tissue have been carried out by various research groups where radiation induced genomic instability (RIGI), bystander effect (RIBE) and abscopal effect (AE) are the effects with most evidence available. This thesis addresses the question of whether low dose ionizing radiation (LDIR) stimulates or suppresses the immune system and how NTEs contribute to this immune modulation by adopting a two-pronged approach where first a narrative review constituting the introduction and literature review was performed followed by a systematic review using PRISMA guidelines to synthesize existing LDIR literature.

This was prompted by our recent discovery that UVA photons are emitted by the irradiated cells and that these photons can trigger bystander effects in unirradiated recipients of these photons. Given the well-known association between UV radiation and the immune response, where these biphotons may pose as bystander signals potentiating processes in deep tissues as a consequence of ionising radiation, it is timely to revisit the field with a fresh lens. After reviewing various pathways and immune components that contribute to the beneficial and adverse effects induced by LDIR, it was found that these modulations can occur by way of NTE. However, the exact mechanistic underpinnings are still unclear and the literature examining low to medium dose effects of ionising radiation on the immune system is complex and controversial. Early work was compromised by lack of good dosimetry while later work mainly focuses on the involvement of immune responses in radiotherapy which typically uses high dose radiation. There is a lack of research in the LDIR/NTE field focussing on immune responses although bone marrow stem cells and lineages were critical in the identification and characterisation of NTE. This may be in part, a result of the difficulty of isolating NTE in whole organisms which are essential for good immune response studies. Models involving inter organism transmission of NTE are a promising route to overcome these

issues. It is concluded that the simple question of whether LDIR stimulates or suppress the immune system is not as simple as initially hypothesized. An attempt was made to analyze if LDIR shifts the balance to immune suppression or enhancement via systematic review but, due to too many differences in the experimental methods in the current radiation and immune studies, a cookie-cutter answer was not possible. However, this thesis did point out the areas of concern such as lack of standardised tools in the field of radiobiological experimental research and quality of methods used which requires urgent attention.

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CHAPTER 1

1. Introduction

1.1. Brief introduction to radiation

Radiation that leads to an ionization event or deposition of energy in the biological tissue is termed ionizing radiation (IR). This deposited energy is approximated to be 33 eV, which is adequate to disrupt chemical bonds within important biological structures (Hall and Garcia 2012). The most commonly used IR in modern medicine remain X-rays and γ-rays, which are considered the low linear energy transfer (LET) radiation. They are very similar with the only difference that X-rays are produced extra-nuclearly while gamma rays result from events that take place from inside the nucleus (Hall and Giaccia 2012). Radiation initiates a variety of alterations in biological tissue that is directly exposed as well as in regions that are not within the direct field of exposure. The principal target still remains DNA, leading to cell-death, carcinogenesis and mutation, which occurs either directly due to DNA breaks caused by charged-particle tracks or indirectly by the reactive chemical species produced as a result of bond breakage in non-DNA molecules. IR may interact with biological tissue directly by hitting the DNA molecule, which is characteristic of high LET radiation. Alternatively, low LET radiation occurs by way of an indirect mechanism, where interaction with other cell components, such as water may release secondary electrons or free radicals like reactive oxygen species (ROS), inducing damage at the cellular level. The modern literature typically reports radiation measurements in the units of Gray (Gy), which measures the absorbed dose i.e., the

energy deposited per unit mass (1 Gy = 1 Joule/Kg). In order to account for different radiation qualities with different LETs, the unit of Sievert (Sv) is also frequently used, where conversion for low LET radiation is fairly simple as 1 Sv equals 1 Gy but a weighting factor has to be applied for high LET radiations (Sazykina and Kryshev 2016).

1.2. Early Research on Radiation Induced Immune Effects

The earliest report of radiation effects on cells involving the immune response, dates back almost to the time when radiation was first described by Röntgen in 1895. Heineke (1905) reported changes in lymph follicles in the spleen and lymph glands with a decrease in circulating lymphocytes post x-irradiation. This effect was firmly confirmed by James Murphy, who was considered pioneer in identifying the modulation of the immune system by ionizing radiation which resulted in enhanced tumor protection (Schaue 2017). X-ray effects on bone marrow were first reported by Warthin, who was primarily investigating effects on renal tissue but discovered marrow changes (Warthin 1907). The damage was evidenced as fragments of lymphocytes ingested by macrophages at histological level. Mitotic components were also missing or rarely seen after an exposure suggesting loss of reproductive integrity. Murphy and colleagues conducted many interesting experiments on murine models to investigate x-rays and immune system interaction. In one study, a tumor was inoculated into mice and caused “lymphocytosis” (production of a more than normal number of lymphocytes) in the blood (Murphy and Morton 1915). They first analyzed the “natural resistance” of mice after a tumor was inoculated in three groups: a) healthy 10 days post-immunized with mouse blood (artificially induced immunity), b) healthy non-immunized (natural immunity) and c)

susceptible with growing tumor as control. Immunized and non-immunized mice were 100% resistant to tumor growth with a significant lymphocytosis as opposed to 60% tumor growth in susceptible controls. Next, they investigated response to tumor inoculation in naturally and artificially induced immunity in mice after their lymphoid tissue was x-irradiated which resulted in reduced lymphocytosis in both groups allowing for tumor suppression. They confirmed the impact of x-rays on lymphocytes by declaring them a "necessary factor" in maintaining host immunity against x-ray induced damage. Another study reported the mechanism by which tumor growth was suppressed in mice groin when exposed to prior erythematic dose of x-rays compared to the contralateral groin that was lead shielded (Murphy et al. 1921). It was suggested that prior irradiation of tissue may allow for such environment or signals which halts tumor growth. However, when the same experimental setup was repeated for cancers inoculated subcutaneously, the tumor growth was the same for shielded or unshielded groins suggesting that the observed effect is restricted to cancers that grow intracutaneously. The study concluded with a hypothesis that the therapeutic role of x-rays in tumors may depend on cellular reactions that induce changes in irradiated tissue hindering subsequent tumor growth. Similar studies followed where serum drawn from low dose irradiated rats when released in non-irradiated lymphocyte suspension caused a 15-30 % increase in the number of these cells as mitotic figures were assessed as an endpoint (Murphy et al. 1922). An increased proliferation and appearance of mitotic figures in the lymphoid tissue (spleen and lymph glands) on day 4 following exposure to a small dose of x-rays is also reported (Nakahara and Murphy 1920). On the other hand, tumors grew after inoculation into mice

that had artificially induced immunity using defibrinated blood if they were exposed to small, repeated doses of x-rays (Murphy and Taylor 1918). It is important to note that the studies by Murphy and colleagues used spark gap which is an ambiguous quantity. It is speculated that it may refer to the distance a spark can cross depending on the voltage difference between the terminals and the medium (air, vacuum, air pressure etc.) between them. Moreover, most studies employed 10 milliamperes with exposure time in minutes which leads to an extremely high dose undermining claims about low dose radiation effects.

Russ et al. (1919) investigated the effects of small doses of radiation (1/200 rad) in rats where the whole body was shielded and only the rat's heart was irradiated, which decreased the lymphocyte number in blood circulation. This was presumed to be an indirect effect on lymphocytes as no aggregate or alteration was observed in directly irradiated tissue. A low dose was used in this study, but whether dose units of 1/200 rad for 12 seconds were delivered daily for two months remains unclear and roughly converts to a cumulative equivalent dose of 5 mrad or 0.05 mGy according to modern dosimetry. Compared to other work around this time, this study was better with regards to the reporting of units of Rad which can be directly converted to the modern unit, Gray, since both are measures of energy absorbed per unit mass. The authors also translated these results to human subjects by suggesting that normal tissue that is nearby or distant to a tumor site in patients undergoing x-ray therapy, may manifest unfavorable effects such as lymphocytes destruction in the normal blood vessels and tissues that may not be directly irradiated. Therefore, they suggested that caution and high clinical acumen must be

exercised by clinicians and radiologists before delivering x-rays to patients, to prevent effects at a distant site. Seen with a modern lens, this study seems to suggest the present-day understanding of out-of-field "non-targeted" radiation effect which will be discussed later in the thesis.

As for the timing and degree of damage, Taylor et al. (1919) reported that bone marrow was affected late and to a lesser degree compared to spleen, lymph glands and blood. Moreover, the destructive action of x-rays with a dose of 3-160 Holzknecht (H) units on blood cells in a variety of mammals was investigated which led to a reduction in both small and large lymphocytes (highly radiosensitive) before any other circulating cells. A lack of acknowledgement for ethical board approval for studies on animal models that used radiation in lethal doses, small sample size and usage of H units were identified as some of the weaknesses in this study. H unit operates on the principle of chemiluminescence where a yellow disc becomes darker on x-irradiation. This color is compared to various shades of yellow where darker shades imply higher dose and 1 H equals one third exposure that results in erythema (Ryan and Poston 2006). This dosimetry technique was subject to changes in temperature and humidity which may impact the reproducibility of the device making it obsolete in later years. Radiation measurements with visible physical and biological effects seemed like a convenient or perhaps the only option for nuclear workers when not much was known about x-rays. The use of the depilatory effect of x-rays and radiation inducing a skin erythema dose (SED) for dosimetry (Wambersie et al. 2000) reflects great gaps in the knowledge of the harmful effects associated with the uncontrolled use of x-rays. The onset of erythema or depilation

response varies with dose delivered, species and the type of tissue irradiated which makes usage of these endpoints almost impractical.

Mid-20th century researchers investigated possible mechanisms that bring about a radiation induced immune response. Jacobson et al. (1950) observed histologic regeneration of haematopoiesis in marrow and lymphoid tissue of mice when their spleens were lead protected during whole body irradiation (WBI) (1025r) compared to another group with no lead protection where lymphoid tissue destruction was seen. The authors proposed that protected spleen provided tissue protection due to a humoral mechanism which allowed damaged organs to replenish. In an extensive review on radiobiological effects, Mole gave the name "abscopal" to an out-of-field response observed at a site distant from originally irradiated tissue (Mole 1953). Mole disagreed with using a blood count assessment to make predictions about the extent of radiation damage to bone marrow suggesting it may not be a correct interpretation of the many abscopal processes at play. Parsons et al. (1954) observed changes in sternal marrow's cellularity in a biopsy sample of chronic granulocytic leukaemia patients who received radiotherapy (280 r) localized only to the spleen. A significant reduction in marrow cells was observed, but this change could not be detected with only the peripheral blood count. These studies used Roentgen, which is a standard unit for radiation exposure, although it is not SI and therefore it is considered outdated. It is difficult to convert roentgen as it is ionisation induced (charge released) per unit mass where modern units of Gray and Sievert measure energy absorbed per unit mass. Roentgen can only be converted to rad if one specifies the circumstances in terms of the range of photon energies employed in a

particular medium, which was not done in this study. A neglect of ethics was also noticed as aspiration sternal biopsy is a risky and uncomfortable procedure to be performed on human subjects due to the presence of vital organs just below the sternum.

It is safe to say that up until the 1950s, although attempts were made to explore mechanisms underlying radiation and immune interaction, these processes were not well understood due to lack of advancement in the field of immunology and radiobiology, let alone intersection of both fields. Alvaro (1953) conducted an extensive review of previous literature and carried out multiple experiments to examine mechanisms that led to resolution of pain and inflammation suffered by uveitis patients when treated with X-ray therapy. Uveitis is an eye-infection instigated by microbes upon reaching the uveal tract where it causes inflammation of the surrounding tissues leading to infiltration by lymphocytes, plasma cells and large mononuclear phagocytes and epithelial cells. He advocated for the use of x-rays as it produced positive results in alleviating the symptoms of uveitis. In this context, x-ray was called vasodilating agent and it was adopted as routine practice by many. However, with the new advancements in science and drug methods, x-rays are no longer used for uveitis treatment. (He et al. 2013). Many in-depth reviews and experimental work addressed mechanistic uncertainties associated with the immune system (Taliaferro and Taliaferro 1951; Jacobsons et al. 1949), which will be explored with a focus on facets where ionizing radiation and immune system overlap.

An out-of-field response called ‘clastogenic factor associated effect’ was reported by Souto (1962) in female rats when tumors developed as a result of plasma received from irradiated rats (1250 r) and sheep (1610 r). Hollowell and Littlefield (1968) noticed

an increase in leukocytes' chromosome aberration frequency when they were cultured in a medium containing plasma from cancer patients that received a lethal dose of radiotherapy. The identity of a damaging agent remained unclear, and it could not be determined if the damage were caused due to breaks in chromosome or disturbance in normal repair mechanisms during mitosis. Later, the same group proposed that a damage causing agent was released from the irradiated tumor which migrated to non-irradiated tissue and this hypothesis was based on the lack of observed damage when the tumor tissue shrank (Littlefield et al. 1969). The old literature described all such “out-of-field” and “indirect effects" as responses in the biological tissue that do not receive direct energy deposition and are now termed as non-targeted effects (NTEs). The modern literature considers various phenomena such as radiation induced genomic instability (RIGI), radiation induced bystander effects (RIBE) and abscopal effects (AE) as NTEs.

A manifestation of RIGI was reported for the first time by Seymour et al. (1986), where an increase in lethal mutations was observed in an un-irradiated surviving progeny of cells that received the initial radiation insult. Kadhim et al. (1992) extended this observation using a bone marrow stem cell model and demonstrated an increased frequency of non-clonal lethal chromosomal aberrations in the clonal descendants of α-irradiated murine hematopoietic stem cells where these abnormalities may appear de novo in distant progeny. A similar effect was reported in human bone marrow cells where delayed apoptosis was also reported (Kadhim et al. 1995). These processes are therefore of relevance in discussion of the immune system where T-cells expand via clonal expansion upon encountering a relevant antigen and any RIGI may be propagated to

future progeny. RIGI in an un-irradiated progeny may manifest phenotypes which include but are not limited to delayed lethal mutations, delayed cell differentiation and delayed reproductive death (Clutton et al. 1996; Watson et al. 2000). Biochemically, an increase in DNA base oxidation and fragmentation, lipid peroxidation and superoxide formation led to a pro-oxidative environment disrupting the normal repair mechanism and genome's integrity in the progeny of irradiated haematopoietic cells (Clutton et al. 1996). It is important to appreciate that the concept of genomic instability may have different connotations in the field of cancer where it facilitates cancer progression as tumor cells accrue several lethal mutations leading to formation of resistant clones (Andor et al. 2017). A study reported that fractionated radiotherapy (2 Gy per fraction), in fact, increased genomic stability (decreased genomic instability) in medulloblastoma in mice which led to enhanced treatment efficacy (Morrissy et al. 2016).

RIBE includes responses in unirradiated cells facilitated by signals released from nearby irradiated cells where these effects are reviewed in great detail by Mothersill et al. (2018). A study by Nagasawa and Little (1992) stimulated interest in RIBE with DNA aberration occurring in 30% of mammalian ovary cells when only less than 1% of nuclei received a direct hit from an alpha particle. The underlying mechanism was not discussed in detail, but later studies confirmed the involvement of gap junctions (Azzam et al. 2001); calcium fluxes, loss of mitochondrial membrane potential, and an increase in reactive oxygen species (ROS) (Lyng et al. 2001, 2006). Fernandez-Palomo et al. (2016) observed strong bystander/ abscopal signals in athymic (immunocompromised) mice, mediated via innate immune response involving macrophages or neutrophils. Since

thymus is critical to adaptive immune response, its absence raised questions about the involvement of an adaptive component and it was suggested that a RIBE might not require the initiation of adaptive immunity. The transmission of bystander signals from irradiated to an unirradiated tissue include extracellular factors (Mothersill and Seymour 1997, Mothersill and Seymour 1998, Lyng et al. 2000) and intercellular communication via cell gap junctions (Azzam and Little 2004). Although not considered NTEs, non-linear low dose radiation effects such as low dose hypersensitivity, enhanced cell differentiation and radio-adaptation often appear in the discussion of NTE (Mothersill et al. 2002; Maguire et al. 2007; Ilnytskyy and Kovalchuk 2011) and sometimes incorrectly considered an NTE (for examples see Szumiel 2014; Shimura and Kunugita 2016). The adaptive response is a result of an efficient DNA damage repair mechanism in place which, on subsequent exposure, reduces the burden on cells or tissues irradiated (Coates et al. 2004).

1.3. Low dose ionizing radiation (LDIR) and immune stimulation

Hektoen (1918) reported an increase in antibody formation when dogs received a sequential increase in radiation dose as it enhanced immunity since antibody producing enzymes accumulated and protected the animal from radiative damage. Olivieri et al. (1984) demonstrated for the first time an adaptive response to radiation in human lymphocytes culture when a prior exposure to LDIR led to a marked decrease in chromosomal aberrations caused by a subsequent high dose. The stimulative effect of LDIR on the immune system still remains unclear, but attempts have been made to explain its role in antigen presenting cells (APCs) and T lymphocytes interaction

facilitated via surface molecules and cytokine secretion (Liu 2003). Cytokines are small proteinaceous molecules facilitating many immune functions and these were reviewed in detail with a radiobiological lens by Schaue et al. (2012). Promotion of antioxidants, DNA damage repair and increased immune surveillance for anti-cancer properties are mainly considered the putative mechanisms underlying immune enhancing pathways. The response heavily depends on the dose where the number of T-lymphocytes and macrophages increase at both a high and low dose of radiation (Liu 2003). The various interweaving biochemical pathways involved in cellular signaling that define the outcomes of this biphasic immune response are outlined by Liu (2003).

Radiation risk in the low dose zone remains uncertain where models such as Linear-Non-Threshold Model (LNT) are currently used to make assessments about the carcinogenic effect of radiation. Many reviews have been written to discuss this model and how its applicability in the low dose zone may need re-evaluation (Prise et al. 2003; Clarke 2007; Little 2010; Calabrese 2017; Calabrese 2018). The anti-tumor effect of LDIR has been reported by many (Portess et al. 2007; Csaba 2019), potentiating it as a viable immunotherapeutic treatment option (Janiak et al. 2017). Scott (2017) asserted that in addition to cancer, LDIR may also impart protection against other life-threatening conditions. Some suggested LDIR induced stimulation of a protective biological mechanism by way of an adaptive response (Feinendegen 2005; Scott 2008; Jargin 2012), such as activating bone marrow cells to differentiate into dendritic cells, thereby enhancing DC cells' antigen uptake capacity and cytokine release (Chun et al. 2012). Tsukimoto et al. (2009) outlines an anti-inflammatory mechanism for gamma radiation

(0.5–1 Gy) which alters expression of two proteins mitogen-activated protein kinases (MAPKs) and MAPK phosphatase 1 (MKP-1) where the latter is upregulated decreasing MAPK expression by dephosphorylating it. MKP-1 is thus a crucial regulator of MAPK-mediated cytokine production and immune system homeostasis. MAPKs may be broadly categorized into three subfamilies: extracellular signal regulated protein kinase (ERK), C-Jun amino-terminal kinase (JNK), and p38 (Wancket et al. 2013). MKP-1 can modulate the pro-inflammatory activity of p38, which mediates production of cytokines like TNF-α and IL-1β in macrophages. These anti-inflammatory properties of radiation can inactivate p38 MAPK and upregulate MKP-1 which may enhance innate immunity (Wang and Liu 2007).

1.4. Low dose ionizing radiation (LDIR) and immune suppression

LDIR may also trigger immunosuppressive responses. Atomic veterans involved in U.S. nuclear testing (1945-1962) were exposed to chronic LDIR (Beck et al. 2017; Till et al. 2018). These research groups collaborated to determine scientifically valid and precise estimates of radiation risk for atomic veterans who were concerned about predisposition to cancer and leukaemia mortality. Data on dose delivered to red bone marrow and male breast were collected and results were found to be comparable (low coefficient of uncertainty ~0.5) to the Japanese Life Span Study conducted for A-bomb survivors who received acute and high doses of radiation. LDIR is known to cause fatigue symptoms with prolonged exposures leading to Chronic Fatigue Syndrome (CFS), which may go unnoticed and unreported due to the idiopathic nature of symptoms (York et al. 2014). When fatigue symptoms appeared in veterans many years later, it was difficult to

associate these with LDIR definitively as the veterans were reported to have received doses within safe limits (Rusin et al. 2018). This can possibly be the result of the lack of modern dosimetry methods, lack of accurate epidemiological data and a poor radiobiological understanding of LDIR effects.

A damaging consequence of ionizing radiation is oxidative stress which can occur directly due to breaks in DNA structure by charged particles or indirectly via reactive chemical species generated due to bond breakage in non-DNA macromolecules (McMahon 2018) or via by-products from a water radiolysis reaction also generating free radicals and ROS (Azzam et al. 2012). This damage may migrate from the original site of radiation to nearby bystander cells through "redox-modulated intercellular communication mechanisms." (Azzam et al. 2012). LDIR induced oxidative stress may generate short and long-term ROS/RNS which can damage mitochondrial DNA (mtDNA) where genome alterations may be translated to various electron-transport chain subunits and relevant proteins (Rusin et al. 2018). When perturbations in oxidative metabolism reach abnormal levels, chronic inflammatory processes may initiate the recruitment of macrophages and neutrophils to the inflammation site where these immune cells release more ROS (Azzam et al. 2012). An increase in size and number of mitochondria per cell was also associated with radiation induced stress response leading to mitochondrial dysfunction upon direct irradiation (5 Gy) or through bystander factors that linger in the irradiated cell conditioned medium (ICCM) (Nugent et al. 2007) which may cause genomic instability in the future progeny (Kim et al. 2006).

1.5. Brief review of immune components relevant to radiation response

The main function of the mammalian immune system is to monitor tissue homeostasis, protect against invading infections, and eliminate damaged cells. The immune system cells are dispersed all over an organism's body for constant surveillance, homeostasis and protection against non-self (Nicholson 2016). The immune system also eliminates non-functioning cells that are damaged or dying. Although it is not a solid visceral organ, the immune system produces the largest number of cells that carry out various defense functions. Its function complexity was compared to the nervous system by Jerne (1973) with about a trillion (10^{12}) immune cells that secrete 100 million trillion (10^{20}) molecules. This system can be divided into two complementary arms: the innate arm and the adaptive or inducible arm, each performing distinct roles or working in collaboration. The site of immune cells production changes location throughout one's development beginning in yolk sac which shifts to the liver and spleen, and thymus after early childhood, the primary site of production of immune cells is the bone marrow (Nicholson 2016). All immune cells originate from multipotent stem cells in bone marrow which differentiate into the lymphoid and myeloid lineages as signals such as cytokines and growth factors are received. The lymphoid progenitor differentiates into T-lymphocytes, B-lymphocytes and natural killer (NK) cells whereas myeloid progenitor differentiates into erythrocytes, thrombocytes, mast cells, eosinophils, basophils, neutrophils, monocytes/macrophages, and dendritic cells. The B progenitor in bone marrow gives rise to B lymphocytes and plasmocytes which are a part of humoral immunity with a life span of 7 weeks and 2 to 3 days, respectively. T-cell precursor cells from bone marrow migrate

to thymus, where they undergo maturation into T-lymphocytes with a life span of approximately 5 months and classified as cell mediated immunity. In thymus, they are referred to as double negative since they express neither of the cluster of differentiation (CD) cell surface markers i.e., CD4 and CD8. The selection of CD markers is determined by the affinity of the T-cell receptor (TCR) for the cell surface self-peptide i.e., major histocompatibility complex (MHC) ligands resulting in T-cell maturation (Kurd and Robey 2016). MHC exists in two classes i.e., MHC I and MHC II which positively selects T-cells to either CD8+ or CD4+ respectively. Although, all nucleated cells can be considered antigen presenting cells (APCs) as they bear endogenous antigens through MHC but macrophages, DCs and B-cells are 'professional' APCs since they can prime naïve T-cells to enable a specific immune response. It is difficult to demarcate a fine border between innate and adaptive arms of the immune system as they work in close harmony.

The innate arm consists of first line defenders that includes anatomical and physiological barriers, blood proteins (complement), phagocytes (dendritic cells DCs, macrophages etc.), granulocytes (neutrophil, eosinophils, basophils) and inflammatory components. It has limited specificity and diversity and does not get enhanced by repeated exposure to stress. The vital function includes maintenance of a homeostatic environment, phagocytosis, secretion of inflammatory factors, chemotaxis upon sensing imbalance and tumoricidal activities (Mohammadi et al. 2019). Monocytes, a precursor for macrophage and DCs, originate and mature in the bone marrow, after which they migrate to the bloodstream. In tissue, monocytes differentiate into macrophages or DCs

(Hume 2006). Macrophages are found in majority in most tissues and viewed as sentinels of the innate arm of the immune system (Schug and Li 2009; Gordon and Martinez-Pomares 2017). Macrophages possess the greatest plasticity, a hallmark characteristic to these cells which greatly influences their response to external stimuli such a radiation. Plasticity is a measure of efficiency of macrophages to switch their functional phenotypes upon sensing danger. The two generally accepted phenotypes are the proinflammatory, formed under the influence of lipopolysaccharides and/or interferon- γ (M1) (Mills et al. 2000) and anti-inflammatory (M2) states formed under the influence of Interleukins such as (IL-4, IL-13, and IL-10) (Martinez et al. 2008), however, research has shown that there exist more than two phenotypes. Interferons are a superfamily of cytokine proteins that play a key role in the first line of host innate defense but also orchestrate innate and adaptive immune response and hence exhibit a variety of immunomodulatory properties as they engage in multiple pathways (Chen et al. 2017).

The adaptive arm can be considered avengers, primarily consisting of T and B lymphocytes with specific and diverse responses. They are self-limiting in terms of growth where a feedback loop mechanism regulates their proliferation into relatively short-lived 'effector cells' i.e., activated state in response to a stimulus. They also demonstrate a unique feature of memory of "self" versus "non-self" antigens on the first encounter which is saved in memory cells and allows for a better host response against a specific antigen on next encounter, even if it happens decades after the initial sensitizing encounter (Chaplin 2010). Cytotoxic T cells (CTLs), also known as CD8+ T-cells destroy tumor cells and are also implicated in transplant rejection. CTLs use their specific T-cell

receptor (TCR) to recognize the targets by binding to antigen associated with MHC class I molecules, which are present on the surface of all nucleated cells. Small proteinaceous factors called cytokines are secreted by regulatory T-cells (Tregs) which inactivate CD8+ cells to an anergic state that prevents an autoimmune reaction. T-helper cells (Th cells), also known as CD4+ T cells, have assistive role in immunological processes such as maturation of B cells into plasma cells and memory B cells, activation of CTLs and macrophages, cell-kill functions by CTLs and natural killer cells (NKs) (Chaplin 2010). Th cells orchestrate almost all effector functions but do require recognition of their specific antigen complexed to MHC class II by their TCR (first signal) and co-stimulation through the binding of B7 molecule on professional APCs by CD28 (costimulatory signal). This leads to CD4+ activation which results in rapid proliferation and secretion of cytokines which mediates further regulative responses. Originating from the same precursor, Th cells differentiate based on the antigen encountered on the APCs, cytokines released, and transcription factors upregulated by the cytokines. Although there are several subtypes, the three classes arising from the same precursor include TH1, TH2, TH17 etc. facilitating different immune responses (Chaplin 2010). The innate and inducible arms synergize closely when specialized antigen specific response is required under stresses such as ionizing radiation (Yahyapour et al. 2018).

1.6. Non-Targeted Effects (NTEs) of radiation and the immune system

It is important to make note of disagreement among researchers about definition of the low and high doses of radiation before the NTE discussion, as varying categorizations

were found in the literature. It seems that context is key and the use of terms “low” and “high” was mostly relative. Hence, no strict categorization was done for the purpose of this thesis. The DNA-centric targeted effects of ionizing radiation do not adequately explain the experimental findings observed in the tissue not directly irradiated. Various NTEs have been reported to date in bystander cells which lie right next to the irradiated target or at a distant site. Effects were seen in nearby or distant unexposed tissue and were variously called “indirect effects” (Russ et al. 1919), “abscopal effects” (Mole 1953), “clastogenic factor associated effects” (Souto 1962). All such effects can be nested under the umbrella term “Non-Targeted effects” (NTE) where some effects may show great similarities in response which makes it difficult to distinguish them as a separate phenomenon. The mechanisms involved and signaling pathways thus far discovered include calcium flux signalling (Lyng et al. 2002; Lyng et al. 2006), production of ROS and direct cell-to-cell communication via intercellular gap junctions (Prise et al. 2002), ROS mediated mitochondria oxidative metabolism disturbances (Lyng et al. 2002; Shao et al. 2006), volatile secretions (Surinov et al. 2004), UVA signals (Le et al. 2015) and bioacoustic signals (Matarèse et al. 2020). Some of these observations overlap and make the understanding of the RIBE rather challenging as they may not manifest as a single effect but as several responses with similar endpoints. In bystander media transfer experiments, signaling factors (chemical) may be released by irradiated cells that may cause a rapid calcium flux which triggers alterations in mitochondrial membrane permeability and increases ROS levels in bystander non-irradiated cells. One such factor that mediates a potential radiation induced bystander response was recently found to be

exosomes, which are endosomal in origin but attach to the plasma membrane and are released into the extracellular environment under normal and stress conditions (Al-Mayah 2012; Jella et al. 2014). Le et al (2017) demonstrated that exosomes released from UV biophoton-exposed bystander cells have the capacity to induce RIBE in recipient cells, confirming their role as a secondary signalling system. The contents of an exosome include proteins, different types of RNAs, mitochondrial and genomic DNA. The role of RNA molecules contained in exosomes in RIBE was identified for the first time by Al-Mayah et al. (2012). Recently, exosomes have also been explored due to their unique ability to transfer biomolecules with evidence of mediation in various NTEs (Tuncay Cagatay et al. 2020), as well as radiation induced immune related signaling pathway at 2 Gy (Szatmari et al. 2019). However, it is yet to be determined if the same response will be elicited at lower doses of radiation (Kadhim et al. 2020).

LDIR can upregulate mitochondria dependent ROS generation in radiosensitive organ systems such as the hematopoietic system, which stimulates inflammasome mediated cytokine maturation (York et al. 2012). Cytokine secretion reflects functional integrity of the immune system as they facilitate communication between the immune system cells (Bogdandi et al. 2010). Physical signals such as sound and UV photons generate responses in bystander cells. Cohen et al. (2020) presented the first proof of two types of UV biophoton emission by gamma radiation of wavelength 340 and 610 nanometre (nm) to elicit a bystander response where previous work had been conducted with beta particles (Ahmad et al. 2013; Le et al. 2015). 340 nm made up the predominant energy UV spectrum, where secondary photons emitted at 610 nm. These secondary

photons can also induce direct or indirect damage via exosome release from bystander cells that cause responses in additional bystander cells and the chain may continue (Le et al. 2017). In our laboratory, the bystander signal mostly involved UVA photon emissions wherever LDIR interacted with cells. There is extensive literature that UV interacts with the immune system, particularly Langerhans cells (Ullrich and Byrne 2012; Moattari and Granstein 2021). While this is outside the scope of this paper, it is interesting to consider that UVA biophotons generated due to ionising radiation interaction in deep tissues could be a mechanism leading to an ionising radiation-induced immune response.

Mothersill et al. (2012) made the first mention of the theoretical possibility of weak acoustic signals as physical signals that can elicit a RIBE. Although a sound signal as a bystander signal lacks evidence at the moment, interest is developing in this topic (Matarese et al. 2021, unpublished data from our lab group). Sound signals are ubiquitous in each biological kingdom including plants, animals and microbes where they may impact molecular and physiological responses (Barbero et al. 2009; Frongia et al. 2020) with a positive role in regulatory functions including the immune system (Choi et al. 2017). The strength of the signal depends on the distance and height between the acoustic signal source and receiver (Padgham 2004). These signals may behave similarly to physical bystander signals that participate or trigger various metabolic pathways and gene expression patterns (Matarèse et al. 2020). The sound signals released from irradiated cells leads to, complements, or interacts with other signaling pathways including endosome release, which may migrate to unirradiated cells to initiate a RIBE. Additionally, both sound and biphotons may contribute to a potential bystander stress

response via apoptotic pathways that involve macrophages (Matarèse et al. 2020), and this may imply that more immune system components are also associated. The relationship between the immune system and various NTE is complex. Figure 1 provides a conceptual Venn diagram of the complexity and inter-relationship of some of the factors involved that can impact an eventual immune effect.

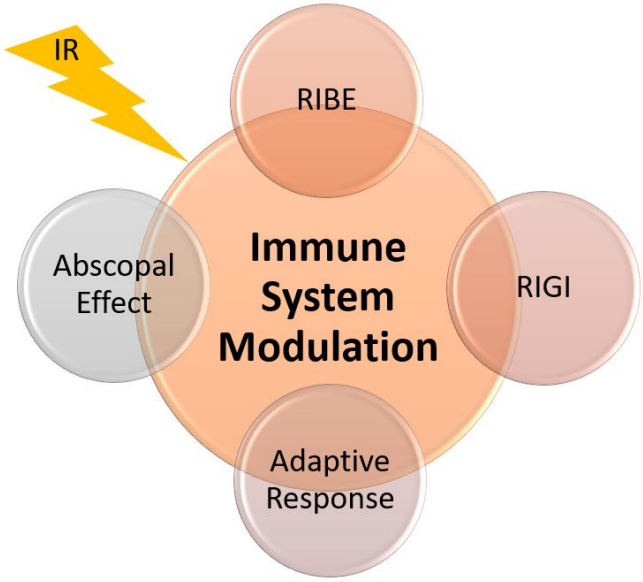

Figure 1: An oversimplified depiction of a few factors, out of the many, that modulate a radiation induced immune response. The four outer circles are the various NTEs (RIBE, RIGI and AE) and low dose radiation effect (Adaptive response) impacting the eventual manifestation of an immune response triggered by LDIR.

1.6.1. Possible mechanism of immune stimulation via RIBE

Seymour and Mothersill (2004) suggested likeness of RIBE with a primitive immunological response where RIBE, at the time, was considered to not involve any known cytokines. However, later literature indicates that bystander signaling is associated

with a radiation triggered cytokine release (Prise and O'Sullivan 2009; Farias et al. 2020). LDIR when applied to the whole system has confirmed reports of immune stimulation by way of increased cytotoxicity and cytokine production in pre-clinical models (Cheda et al. 2008; Nowosielska et al. 2012). There is also evidence that targeting LDIR (2 Gy) to a small region may initiate T-cell cytolytic activity in large established murine, xenotransplanted and primary human tumors (Klug et al. 2013). The proposed mechanism was the recruitment of effector T-cells into the tumor site as polarization of M1 type inducible nitric oxide (iNOS) expressing macrophages are triggered by radiation which induces chemokine release from T-helper cells reversing an immunosuppressive environment (Klug et al. 2013).

Another consideration is the variation in immune response that exists at low and high doses but may also vary amongst two different magnitudes of low doses. El-Saghire et al. (2013) exposed human monocytes to LDIR (0.05 Gy and 0.1 Gy) and HDR (1Gy) to assess central immune pathways including the activation of a toll like receptor (TLR) on antigen presenting cells, MAPKs and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling. A positive effect was demonstrated in the TLR activation at low dose where high dose caused a relatively less significant response. TLRs were upregulated in response to the signaling molecules such as high mobility group box 1 (HMGB1), TLR4, TLR9, myeloid differentiation primary response 88 (MyD88) and interleukin-1 receptor associated kinase (IRAK1). Next, NF-κB and MAPKs are activated where adapter molecule MyD88 plays a crucial role in this activation as it interacts with IRAK1 facilitating NF-κB's translocation to the nucleus as IκBα is phosphorylated and

degraded. Downstream of this pathway, MAPKs (p38, ERK and JNK) are stimulated and pro-inflammatory signals are expressed (See Fig 7 in El-Saghire et al. 2013). The study concluded that low dose (<0.1 Gy) promotes immune-stimulatory and pro-survival responses via TLRs, MAP kinases and positive regulation of NF-κB signaling pathways where high dose (1 Gy) discourages this response which leads to damage-inducing pathways. The relevance of TLR pathway to bystander signaling is reported by Ermakov et al. (2008) who irradiated peripheral human lymphocytes to an adaptive dose of 10cGy and observed fragments of extracellular DNA in the growth media as a result of either spontaneous or radiation induced cell death. The irradiated lymphocytes were removed from media, after which normal lymphocytes (bystander cells) were introduced in it which undergo transposition of the pericentromeric loci of homologous chromosome. This was suggested to be a radiation induced initial adaptive response as loci approach each other to facilitate a possible repair of double-stranded breaks of DNA during recombination. Moreover, authors indicated that modified extracellular DNA induced such changes in it that make its able to bind with toll like receptor 9 (TLR9), where nuclear DNA is not able to accomplish this binding. This bond is crucial for immune cells' activation as MAPK-family protein kinases and NF-κB pathways are triggered leading to an increased cytokine and ROS production. Similar pathways are also reported in macrophages when activated by DNA fragments from irradiated non-surviving cells (Choi et al. 2005). Future experiments on a range of LDIR doses and bystander mediated activation of immune pathways such as the MAPKs and NF-κB may elucidate our understanding of RIBE mediated immune responses.

1.6.2. Possible mechanism of immune suppression via RIBE

One of the most common cytokines released as a result of radiation is tumor growth factor β (TGF-β), which mediates damage caused by reactive oxygen and nitrogen species (ROS/RNS) emitted from various sources (Schoenhals et al. 2017; Jayaraman et al. 2018; Farhood et al. 2019). TGF-β is considered a pleiotropic molecule, that causes suppression of epithelial growth in the early phases (pre-malignant) of tumour growth but manifest tumor-promoting properties in advanced cancer stages facilitating cancer spread and invasion (Formenti et al. 2018). TGF-β can elicit immune mediated bystander responses in nearby tissues (Yahyapour et al. 2018) such as the induction of genetic alterations (Farhood et al. 2020). The proposed mechanism was the activation of macrophages triggered by TGF-β which stimulates iNOS expression. This results in high levels of both nitric oxide (NO) and ROS which migrate to bystander cells and cause DNA damage. NO may also interact with superoxide (O_2^-) and hydrogen peroxidase (H_2O_2) from the mitochondria that leads to a peroxynitrite ($ONOO^-$) free radical which has longer half-life further propagating unfavorable effects (Farhood et al. 2020).

Mitochondria is implicated in RIBE where disruption in oxidative energy metabolism and redox biochemistry modulates apoptotic pathways in non-irradiated bystander cells (Lyng et al. 2011; Mothersill et al. 2000). Biophotons emitted as a bystander signal reduce the activity of a Complex I (NADH dehydrogenase), the main complex involved in proton pumping, eventually compromising ATP yield (Le et al. 2018). The interaction of LDIR elicited bystander biophotons is still a fairly recent discovery where its role in the immune function still remains to be fully explored. An

association of altered mitochondrial metabolism and energy output has been made with chronic immune-deficiency syndrome (CFIDS) (Rusin et al. 2018). Mitochondria may be relevant to immune functions since it is a semi-autonomous cell organelle that adapts and re-programs to the needs of any respective organ (Schirrmacher 2020). This may be of significance to immune functions as immune and stem cells alter between a resting and proliferative phase.

1.6.3. Possible mechanisms for immune stimulation via RIGI

RIGI is a complex phenotype that may occur due to a variety of stresses where elevated levels of genetic alterations that occurs in distant progeny of irradiated cells or in a fraction of clonal progeny that survive the initial exposure. GI as a consequence of radiation, termed as RIGI, was defined by Kadhim et al. (2013) as:

> "Heterogeneous, delayed elevation in the rate of de novo appearance of genetic changes such as mutations, chromosome aberrations or micronuclei, cell death or mitotic failure etc. within clonal descendants of irradiated cells."

The important points are that non-clonal mutations are seen in distant progeny and these mutations are somehow tolerated by the system so that high frequencies are seen compared with the spontaneous mutation frequencies seen in descendants of unirradiated cells (Mothersill and Seymour 2019). The phenomenon was described in a bone marrow stem cell culture model where the lineages could be studied and mapped (Kadhim et al 1992). Mutations induced by way of RIGI might not be all "bad" and can result in natural selection allowing for increased genetic variability (Mothersill et al. 2017). Long-term,

this may allow the evolutionary advantage of "fitter" phenotypes and genotypes that are better adapted to cope with the ever-changing biological macro and microenvironment, as well as ambient stresses. The selection of conformational substrate states by proteins and post translational modifications were considered examples of genomic instability at low doses that induced epigenetic changes (Baverstock and Karotki 2011). Bystander signals allow communication during stress situations by way of stimulating antioxidants and mitophagy (a type of autophagy). The disturbed balance between the oxidants and antioxidants could damage important molecules that are required for cellular functions and result in cell death. But if the cell manages to survive, it has undergone epigenetic changes allowing for increased tolerance or adaptability to such stresses in the future (Szumiel 2014). As for the relevance of the immune system to the above discussion, autophagic proteins may regulate innate responses (Nakahira et al. 2011) and hence, investigating RIGI mediated epigenetic modifications in the immune function and associated proteins (enzymes, cytokines, antibodies) may reveal valuable findings.

1.6.4. Possible mechanisms for immune suppression via RIGI

RIGI may confer immediate and delayed effects in biological tissue, which is an intricate playground of countless inter and intracellular reactions. Mothersill and Seymour (1997) listed the following adverse effects as a result of RIGI: 1) the inability of some descendants to divide in the clonal progeny of irradiated cells, 2) chromosomal instability (mutations) in genome that were not present in the irradiated parent (non-clonal mutations), 3) microsatellite instability and 4) gene amplification. Of these, microsatellite instability and gene amplification were suggested to be of acute nature allowing for

unstable clones which may either develop a selective advantage leading to proliferative success, or disadvantage that leads to death (Mothersill and Seymour 1997). An example of immediate mutation is DNA mismatch repair defect which disrupts protein translation and selects such cells which lack surface antigens enabling immune escape (Branch et al. 1995; Karran 1996), and a possible tumour formation (Karran and Bignami 1994). Many more manifestations categorized as radiation triggered GI include delayed lethal mutations, delayed apoptosis, karyotypic abnormalities, cellular transformation, clonal heterogeneity and the delayed loss of reproductive ability (Tang and Loke 2015; Mothersill et al. 2017). GI is also accepted as one of the hallmarks of cancer with an associated immune-suppression which facilitates and promotes carcinogenesis. Activation of innate immune components by cytokines disrupts physiological homeostasis with a myriad of downstream stress pathways causing cell damage over time which may be associated with a RIGI (Morgan and Sowa 2015). Moreover, radiation induced elevated levels of ROS in directly hit or bystander cells may trigger a cascade of events manifesting RIGI phenotypes in unstable clones where concomitant damage to mitochondria may also add to ROS burden. Details of mitochondria involvement in apoptotic pathways triggered by radiation and how it generates ROS are reviewed by Valerie et al. (2007) and inflammatory cytokine led NTEs was reviewed by Hei et al. (2015) and McBride et al. (2017). Inflammation has also been implicated in RIGI where macrophages were considered a source of bystander signals as they remained active long after the initial exposure ended (Lorimore et al. 2008). The prolonged activation led to increased levels of TNF-α, NO, and superoxide, where these species contributed to a

delayed expression of RIGI. The discussion above is summarized in the graphics below (Figure 1 and Figure 2).

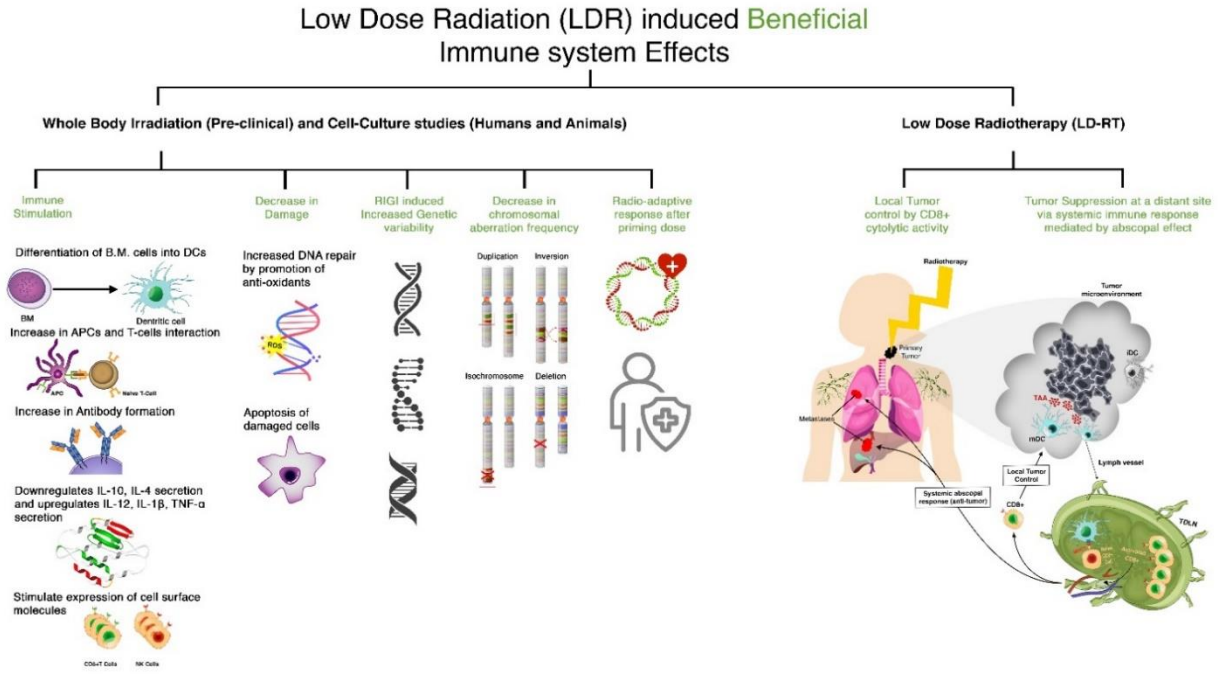

Figure 2: A conceptual flowchart showing the various low dose radiation induced beneficial effects. The reviewed literature suggests that most of these immune responses, if not all, may be elicited by way of a non-targeted mechanism where immune system cytokines, reactive oxygen species, volatile secretions, exosomes and biophotons released by targeted cells may potentiate a possible radiation induced bystander effect (RIBE) or genomic instability (RIGI), described in detail under the NTE discussion. It is important to note that radio-adaptive response (RAR) is not considered an NTE, but rather a low dose radiation effect in which irradiated cells adapt better to higher doses of ionizing radiation when a prior lower priming dose is delivered.

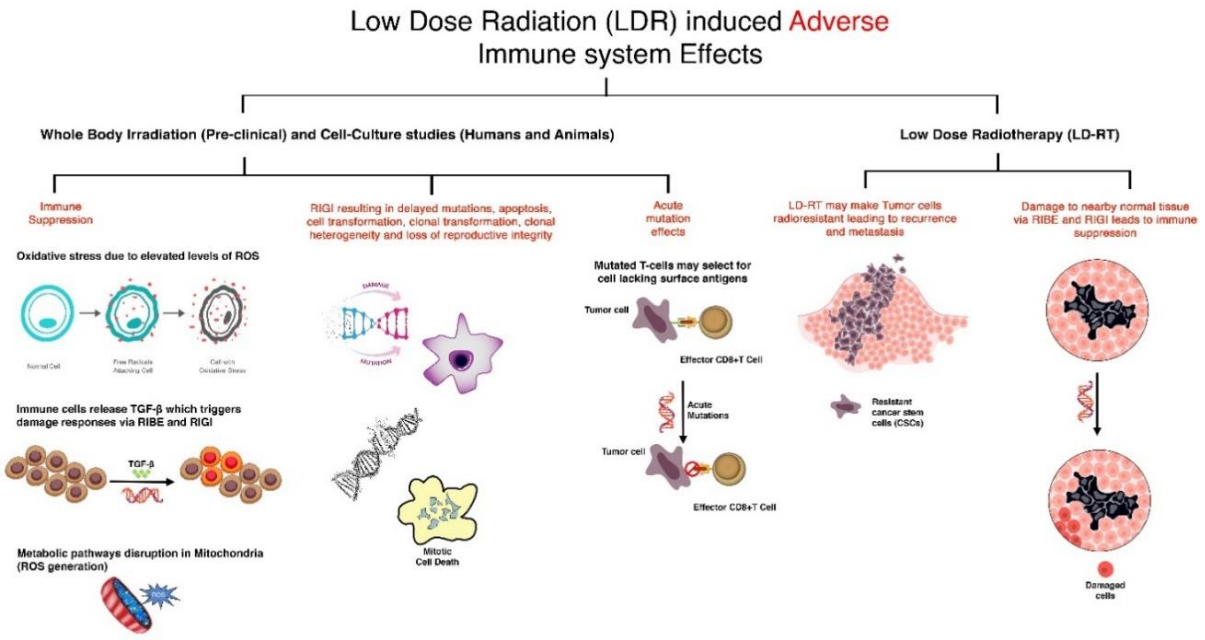

Figure 3: A conceptual flowchart showing the various low dose radiation induced adverse immune effects. It was suggested in the reviewed literature that most of these immune responses, if not all, may be suppressed by way of a non-targeted mechanism where immune system cytokines, reactive oxygen species, volatile secretions, exosomes and biophotons released by targeted cells may potentiate a possible radiation induced bystander effect (RIBE) or genomic instability (RIGI), which are described in detail under the NTE discussion.

1.6.5. Possible mechanism of immune stimulation via abscopal effect

Abscopal effect (AE) is an out-of-field effect which is very similar to a RIBE and mechanisms by which AE and RIBE may overlap, and it may be difficult to identify them as a distinct phenomenon. AE manifests as a systemic effect within the same organism when ionizing radiation is applied locally. Abscopal takes its roots from Latin where "ab-scopus" means away from the target. AE is typically discussed in the context of

radiotherapy and has shown therapeutic promise in cancer care where many clinical trials are ongoing to investigate its full potential (Welsh et al. 2020). Demaria et al. (2004) first introduced the idea that the abscopal effect is immune mediated as suppression of tumor growth at a distant site was observed due to an anti-tumor immune response. Tumor bearing mice were irradiated (2 and 6 Gy) and after a day dendritic cell (DC) maturation was induced by using growth factor Flt3-Ligand (Flt3-L). This step ensured that intrinsic immunosuppressed status of the host did not interfere with investigation of immune mediation of abscopal effect. No response was observed when Flt-3 was administered alone, however, in combination with radiation, relevant T-cell effectors were activated and reached a distant tumor site to repress its growth. The 2 and 6 Gy group showed the same response without any significant difference except the lower dose group's limited capacity to ensure complete eradication of the tumor which raised clinical concerns (Demaria et al. 2004). Immunogenicity (ability to elicit an immune response) is a factor that greatly impacts tumor elimination since a poorly immunogenic tumor is unable to express co-stimulatory molecules needed for the activation of naïve T-cells. Radiation may enhance tumor immunogenicity as it improves cross-priming which is a phenomenon by which DCs uptake tumor associated antigens (TAAs) from tumors and present these to a CD8+ T-cell response via the classical route of MHC class I presentation to induce a cytotoxic response (Melief 2003). When radiation has successfully killed tumor cells, inflammatory signals are released which allows for increased permeability of both DCs and effector T-cells. On the contrary, Camphausen et al. (2003) suggested involvement of the tumor suppressor protein 53 (p53), which is typically upregulated during irradiation.

Authors compared tumor reduction when non-tumor bearing legs in wild-type (WT) and p53 double knockout (dKO) were irradiated after tumor was inoculated at a distant site (mice dorsum) in both groups. The tumor in WT-p53 grew at a significantly slower rate compared to p53-null mice suggesting it as a key mediator in radiation induced abscopal effect which may involve some components of the immune system. p53 acts as a transcription factor that leads to increased expression of cytokines and other signaling factors in response to radiation induced inflammation (Camphausen et al. 2003). This is interesting because p53 is known to be involved in many bystander responses in vitro (Mothersill et al. 2011; Strigari et al. 2014) where cytokines such as IL-6, IL-1α, and TNF-α are known to act as bystander signals (Wang et al. 2018).

1.6.6. Possible mechanism of immune suppression via abscopal effect

Abscopal effect may also induce unfavorable consequences. A review by Farhood et al. (2020) stated how locoregional radiotherapy of tumor-stricken areas (pelvis or chest) led to distant affects in the bone marrow stem cells which resulted in decrease of peripheral blood cells, especially lymphocytes and platelets. The proposed mechanism was systemic activation of TGF-β triggered pro-oxidant and pro-fibrotic genes which leads to fibrosis and RIGI in normal tissue. This weakens the immune function which increases risk of complications such as infection or bleeding. To support their contention, the authors mostly referenced studies or reviews that discussed or employed in-vitro or in-vivo whole-body irradiation (Shao et al. 2008; Wei et al. 2019), raising concerns about the validity of claims about distant effects of targeted irradiation. Although, radiotherapy inevitably damages the normal tissue surrounding tumor site primarily by direct cell kill

or by affecting replenishment pathways in rapidly proliferating tissue, but tumor itself also contributes to this adverse effect in normal tissue (Siva et al. 2015). Cancer cells communicate with nearby normal tissue via molecules like ROS such as hydrogen peroxide ($H_20_2^-$), cytokines and growth factors causing long-lasting inflammatory state, genomic instability and radiation damage which may extend to the distant normal tissue via abscopal effect. Some have disagreed and stated that abscopal effect is multifactorial where variables like irradiated tissue's volume, number of surviving cells and their capacity to migrate and lodge in the tumor microenvironment may greatly impact its final outcome where in some cases, it may not be clinically significant (Rodríguez-Ruiz et al. 2018; Demaria et al. 2016). Moreover, efficacy of an abscopally elicited anti-tumor immune response triggered after radiotherapy may be affected by radioresistance developed by tumor cells. Cancer stem cells (CSCs) are such radioresistant cells which disseminate from the tumor site leading to an increased risk of tumor recurrence and metastasis (Prasanna et al. 2014; Ashrafizadeh et al. 2020; Yoshida and Saya 2021).

In some cases, radiation may not achieve complete tumor elimination despite initiation of an efficient immune response. There are many reasons for this but one involving the immune response was described by Hellstrom and Hellstrom (1993) who outlined a mechanism which begins with recognition of target peptides by T-cells in the context of an MHC molecule with an additional need of a secondary co-stimulatory signal where failure of either may result in an inadequate anti-tumor response. The interaction between an adhesion molecule such CD28, CD152 (CTLA-4) expressed on T-cells and their ligand CD80 or CD86 (the two major types of B7 protein) expressed on the APCs

such as macrophage, tumor cells etc. establishes a co-stimulatory molecular bond. Any disruption in this bond may hinder generation of an appropriate immune response as some mechanisms might not turn on where a long-term immunological nonreactivity may also result. Belehradek et al. (1972) highlighted a phenomenon called “eclipse state” in which the host is essentially immunologically unresponsive to the tumor. It was proposed that injection of irradiated tumor cells into peritoneal cells reversed this eclipse state to an immunological “booster” effect, although no recent studies were found confirming this effect.

1.7. Other radiation effects relevant to immune system discussion

1.7.1. LDIR induced immune priming: Radio-adaptive response (RAR)

The adaptive response refers to the ability of irradiated cells to survive better when exposed to high doses of ionizing radiation if a prior low dose is applied to prime the system under consideration (Olivieri et al. 1984). DNA repair pathways and modulation of associated genes is the responsible mechanism for this response (Coleman 2005). A recent study suggests that radiation induced oxidative stress is a master regulator of adaptive radiation responses (Sisakht et al. 2020) although this response may vary at low and high doses of radiation (Mothersill and Seymour 2006; Hlatky and Hahnfeldt 2014; Scott 2017). RAR is typically induced at low doses after initiation of various signaling pathways which include DNA repair response, beneficial stress signalling, cell cycle regulation and physiological apoptosis in normal cells (Zhao et al. 2017). Low dose radiation promotes antioxidant environments (e.g., increased glutathione levels) which

improves immune function (Liu 2003). A dose of ≤ 0.1 Gy leads to an adaptive response which increased proliferation in normal and bone-marrow stem cells but not in tumor cells (Jiang et al. 2008). This stimulates growth in normal tissue as damaged cells adjacent to tumor cells are replenished enhancing radiotherapy outcomes.

Bravard et al. (1999a) investigated if protective response was due to upregulation of antioxidant enzymes in human lymphoblasts after a priming dose (0.02 Gy) followed by a larger dose (3 Gy). An increase, although not significant, was observed in the antioxidant enzyme activity that scavenged free radicals in cells that received prior priming exposure compared to unprimed cells. Authors stated that factors like the cell-cycle phase of irradiated cells may modulate the final adaptive response as enzyme function varies with cell proliferation and differentiation. Since immune system is comprised of many proteins, research on RAR with a focus on immune-specific proteins may unravel useful discoveries. Mitchel (2010) also confirmed the variable nature of RAR where it depends on priming and challenge dose, cell cycle stage and dose rate employed. RAR is not observed in all biological systems and when it occurs, the priming effect occurs between 4-6 hours and roughly lasts for three cell cycles (Shadley et al. 1987). While the mechanisms underlying RAR remain to be fully elucidated, Kadhim et al. (2013) states that that bystander signals can lead to adaptive responses. This hypothesis lacks evidence in in-vivo models and the existing findings were primarily observed in medium transfer experiments (Maguire et al. 2007). Premkumar et al. (2019) demonstrated immune relevant RAR in two mice strains that showed variable response in splenic lymphocytes. The endpoints assessed were DNA damage, early activation marker

(CD69), cytokines (IL-2, IFN-γ) and the proliferation status. RAR was observed in one strain of mice after irradiation (priming dose: 0.1 Gy; challenge dose: 2 Gy) as ROS levels were reduced which effected downstream MAPK activation pathway. Authors also suggested that RAR signals may travel to neighboring cells and hence participate in a protective RIBE.

1.7.2. Hormesis effect

Any stress response may be initiated either by a negative inhibitor or a positive stimulating factor formally termed as distress or eustress respectively (Bienertova-Vasku et al. 2020). Hormesis occurs when a biochemical or radiological stress leads to biphasic biological response where beneficial pathways are typically upregulated at low dose and downregulated at high dose of stress (Agathokleous et al. 2020). Liu (2003) reviewed LDIR radiation effects with a focus on immune endpoints such as inflammatory factors, genomic instability and ROS which leads to secretion of cytokines in the immunologic synapse followed by upregulation of PKC/$[Ca^{2+}]$ ratio increasing expression of the NF-κB. The role of NF-κB pathway in radiation induced immune response is paradoxical in nature and it is considered an important regulator of both innate and adaptive immune responses (Spiotto et al. 2016). Radiation stimulates the functional form of NF-κB found inactive in the cytoplasm by translocating it from cytoplasm to the nucleus where it initiates gene expression of a variety of pro-inflammatory cytokines such as TNFα, IL-6, IL-1α and IL-1β etc (Spiotto et al. 2016). During radiation induced DNA damage, the genotoxic stress arm of NF-κB pathway is switched on, translocating it back to cytoplasm

where they activate proteins such as p50/p65 of the NF-κB/Rel family. The effect of NF-κB as radioprotective (resistant) or radiosensitizing is currently a matter of debate as its activation has induced expression of both anti-apoptotic proteins as well as proteins that promote tumor growth (Hellweg 2015).

1.7.3. Hyper-radiosensitivity (HRS) and induced radioresistance (IRR)

With the improvement in modern techniques such as dynamic microscopic imaging, improved clonogenic assays, and others in the last three decades, it has become easier to investigate cell responses in the low dose region where phenomena like hyper radiosensitivity (HRS) and increased radioresistance (IRR) became apparent. At doses below 0.5 Gy, there is a region of high sensitivity in the cell survival curve, following which, relative resistance to radiation induced cell death develops at the dose range 0.5–1 Gy which is termed as IRR (Joiner et al. 2001; Prasanna et al. 2014; Brooks et al. 2016). The interrelationship of HRS/IRR with the RIBE is elusive, Mothersill et al. (2002) investigated 13 cell lines to explore this relationship and reported that significant HRS response occurred in cellsthat did not induce RIBE (Whereas, Fernandez-Palomo et al. (2016) suggested that RIBE may only occur in the HRS region of the dose response curve and disappear with increasing dose to a point where IRR is the dominant process at play. Typically, cells in the G2 phase of the cell cycle are considered most susceptible to HRS/IRR response (Marples et al. 2003). IRR and adaptive response share similar mechanisms, but this may only be true for a short time scale and further investigation is needed to clearly define the border between the two (Joiner et al. 2001). In another study Joiner et al. (1999) states that a “synchrony” phenomenon prevails after initial priming

dose that eliminates cells in the sensitive phases of cell cycle and the surviving cells are mostly resistant exhibiting IRR upon subsequent exposure. The interval between the first low dose and the second dose may act as a buffer for repair mechanism which may enhance the immune response. Mothersill and Seymour (2019) suggests as the dose increases, tissue level repair mechanisms dominate as opposed to an individual cell complicating interpretation of the low dose response curve. This may have relevance to low dose radiotherapy as irradiation of diseased tissue may also inadvertently deliver radiation to the normal tissue in the range where these phenomena occur.

Another factor bearing implications for an anti-tumor immune response is the hypersensitivity acquired by radioresistant tumour cells when multiple small doses of radiation are delivered in fractions ensuring increased cell kill (Short et al. 2001). Timing between these fractions is critical as radioresistance was developed when a short time interval technique was used which leading author to believe that increased radioresistance (survival) was similar to a radio adaptive effect. A study reports that radioresistance and increased survival of immune cells after local radiotherapy may contribute to late radiation effects being transmitted via patient's T cells (Lippitz and Harris 2019).

1.7.4. Fractionation in radiotherapy

Fractionation refers to dose sparing when the two doses are both large but have a less severe effect than the total dose given as a single dose. Mothersill and Seymour (2002) demonstrated that fractionation of an actual dose of radiotherapy manifested a dose-sparing effect, where this effect diminished when medium harvested from cells irradiated with the same fractionation protocol was introduced to unirradiated cells. This

implies that fractionation and the time interval between each treatment may impact the manifestation of adaptive or bystander responses. Scott and Tharmalingam (2019) discussed how fractionation activates immune cells such as NK cells, DCs, macrophages and T-cells where T-regulatory cells (Tregs) production is reduced at low dose radiation (LDIR). Moreover, LDIR alters secretory components of the immune system where cytokines such as IL-2, IL-12, TNF-α and IFN-γ are upregulated while TGF-β and IL-10 are decreased. Fractionation into 10 equal doses in mice resulted in life shortening compared to when the dose was delivered as a single exposure due to possible radiation induced sensitization of mice to cancer induction (Maisin et al. 1983).

1.7.5. Key players in radiotherapy induced anti-tumor immune response

T and B-lymphocytes

T-lymphocytes and their sub-types exist in a dormant state where activation of naïve T-cells into specialized memory and effector T-cells must be accomplished by an antigen. Radiation triggers release of tumor associated antigens (TAA) from tumor tissue which can activate CD8+ T-cells. It is crucial that radiation induces the right type of signal to initiate maturation of professional antigen presenting cells (APCs), such as DCs, within the context of a major histocompatibility complex (MHC) molecule (Demaria and Formenti 2007). Tumor cells may also escape CD8+ T-cell kill by down-regulating expression of MHC class I molecules. If tumor begins to spread, the initial line of defence such as the such as the natural killer (NK) cells (innate) and natural killer T (NKT) cells (adaptive) are alarmed, an instance of the innate-adaptive coordination. NKT cells are

specific T-cells characterized by the expression of the T-cell receptor where they also resemble NK cells features (Kriegsmann et al. 2018). These first responder cells then release various cytokines and chemokines attracting APCs into the site of damage where they uptake TAAs to present these to CD8+ T-cells in the tumor draining lymph nodes (TDLN) to activate them. At this stage, CD8+ T-cells are ready to depart the TDLN to reach the tumor site where they perform effector functions with high specificity and efficiency also known as 'immune-surveillance' (Demaria and Formenti 2007). Despite this surveillance mechanism in place, there are still occurrences of cancer development prompting researchers to coin a new term i.e., 'immuno-editing' to explain altered tumor immunogenicity enabling its growth that escapes the T-cells cytotoxicity (Dunn et al. 2002 reviewed in Demaria and Formenti 2007; Janiak et al. 2017).

Older research around radiation induced immune effects in the earlier part of 20th century lacked focus on B-cells and this continues to be the case even today. B-cells express diverse cell surface immunoglobulin (Ig) receptors which recognize specific antigens which are then presented to T-cells similar to the role played by the APCs. Additionally, B-cells also produce antibodies that attack the tumor tissue (Namm et al. 2015). B effector cells mediate the anti-tumor immune response and collaborate with various T- cells and this understanding may be a steppingstone for future research on B-cells with a radiobiological perspective which currently lacks literature.

Natural killer (NK) Cells

NK cells can trigger an acute antitumor response by releasing inflammatory cytokines and chemokines and becomes one of the early responders of stress where they also coordinate

with other immune cells (Sonn et al. 2012). Ionizing radiation significantly affects the NK cell biology either by way of a direct or an indirect mechanism which enhances antitumor immunity (Chen et al. 2020). NKs are stimulated at low doses while a high dose typically impairs their function except in cases where pre-treatment with IL-2 takes place. Moreover, it was suggested that NK function may be adjusted by other immune cells (such as TAMs, DCs, Tregs, MDSC etc.) after radiation exposure which alters tumor immunogenicity. NK cells' role is supplementary to T-cell mediated immune response where they directly kill the tumor without relying on antigen presentation mechanism in a major histocompatibility complex dependent way. This makes them a novel and promising target for radio-immunotherapy tools. A study by Canter et al. (2017) conducted a first clinical trial on dogs to investigate if prior focal radiotherapy (10-20 Gy) of sarcoma in dogs would enhance the homing and cytotoxicity of NK cells. A sensitizing effect was induced by radiotherapy in conjunction with NK cell therapy, which greatly improved cancer treatment outcomes at the local and distant site by way of an abscopal effect.

IFN/STAT1 pathway

IFNs are signaling proteins of the innate immune system classically associated with first line of anti-viral defence response (Chen et al. 2017). Their role in radiation induced immune modulation recently emerged with crucial role in radiotherapy (Liu et al. 2020). Radiation effects the expression of interferon-related genes such as IFNα, IFNβ, STAT1 and IFNγ (Spiotto et al. 2016). Classified into two types, Type I interferon (i.e., IFNα and IFNβ) facilitate DCs maturation which kickstarts effector T-cells responses as they reach

tumor site to release Type II interferon (i.e., IFNγ) where tumor vasculature is obliterated sensitizing it to cytolytic T-cells. IFNs initiate intracellular signaling cascades via STAT pathway which leads to a wide variety of immunological response (Chen et al. 2017). Signal transducer and activator of transcriptions (STATs) are a protein family where STAT1 and STAT2 have critical role in sensitizing cells to overcome radioresistance during radiotherapy (Liu et al. 2020). They accomplish this by inhibiting cell proliferation, differentiation, apoptosis, and angiogenesis while STAT3 and STAT5 have been associated to cancer progression

Danger signals

Danger signals initiate nonspecific immune responses at the site of stress with the activation of an appropriate and specific adaptive response (McBride et al. 2004). They distinguish between apoptotic and necrotic death. Previously, it was believed that programmed death is dealt locally with minimal immune involvement. Inflammation (or immune system) was only triggered by pathological necrosis inducing an antigen-specific response. Researchers have challenged this notion and established that radiation induced programmed cell death not only involves immune system but may also trigger an effective anti-inflammatory response mediated by cytokines (i.e., IL-10 and TGF-β) (Lorimore et al. 2001; Apetoh et al. 2007). Danger signals are received from the dying tumor cells that leads to DCs maturation followed by phagocytosis which initiates cytotoxic activity by T-cells at local and distant sites. Sufficient release of these danger signals by dying cells is a prerequisite for detection by DCs to initiate a suitable immune response (Apetoh et al. 2007). Moreover, interactions between unirradiated and irradiated

hematopoietic cells via danger signals have also been reported to occur by way of a bystander mechanism producing genomic instability (Lorimore et al. 2001). Activated macrophages initiate this response by producing ROS which mediate clastogenic factors capable to induce gene mutations, DNA base modifications, DNA strand breaks, and damage in nearby cells.

Toll-like receptors (TLRs) are a type of membrane bound pattern recognition receptors (PRP) on APCs which detect danger signals such as Pathogen associated molecular patterns (PAMPs) from microbes and damage associated molecular patterns (DAMPs) released from dying or damaged tissue (Brandmaier and Formenti 2020). Once these signals initiate inflammatory processes, innate and adaptive immune responses coordinate as APCs are activated to secrete pro-inflammatory cytokines (IL-12, IL-18) (Krutzik et al. 2005; Shan et al. 2007). Humans have ten characteristic TLRs (TLR 1–10), where TLR2, TLR4 and TLR9, can bind to a common DAMP making them relevant to an innate immune response triggered by LDIR (El-Saghire et al. 2013). Tumor irradiation (2-20 Gy) also generates danger signals DAMPs from dying cells which are critical for DC maturation (Golden et al. 2014). Once matured, DCs uptake and process antigens to cross-present them to naïve T cells in a tumor-draining lymph node. A high concentration of these antigens is needed for successful cross-presentation, optimum radiation will ensure that a sufficient quantity and quality of danger signals are released to initiate an appropriate cytolytic T-cell activity that exit the lymph node (Spiotto et al. 2016). The characteristic release of DAMPs that triggers a radiotherapy induced immune response as tumor cells, specifically DNA damage, is termed as immunogenic cell death (ICD)

(Rapoport and Anderson 2019). ICD is different from classic apoptosis which is immunoquiescent and does not release chemical signals to initiate an immune response. ICD is typically observed upon irradiation of various tumors with the involvement of three molecules as listed below (Apetoh et al. 2007).

1) Calreticulin (CRT) is a protein attached to the inner membrane of endoplasmic reticulum (ER) under normal conditions. When ER stress is induced by radiation, CRT translocates externally to the surface of damaged cell where it must emit death signals in order to trigger an efficient immune response (Apetoh et al. 2007). A study reported a higher incidence of type 1 CALR mutation (gene that code for CRT protein) in cancer patients' genomic profile who were exposed to IR (clean-up workers: 20 – 500 mSv; permanent residents: 5.9 – 31 mSv) after the Chernobyl accident (Poluben et al. 2019). Mutant CRT triggers the JAK/STAT signaling pathways (Klampfl et al. 2013; Milosevic Feenstra et al. 2016), which has great implications for both immune-stimulatory and immune-suppressive pathways (Owen et al. 2019).
2) HMGB1 is a chromatin protein within nucleus which was also identified as one of the first radiotherapy induced inflammatory molecule and its depletion reduced RT success (Dar et al. 2018). HMGB1 transforms tumor environment into an "acute inflammatory" tissue via cytokine release which activates the adaptive immune response (Liao et al. 2020).
3) Adenosine triphosphate (ATP) release from irradiated tumor recruits DCs which activates CD8+ T-cell responses (Golden and Apoteh 2015)

The three molecules discussed above contribute to recruitment and maturation of DCs and allow them to cross-present tumor-antigens to activate innate and adaptive immune components (Portella and Scala 2019). Golden and Apoteh (2015) proposed that this immune response transforms the malignant cells into endogenous in-situ tumor vaccine. The current literature largely focuses on CRT, HMGB1 and ATP's response at radiotherapeutic high doses (>10 Gy) with the exception of one study that discusses HMGB1 at low dose (El-Saghire et al. 2013).

Tregs and Bregs

T regulatory cells (Tregs) perform diverse functions and regulate the immune and non-immune cells' population (Schaue et al. 2012). Tregs dampen an overzealous immune response which prevents an attack on self (Mauri and Ehrenstein 2008). They are also involved in tumor progression while excessive radiation induced depletion may lead to chronic inflammation, autoimmune and allergic reactions (Schaue et al. 2012). Considered as one of the most common immunosuppressive cells in the tumor microenvironment, they have been reported to encourage tumor growth (Shitara and Nishikawa 2018). Hence, a paradox exists with respect to radiation effect on these cells as both beneficial regulatory and tumor promoting functions are reported. A study retrospectively analysed biopsy samples from rectal cancer patients who underwent RT (30Gy/10 fraction) and reported a high proportion of Tregs which was negatively correlated with prognosis (Ji et al. 2020). It was proposed that Tregs inhibit APC's maturation by increased expression of high-affinity CD25 receptors that binds to IL-2 and release of inhibitory cytokines (such as perforin) that destroys effector T-cells and APCs

(Ji et al. 2020). Reduction in Tregs activity leads to an improved anti-tumor immune response and targeting their subpopulations holds considerable potential in immune-radiotherapeutic strategies (Li et al. 2003). On the other hand, Tregs suppress auto-reactive T-cells that escape the process of negative selection i.e., elimination of cells with no surface markers (Cao et al. 2009). Brandmaier and Formenti (2020) states tumor irradiation leads to an increased expression of TGF-β, which promotes T regulatory (Treg) cells and hypoxia inducible factor 1 subunit α (HIF-α) function. HIF-α triggers myeloid derived suppressor cells (MDSCs) cells activation and induces M2 phenotype in macrophages which initiates proinflammatory response, as well as tumor progression. Tregs are more radioresistant compared to other effector T-cells as their number was markedly increased in the peripheral blood and thymus after local irradiation suggesting an abscopal effect involving immune components (Qu et al. 2010; Kachikwu et al. 2011).

Similar to Treg cells, B regulatory cells (Bregs) regulate the functions of B-cells with the release of suppressive cytokines such as IL-10 and TGF-β in the tumor microenvironment (Mauri and Ehrenstein 2008). A study by Li et al. (2019) reported a significant increase in Breg sub-sets (CD19+, $CD24^{hi}$, CD27+) when blood samples were recollected from 24 nasopharyngeal cancer (NPC) patients post radiotherapy (66 Gy). The cytokine expression profile and ROS release remained vague for Bregs compared to other plasma B cells investigated in this study. Research on ionizing radiation (both low and high) effects on Bregs is essentially non-existent, but their role in immunotherapy and nuclear medicine therapy has gained a lot of recent interest (Jiang et al. 2013; Chen et al.

2019). Expanding on this knowledge may assist in future research on radiation modulation in Bregs.

Cytosolic DNA Sensor: cGAS–STING Pathway

If found outside the nucleus, damaged DNA fragments in the cytoplasm pose danger which triggers release of interferons initiating an innate immune response. Cyclic guanosine monophosphate-adenosine monophosphate synthase- stimulator of interferon genes (cGAS-STING) detect cytosolic DNA fragments released in response to ionizing radiation forming micronuclei outside the nucleus (Brandmaier and Formenti 2020). In-vitro x-irradiation (1 Gy), led to frequent association of cGAS with micronuclei with a proinflammatory response as seen by elevated levels of cytokine (CCL5) production (Mackenzie et al. 2017). Additionally, this study also investigated if cGAS pathway and cytokine release was sufficient to activate innate immunity by comparing cell-cycle-arrested with actively cycling mouse fibroblasts post irradiation. The presence of functional cGAS signalling that generated similar amounts of cytokine in cell-arrested fibroblasts compared to cycling cells was observed but did not lead to micronuclei aggregate or innate immune activation, despite the DNA damage suggesting cell-cycle kinetics during mitosis are a crucial consideration. A typical radiation induced cGAS pathway initiates with catalysis of cyclic GMP-AMP, a second messenger which binds and activates STING on the ER membrane (Brandmaier and Formenti 2020). Next, transcription factors NF-kB and interferon regulatory factor 3 (IRF3) are upregulated with an increase in type 1 IFN expression which recruits DCs into the tumor microenvironment initiating cytolytic T-cell response. Ablasser et al. (2013) stated that

cGAMP may migrate via inter-cellular gap junctions to stimulate the STING pathway in bystander cells. A study by Liang et al. (2017) declared cGAS/STING activation by radiation as "double-edged" as STING agonist promotes IFN production leading to a favorable anti-tumor immune response on one hand, but on the other, if IFN levels remain chronically high, it leads to an immunosuppressive environment. Authors suggested the combined effect of immune-radiotherapy in alleviating immunosuppression and radioresistance in mice tumor where novel strategies such as targeting STING agonist functions may prove to be valuable.

1.8.7. Myeloid Derived Cells: Dendritic Cells, Macrophages, Neutrophils and Myeloid Derived Suppressor Cells (MDSCs)

Radiotherapy allows for dendritic cells (DCs) maturation which enables them to cross-present tumor antigens to the cytotoxic T-cells in the tumor draining lymph node (Turgeon et al. 2019). This is accomplished by various cytokines release in the tumor microenvironment that initiate DCs recruitment followed by proliferation and priming of cytotoxic T-cells (Burnette et al. 2011). Radiotherapy (15 Gy) can induce vascular or lymphatic alterations which lead to an increased number of DCs from the tumor site to the draining lymph nodes forming conduits which greatly enhance immune responses (Lugade et al. 2005).

Macrophages, dendritic cells, neutrophils and MDSCs originate from the same myeloid precursor in the bone marrow where radiation may modulate variable phenotypes of macrophages and neutrophils (Vatner and Formenti 2015). Similarities have been reported between neutrophils and MDSCs and it is unclear if these are separate or same

population complicating the understanding of these cells' function. Although, ample data on radiation induced effects of macrophages is available which makes them one of the widely researched cells with potential overshadowing of radiation effects in neutrophils. Nonetheless, neutrophils have shown potential since the recognition of their role in cytokine release with possible antigen presentation functions (Silva 2010). Schernberg et al. (2017) stated neutrophils' "double-deal" as radiotherapy induces either an anti-tumor N1 phenotype that occurs in the presence of IFN-β or an N2 pro-tumor phenotype which is stimulated by TGF-β upregulation. Calabrese et al. (2019) reports evidence in the literature where macrophage polarization between two phenotypes dependent on radiation dose induces a M1 (pro-inflammatory) state at doses above 1 Gy and M2 (anti-inflammatory) state at doses below 1 Gy. Interestingly, Ruffell and Coussens (2015) describes the macrophage polarization system as a continuum contrary to a commonly accepted binary categorization of pro- or anti-inflammatory. Authors suggests a possibility that radiation may act as an external stimulus that puts macrophage polarization into a spectrum of functional phenotypes rather than a fixed pro- or anti-inflammatory state.

Teresa Pinto et al. (2016) simulated radiotherapy clinical dose (10 Gy) in human macrophages to investigate various bio-chemical and morphological responses like NF-κB signaling pathway, polarization, phagocytosis, and cancer promoting properties. Irradiated macrophages were found to linger in the tumor microenvironment supporting tumor invasion and angiogenesis after radiotherapy where non-irradiated macrophages were also capable of accomplishing the same actions which raised concerns about

treatment efficacy. Possible reasons for this response include sustained DNA damage originally induced by radiation, upregulated metabolic activity and pro-survival pathways mediated by NF-κB. Moreover, although macrophages exhibited a pro-inflammatory phenotype, but they did not qualify for a classical pro-inflammatory profile as cytokines such as IL-1β, TNF-α and IL-6 were not observed.

MDSCs are a heterogeneous population of immature myeloid cells that fail to differentiate and suppress activation of T-cells (Youn and Gabrilovich 2010). Radiation induces migration of MDSCs to tumor site where they can exhibit variable response (Vatner and Formenti 2015) where physiological conditions such as hypoxia and pH levels regulate their survival at the tumor site (Youn and Gabrilovich 2010). Yin et al. (2019) reports several crucial MDSCs pathways such as STAT signaling pathway NF-κB that inhibit T cells, B cells, NK cells, and DCs whereas tumor associated macrophages, Tregs, Th17 cells, as well as angiogenesis and tumor spread exerting a “yin-yang" effect. Small cytokine molecules called chemokine ligand 5 (CCL5) trigger their recruitment into tumor microenvironment, where MDSCs suppress the CD8+ T-cell function by downregulating T-cell receptors (Hemmatazad and Berger 2021). MDSCs secrete IL-10 and TGF-β which hinders an efficient antitumor immune response as activity of effector T cells is reduced and Tregs are recruited (Fleming et al. 2018). The illustration below consolidates the discussion above (Figure 4).

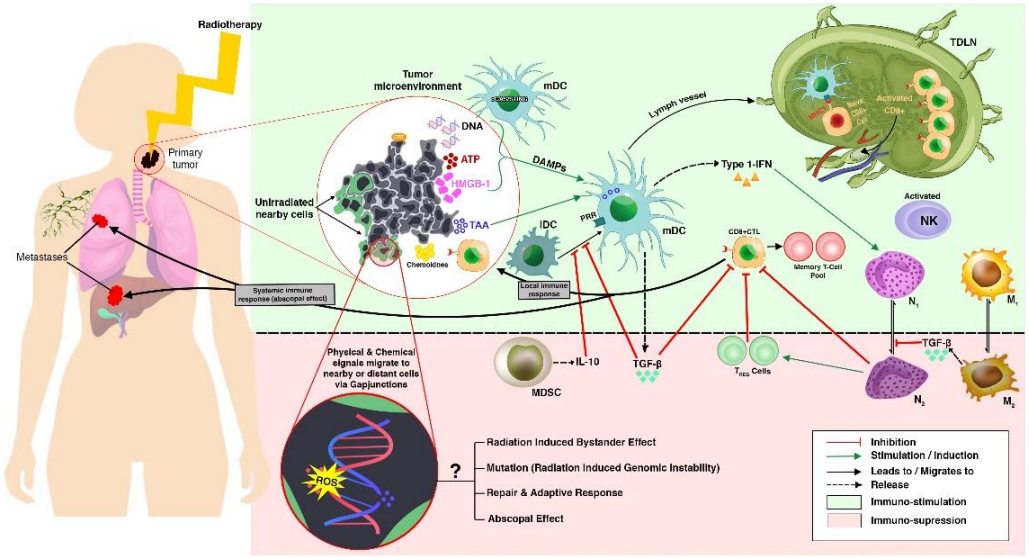

Figure 4: Moderate-to-high dose radiotherapy induced effects in the immune system. Irradiated tumor cells release molecules such as cytokines or danger signals that initiate antigen presentation by dendritic cells as they migrate to tumor draining lymph node where CD8+ T-cells are activated for cytolytic activity at local and distant tissue. Damage-associated molecular patterns (DAMPs), Tumor-associated antigens (TAAs), Interferon type 1 (IFN), Pattern recognition receptor (PRR), Regulatory T-cells (Tregs), inactive dendritic cell (iDC), Mature dendritic cell (mDC), High mobility group box 1 (HMGB1), Tumor growth factor beta (TGF-β), Natural killer cells (NK), Myeloid derived suppressor cells (MDSCs), Reactive oxygen species (ROS), Tumor draining lymph node (TDLN), Interleukin-10 (IL-10), Cytotoxic T-cells (CTLs), Neutrophils (N1), Macrophage (M1).

1.9. Radiation induced immune effects in humans

The non-linear responses that occur in biological tissue as a result of non-targeted effects of radiation warranted re-evaluation of the LNT model where involvement of immune system also contributes to deviation from linearity. A study reviewed 52

immunological endpoints where low dose radiation either upregulated the favorable responses or downregulated the unfavorable responses which led to an inverted J- shaped or a J shaped curve respectively (Liu 2003). The fact that radiation responses in biological tissue occur in more ways than the previously believed notion of only direct cell hit has gained unanimous acceptance from all in the field of radiation biology. However, there is some conflict about the right model to adequately explain the NTEs that predominate at low doses with variable responses at different dose magnitudes. Calabrese and Baldwin (2002) proposed a U-shaped curve characteristic of hormesis effect where stimulation is achieved at low, and an inhibitory effect prevails at high doses. Although mathematical equation like linear quadratic (LQ) may satisfy the LNT response (Pawel and Boyd 2019), there are still concerns about lack of models that can sufficiently account for the in-vivo deep mechanistic and physiological underpinnings including effects that are vascular and immunological in nature (Finkelstein et al. 2011). Furthermore, different radiosensitivities of cells and tissues makes it challenging to understand the effects that vary with "radiation dose, delivery methods on systemic and locoregional naïve, effector or regulatory T-cells" leading to different rationalizations that currently lack clarity (Demaria et al. 2005; Finkelstein et al. 2011).

Blood transfusion is the only treatment that employs total body irradiation (TBI) in humans with an aim to repress the immune system in preparation for an organ transplant (e.g., kidney, bone marrow). A dose of 3.5 to 4.5 Gy for TBI is used to avoid an immune reaction against the new antigen, although this dose may be less effective in cases where a patient is already sensitized to an antigen (Hall and Garcia 2012). A very

common type of this immune reaction is termed as the graft versus host disease (GVHD) which increases post-transplant morbidity and mortality as its management options are currently limited (Manjappa et al. 2019). Lymphocytes within the donated graft recognizes the recipient host as foreign which triggers an immune response in the host. To prevent this, irradiation of the blood products prior to transfusion has proven beneficial, specifically for patient population at risk of GVHD. Ejected electron as a result of ionization damages the lymphocyte DNA, making it incapable of proliferation in the graft tissue which allows for successful transfusion in the host (Francis 2019). Pritchard and Shaz (2016) emphasizes that transfusion candidates that are human leucocyte antigen (HLA) matched or receive donation from immediate blood relatives must have the transfusable products irradiated as they are at a high risk of GVHD due to the HLA similarity that allows donor lymphocytes to proliferate and attack the host.

1.9.1. Occupational data

An epidemiological study assessing the radiation risk in British radiologists over the period of 100 years (1897–1997) reported a decrease in cancer incidence after exposure to low levels of occupational doses of radiation (Berrington et al. 2000). The study findings suggest a possible radio-adaptive response, however, “healthy worker effect” must also be accounted for, a term coined by McMichael (1976) who defined it as a natural tendency of being active and healthy when employed resulting in a favorable mortality status compared to the unemployed population. Alternatively, there is also evidence that suggests an increased cancer risk due to medical use of radiation where safety guidelines must be re-evaluated with new radio-biological understanding in the recent years. A study

that investigated occupational hazard in X-ray technicians found atypical changes in the lymphocyte morphology (Mohammed et al. 2013). The small sample size (47 subjects) of this study, however, weakens the generalizability to a wider population. A similar study done on X-ray technicians confirmed impaired phagocytic function in blood as well as isolated polymorphonuclear neutrophils (PMN) compared to controls (Meo et al. 2006).

1.9.2. Psycho-emotional factors

A relatively understudied factor in radiobiology is the effect of psycho-emotional influences on the immune system and how they exacerbate radiation stress. Wang et al. (2016) states that humans are at an inevitable risk of both psychological and radiation stresses as society has become extremely competitive putting many strains on an individual. All life forms get exposed to constant natural background radiation from cosmic, terrestrial, and internal sources. Some population may be at high risk due to their location, adopted occupation, lifestyle, socio-economic status and health conditions e.g., nuclear facility workers, miners, radiology workers, patients undergoing radiotherapy, astronauts, airplane crew, residents near the sources of the high radiation such as nuclear power plants (UNSCEAR, 2000). For instance, a patient undergoing radiation therapy is bound to experience high levels of anxiety due to fear of the disease severity and apprehensions about prognosis in addition to the direct inevitable hazards from radiation. Wang et al. (2016) reviewed such stressors which lead to an additive effect bolstering radiation damage in humans. This group declared work of Feng et al. as a “milestone” in simultaneous investigation of psychological and radiation stress related effects. Feng et al. (2012) induced chronic stress by immobilizing mice and observed that radiation (4 Gy)

induced attenuation of p53 function which is a crucial tumor suppressor protein. Elevated glucocorticoid levels (stress hormones) mediated this suppression of p53 activity suggesting endocrine involvement. Glucocorticoids prevent an excessive inflammatory immune response where their role is also implicated in radio adaptive response in-vivo following chronic low dose exposures (Nenoi et al. 2015).

Ilderbayev et al. (2020) investigated the combined effect of emotional and radiation stress on immunological reactivity which is considered one of the most radiosensitive functions in human and animal species. Authors shone a bright light on immobilized mice after irradiation (6 Gy). The results reported following pathological processes as a result of exposure to two combined stressors: the absolute reduction in the CD3, CD4+/CD8+ T-cells, decrease in leukocytes and mononuclear phagocytic system's function and failure of switch from IgM synthesis to immunoglobulins A and G (IgA and IgG) due to an increase in CD19+ (B-lymphocyte surface antigen). The decrease in IgA and IgG levels leads to immunodeficiency classically accompanied by chronic inflammation which allows for a conducive environment for cancer. It was reported that radiation took dominance in the effects reported in this study and possible involvement of NTE mechanism via intercellular interaction or by way of some "secret" factors which can transmit to a distance was also mentioned. Currently, there is not enough investigations on the combined effects of radiation and psycho-emotional impacts on immune system and the references mentioned above used moderate-high doses of radiation applicable more in a clinical or nuclear power setting. Exploring this branch of radiobiology may provide valuable insights in the assessment of health effects in a

holistic way where qualitative factors like stress and anxiety are also considered. This will improve radiotherapy outcomes and community health at large.

1.9.3. Epidemiology from nuclear plants and accidents

Earlier epidemiological data showed an increase in the incidence of leukaemia in children exposed prenatally as their mothers received radiation from nuclear sources, but this finding did not corroborate with results of Japanese children exposed in-utero to radiation from Hiroshima and Nagasaki disasters (Devi 2003). One possible reason for this discrepancy could be the fatal nature of leukaemia where most Japanese children did not survive, or due to the inability of researchers to conduct immediate studies as resources and politico-economic situation in the country was not favorable (Devi 2003). Researchers surmised the acute effects that might have occurred in those exposed to radiation from A-bombings (Akiyama 1995). A rapid decrease and delayed recovery of lymphocytes (Snell 1949) that impacts production of various regulatory cytokines (Bogdandi et al. 2010) interferes with immune functions such as antigen recognition by T-cells and antibody production by B-cells, as well as the activation of neutrophils and monocytes. Additionally, humoral factors, such as antibodies and complements (Kimura et al. 1953) subsequently decrease with the declining number and function of neutrophils and monocytes (Ohkita 1975).

Soon after nuclear war, it became imperative to address health concerns that occurred in staff who worked in atomic laboratories or nuclear power plants (especially the clean-up and the decommissioning staff). Jacobson and Mark (1947) drew attention to the lack of experimental and clinical data regarding acceptable dose limits that could be

applied to individuals who work in such environments. Although evidence from earlier literature quantified clinical signs such as lymphocytosis, monocytosis, eosinophilia, or leukopenia in the peripheral blood as chronic effects in radiation workers but these studies were poorly controlled where individual variation, small sample size and lack of advanced methods to measure dose confounded results. (Murphy 1926; Wetterer 1922; Aubertin and Beaujard 1908; Linser and Helber 1905; cited in Jacobson and Mark 1947). It was realised that scientifically sound and reliable hematological studies that evaluated acute and chronic effects to nuclear workers consistently exposed to radiation are vital to ensure timely management of radiation injuries. Current guidelines by International Commission of Radiation Protection (ICRP) puts an annual occupational dose limit of 50 mSv for the whole body or trunk of body for radiation workers and 1 mSv for general public (Wrixon 2008; Bartal et al.2021). Calabrese (2021) suggests that key features from models like threshold, hormesis and LNT should be incorporated at the time of developing radiation protection guidelines. This will reduce uncertainty associated with exclusive use of the LNT model, which extrapolates data from animals to human responses for chronic doses of radiation which may not be appropriate.

Individuals within the 2500-meter perimeter of the hypocenter receiving approximately less than 100 rad who later immigrated to the US were compared with individuals located more than 2500-meter receiving zero rad (Bloom et al. 1983). Statistically significant difference was observed in cell-mediated cytotoxicity where individuals who were closer to the hypocenter suggesting a possible radio-adaptive effect. This study selectively recruited subjects who currently reside in the U.S. and perhaps

received very low doses for shorter periods of time whereas Japanese who received higher doses were perhaps not able to leave the country due to acute radiation induced conditions or other co-morbidities. Age of subjects at the time of exposure may also modulate radiation and immune responses as age-related changes occur in the thymus which mimics responses similar to when this organ is irradiated. A study by Kusunoki et al. (1998) found decrease in CD4+ T-cells with no change in CD8+ T-cells in the older A-bomb survivors where T-cell production was likely affected due to age related degradation of thymus as these cells were not replenished efficiently compared to younger survivors. This introduces an age-dependent confounding variable. The study also reported an increase in B-cells subsets (CD5+, CD5-, CD23+ and CD23-) which was linked with radiation induced B-cell proliferation from stem cells instead of peripheral B-cell activation, the reason for this effect was unclear. Imbalance in the T helper (Th) subsets, i.e., Th 1 and Th 2 cells may be associated to elevated B-levels. Radiation may cause damage to mature lymphocytes in both young and old survivors, but repair pathways differed in the two age groups (Akiyama 1995). In addition to deterioration associated with age, elevated levels of CD4- and CD8- T cells in the peripheral blood may also impact functioning of the thymus. CD4- and CD8- T cells escape the negative selection process which ensures elimination of cells that express neither CD4 nor CD8 surface marker. Another study by Osajima and Tomonaga (1960) collected data from the year 1956 (eleven years after the bomb dropping in Nagasaki) where blood work from A-bomb survivors showed restoration of immune system dysfunction such as anemia, eosinophilia, impaired haemoglobin levels etc. This agrees with a report of Belsky et al.

(1973) who compared morbidity and mortality in individuals exposed to A-bomb radiation to the one who were not present (unexposed) at the time of explosion. They found that A-bomb survivors and the non-exposed groups were in good physical health but risks of delayed radiation effects cannot be ruled out, especially for those who were children at the time of exposure.

Wall et al. (2006) reviewed several radiobiological studies (Wolff et al. 1988; Olivieri et al. 1984; Pohl-Rueling et al. 1983) and reported lack of validity and reproducibility. The main claim in these studies was about the beneficial effects at low doses where prior exposure to low dose of radiation primes the human lymphocytes as they become more tolerant of the radiation injury (chromosomal damage) on next exposure. Although support for adaptive responses did exist in the above studies, serious limitations were identified later (Wojcik et al. 1996). The major concern was related to the scoring of aberrations performed on asynchronous lymphocytes manifesting different radio sensitivity at a different cell cycle phase which may skew results' interpretation.

Data on radiation effects on the human immune system was mostly derived from nuclear disaster and accident survivors, individuals occupationally exposed to radiation and those undergoing radiotherapy (Mosse 2012). However, a lot of in-vitro lab work on normal and cancerous human cell lines has provided some valuable insights into radiation induced immune effects. An increased cancer and leukaemia risk has been established as one of the late radiation effects in Nagasaki and Hiroshima's atomic bomb survivors (Folley et al. 1952; Hsu et al. 2013; United Nations Scientific Committee on the Effects of Atomic Radiation 2017). Radiation risk for acute moderate to high doses are well

documented in the literature from both epidemiological and experimental studies, whereas data on chronic low-dose effects remain elusive. A standard approach for radiation risk assessment at LDIR is by back-extrapolating responses from high doses depicted in linear model (Laurier et al. 2017). There have been concerns about the appropriateness of this approach as LDIR effects largely remain uncertain (Joiner 2001, Liu 2003; Calabrese 2005). Nakanishi et al. (1999) reported a significant difference in chromosomal aberration observed in A-bomb survivors exposed to a dose of less than 1 Gy (6.2 aberrations per leukemia) compared to non-exposed controls (2.6 aberrations per leukaemia). Lord (1999) presented an interesting hypothesis after investigating the cause of increased incidence of childhood leukaemia and non-Hodgkin's lymphoma in a region close to a Sellafield nuclear reprocessing plant. Contrary to a commonly accepted mechanism of radiation induced bone marrow changes and chromosomal instability, these conditions were considered to be a result of viral infection which increased with population mixing. A similar study in Seascale (a village close to Sellafield) reported a correlation between high incidence of leukaemia in children born to fathers who worked at Sellafield before their child's birth (Gardner 1991). A review by Dubrova (2003) reviewed epidemiological data that showed increased cancer incidence in the offspring of irradiated parents due to high mutation rate in their germline and somatic tissue. Previous researchers reported that high concentration of free radicals in the somatic cells cause such molecular events that lead to RIGI (Clutton et al. 1996). However, this might not be true for sperm cells which has very little cytoplasm content and may not be able to carry free radical species to zygote, suggesting that damage occurs in the DNA of the exposed

father (Dubrova 2003). Altered gene expression, epigenetic events such as DNA methylation and chromatin condensation were suggested to be putative mechanisms for RIGI in the unirradiated offspring. These studies motivated researchers internationally to investigate health risks in their areas within the close proximity of nuclear power plants where an investigation on 20 power plants in Germany reported a high incidence of acute leukemia (Relative risk: 4.07, Confidence interval: 1.33-12.45). These cases were mostly reported for children under the age of 15 who lived within a 5 km radius of Krummel Power Plant compared to the control population while the rest of nuclear installations showed a relative risk (incidence) around 1 (Spix et al. 2008).

Marozik et al. (2007) determined the influence of bystander/clastogenic factors from serums of Chernobyl accident survivors on HPV-G cell line (human keratinocyte cell line immortalized by transfection with human papilloma virus making the cell p53 deficient). The serum donor survivors were divided into groups based on dose received, liver cirrhosis and acute viral infection-stricken patients residing in contaminated areas. This was done to ensure accurate measurement of bystander signal strength and avoid quantification of signal production and response in the same genotype. An interesting finding was the highest number of micronuclei in cells treated with a serum from patients with acute viral infections compared to other groups. It was hypothesized that RIBE may be correlated to the level of simultaneous pathological process accompanied with elevated levels of oxidative stress which may significantly influence this response, also posing a risk of RIGI in somatic cells. Alternatively, Sutou (2018) reports that chronic LDIR received by A-bomb survivors in Japan demonstrated an increased lifespan and reduced

average cancer mortality. An interesting comparison was drawn with inhaled oxygen and was considered more detrimental than LDIR as oxidative phosphorylation leads to a higher number of ROS putting a higher strain on the body's defense system compared to what is released as a result of radiation. Currently the data is conflicting for LDIR induced effects in the biological tissue and on balance, it may shift more to being detrimental rather than beneficial. This explains the universal acceptance of the LNT model for the radiation risk assessment that considers no dose of radiation as safe, let alone the beneficial effects on the human immune system. Substantial evidence exists to support the radiation induced modulation of immune function, as well as non-immune responses in biological tissue occurring at variable intensities, which may lead to interesting benefit vs. risk identifications paving the way for valuable discoveries.

1.9.4. Low dose radiotherapy for SARS-CoV-2

Recently there has been considerable renewed interest in using LDIR for COVID-19 as it has been used in the past for the treatment of skin disorders, infectious and other inflammatory diseases (Pusey and Caldwell 1903; Mottram and Hill 1949; Crossland 1956; Calabrese et al. 2019). A mortality rate of 13-25% on day 28 for a severely ill COVID-19 patient is higher than any known cancer where radiotherapy is frequently used, employing much higher doses than what is recommended for COVID-19 (Hanekamp et al. 2020). The pneumonia associated with SARS-CoV-2 leads to a devastating condition with a hyper-inflammatory state, known as a cytokine storm, characterised by increased levels of cytokines such as IL-6, TNF-α, IL-1β, IL-8 and IFN-γ (Hanekamp et al. 2020). IL-6 is the primary constituent of this cytokine storm which

may be downregulated by LDIR <1.5 Gy exerting a potent anti-inflammatory effect. Concerns such as the possibility of suppression of the host's own immunity leading to a worsened patient's condition and a lack of understanding on interaction between radiation and the SARS-CoV-2 virus currently limits its use where LDIR may induce virus activation and replication that strengthens its spread. (Kefayat and Ghahremani 2020). Nevertheless, there is emerging support for use of LDIR (0.3-1.5 Gy) for COVID-19 (Prasanna et al. 2020; Salomaa 2020). A thorough investigation is required into the pathophysiological aspects of the disease by examining autopsy reports of patients who died due to COVID-19 (Maiese et al. 2020).

The temptation to quickly reap the foreseeable benefits of a medical discovery may lead to adoption of a heuristic approach overlooking the finer mechanistic details of a disease process. Therefore, it is critical not to rush the developmental stages of gaining an in-depth understanding of these mechanisms before any attempts are made on human subjects. This would avoid accidents like the failure of an immuno-modulatory drug (TGN1412) which led to devastating consequences costing human lives (Attarwala 2010). Humanized CD28 superagonist TGN1412 was infused to 6 patients, who experienced an adverse reaction which resulted from rapid cytokine release from activated T-cells. Although sufficient preclinical data was available, factors such as the appearance of low-level cytokine release in primates, insufficient in-vitro human studies, choice, and the interval of dose delivery between subjects were neglected. With the advent and availability of FDA approved vaccines, there is less interest in radiotherapy and its use was discouraged (Das et al. 2020). However, in the case of an end stage scenario of

COVID-19, radiotherapy might still be of value in reversing a poor prognostic status (Kumar et al. 2021).

1.9.5. Low dose radiotherapy for pneumonia

Scott (1939) used x-rays for acutely ill pneumonia patients where the length of patient's stay decreased, body temperature returned to normal, and complications reduced in 54 out of the 88 cases that received radiotherapy (200 r). The author supported radiotherapy for pneumonia and showed concern that it was ignored by other clinicians, despite favorable patient outcomes. This seems like a suggestion that lacked prudence as it overlooks many complex factors at play that must be considered in order to exploit the beneficial effects of LDIR. Moreover, the study used a small sample size of 88 patients with no mention of consent and ethical board approval. A lack of systematic, blinded, and modern randomization technique was also not followed. A comprehensive report on how radiotherapy was historically used to treat pneumonia suggests that LDIR may suppress inflammatory responses with widespread therapeutic avenues (Calabrese and Dhawan 2013).

1.9.6. Combined radio-immunotherapy

The combination of radiation and immune therapy has revolutionized the cancer care where exciting possibilities have been projected by great many reviewers (Turgeon et al. 2018, Formenti and Demaria 2009, Formenti and Demaria 2013; Rodríguez-Ruiz et al. 2018). It must be noted that it is not a simple combination of two treatment modalities where each eliminates tumor in a canonical manner, but instead it is optimization of a

great network of reactions occurring at the sub-cellular level. Various examples of immune modulating drugs were reported in this review, where the most commonly accepted and widely employed approach is immune checkpoint inhibitors (ICI), such as the cytotoxic T-lymphocyte antigen 4 (CTLA-4) blockade that is used with radiotherapy (Dewan et al. 2009). Researchers are now exploring to target other immune components where prior priming may significantly enhance radiotherapy outcomes. One such target, although still unclear, is anti-CCR2 antibody that inhibits MDSC infiltration induced by radiotherapy. CCR2 is a C-C chemokine receptor for monocyte chemoattractant protein, expressed on the surface of MDSCs which mitigate the immunosuppressive effects triggered by STING pathway (Liang et al. 2017). Additionally, Formenti et al. (2018) stated that the dual effect demonstrated by TGF-β upon irradiation makes it hard to target this molecule solely through radiotherapy and combining the treatment with immunotherapy becomes an absolute imperative. They investigated TGF-β blockade during radiotherapy (cumulative dose 22.5 Gy) where patients that received this treatment regimen in high dose (10 mg) showed favorable systemic immune response with an overall increase in survival compared to the lower dose group (1 mg) and an improved CD8 central memory pool. The authors did state that a small sample size (23 patients) and relatively short survival time raise some concerns about the efficiency of TGF-β blockade which may not be able to control tumor growth even in conjunction with radiation. The concept of transforming a tumor site into an in-situ vaccine has gained considerable attention as it elicits a systemic anti-tumor abscopal effect when radiotherapy is combined with single or multiple immunotherapeutic tools (Rodríguez-Ruiz et al. 2018). However,

researchers are still struggling to determine optimal radiotherapy technical factors (dose plan and schedule) that will be cytotoxic to the tumor and not damage the surrounding healthy tissue. This thesis maybe a good starting point to analyse the connections and converging points of immunology and radiobiology that will have bearings for the field of oncology.

1.10. Immune effects in non-human species

Protochordates and primitive chordate such as fish are at the junction in evolution where adaptive immune system began to develop (Litman et al. 1993; Smith and Davidson 1994). The earlier reports of immunoglobulins in fish that were homologous to those found in the mammalian immune system were presented by Shamblott and Litman (1989). Müller et al. (1999) demonstrated, for the first time, that sponges are capable of both innate and adaptive immune responses when they presented evidence for the existence of important immune signaling molecules like cytokine. The same group suggested that in addition to cytokines, components like the cell adhesion molecules (CAMs) and immunoglobulins (Igs) were also observed in sponges (Müller 2001; Müller et al. 2004). They proposed that sponges may be good model organisms to investigate origin of vertebrate immunity and associated immune responses. Simple chordates like tunicates, ascidians in particular, have attracted a lot of interest recently as they are considered ideal models for innate immunity investigations within the context of evolutionary events that resulted in invertebrate–vertebrate transition (Franchi and Ballarin 2017). A typical example of this is the emergence of lymphocytes and diversification in receptors via somatic recombination in protochordates such as tunicates

(Litman et al. 2005). Inflammatory response triggers cytotoxic and phenoloxidase (PO) containing cells to the site of damage where granules, cytokines, complement factors and PO are released (Franchi and Ballarin 2017). The enzyme PO acts on a polyphenol substrate which results in elevated levels of ROS. Contrary to common perceptions that invertebrate cytokines share no homologs with vertebrates, genes responsible for cytokines (IL-1 and TNF-α) receptors were found in the genome of tunicates and hemocyte recruitment to the site of inflammation via cross-talk is also reported (Nicolò et al. 2010). Damage decrease (micronuclei formation, comet parameters and DNA fragmentation) in human lymphocytes was observed upon irradiation (4 Gy) after pre-treatment with Dendrodoine analog (DA) which is extracted from tunicates (Kalpana et al. 2010). Colonial organisms like tunicates may release cytotoxins or other signals in a stress-inducing environment as a defence response against non-self colonies (Rinkevich and Weissman 1992). These signals were proposed to have similarities to a radiation induced bystander signaling response (Mothersill and Seymour 2013).

The concept of danger signals also exists in human and animal models where these signals may be biphasic in nature as they were seen to be involved in pathogenesis promotion as well as radiation repair mechanisms (McBride et al. 2004). They also migrate from where the initial damage took place to a distant site and contribute towards a bystander or a systemic abscopal effect as they trigger innate and adaptive immune responses. The difficulty in determining the spatial and temporal orientation of these signals make it hard to isolate the pathways that they facilitate or participate in (McBride et al. 2004). Furthermore, variety of complex interactions at the cellular level within each

tissue and intrinsic cellular radiosensitivities also adds to this difficulty. The presence of extracellular matrix, cell adhesion molecules (CAM), stem cell content and other physiological factors also interfere with how these signals release and respond to radiation (McBride et al. 2004). A lack of literature was found where only one recent review specifically looked at bystander and danger signaling (Nikitaki et al. 2016), warranting future research focusing on LDIR induced danger signals and how they interact or associate with bystander response. Species like tunicates and pre-clinical models may be a good starting point. A study irradiated posterior pharynx bulbs and tails of tunicates which led to an increased germ cell apoptosis, DNA damage in bystander gonads as well as genomic instability in the unirradiated F1 progeny (Guo et al. 2013). DNA damage-induced germ cell death machinery and MAPK signaling pathway were suggested as underlying mechanisms for germ cell apoptosis from the irradiation of somatic cells. Bertucci et al. (2009) exposed caenorhabditis elegans (C.elegans) with 3 MeV protons targeted to the tail as different organs and cell populations are located in this region, triggering a local and distal expression of heat shock proteins (HSPs) which play a critical role in the activation of innate immune responses in certain diseases. This study did not clearly suggest an immune enhancing or suppressive effect in C.elegans, but further research on this species may provide useful insights that may be translated to in-vivo mammalian models.

Mothersill and Seymour (2009) outlined NTEs such as the RIGI and RIBE observed in many fish studies conducted in-vitro and in-vivo. These effects may transmit via chemical signals from cells to the tissue, from tissue to the organism, from the

organism to the population and a speculated effect from the population to the ecosystem. The relationship of how these chemical signals integrate with the immune system's molecules of chemical nature such as complement proteins and cytokines is possible at low doses, whereas in humans it remains restricted due to ethical considerations. A study on leopard frog's liver cells inhabiting ponds where they are naturally exposed to radioactive contamination (1 mGy/y) revealed a decrease in unrepaired chromosomes when irradiated with a challenge dose (4 Gy) compared to controls who lived in clean ponds implicating a radio adaptive response (RAR) by limiting the error prone DNA repair which increases cancer risk (Mitchel 2006). A similar effect was seen in fish cell lines by Ryan et al. (2008) when a priming low dose of radiation (0.1 Gy) favourably sensitized these cells to a future higher dose (2 Gy). These studies did not equate RAR with the immune system, but there is emerging evidence that RAR may have implications for immune functions (Sisakht et al. 2020).

1.11. Other relevant factors that impact radiation and immune interaction

In addition to the discussion on immune system key players involved in a radiation response in biological tissues, it is also crucial to look at factors such as age, overall immune status, environment, and lifestyle of the irradiated organism under study. An in-depth investigation of all relevant variables may not be possible in one thesis project but some of these factors recurred in various studies referenced for the purpose of this thesis. Of note were the acquired strong immunity in species residing in the high background radiation areas due to a possible adaptive response, however, inter-individual variabilities must also be considered that depends not only on radiation quality, dose and dose rate,

but also the cell type, tissue, system and organism that is irradiated with additional role played by genetics (Bannister et al. 2016). Age at the time of irradiation and presence of tumor and its stage greatly influences the outcome of an immune mediated abscopal response (Brix et al. 2017). Young subjects have strong immune function which allows for better utilization of immune-enhancing properties induced by radiation as opposed to older group. Another factor which may modulate radiation response in mammals is gender of the exposed specie. Ovarian tumour in female mice was detected when exposed to low-dose radiation as opposed to their male counterparts who showed no effect (Surinov et al. 2000 reviewed in Mothersill et al. 2018). It is reported that females have a greater degree of radiation tolerance compared to males (Hall and Garcia 2012). Sex hormones, like estrogen has been associated with T-cell development and stimulation in female mice where radiation may alter this response (Kusunoki 1994). However, no new studies were found confirming this association. Chronic myeloid leukemia (CML) primarily occur due to oncogene formation in a hematopoietic stem cell (HSC) and the risk increased with age and radiation exposure which is reported to be higher in males compared to females (Radivoyevitch et al. 2014). The reason for this sex difference was associated with a high number of target cells (such as HSC) found in males where other cancer such as lung cancer might not manifest this difference. It may be inferred that although gender may impact the radiation induced immune response but factors like tumor under consideration, animal-human differences and the intrinsic immune competence of irradiated organism must also be considered.

Existing tumor independent comorbidities also affect the clinical outcomes of radiation induced immune response (Bolla et al. 2019), where factors like genetic makeup of the patient, the cancer associated genomic instability and the evolution of cancer induced phenotypes alters immune homeostasis (Bedognetti 2019). While age, gender, diet, ethnicity and co-morbidities are typical confounding factors, smoking status, alcohol consumption, exposure to occupational carcinogens, exposure from medical x-rays and other environment radiation pollutants are also factors that deserve attention (Pernot et al. 2012; Akiyama 1995). Specifically, environmental radiation contamination from radium and tritium typically increase bystander signalling and RIGI when exposed to a second radiation stressor (Mothersill et al. 2019).

CHAPTER 2

2. Materials and methods:

In view of the literature examined above, the author set out to explore the evidence in a systematic way to evaluate the degree to which LDIR stimulates or suppresses the immune system. The first step to this process was formulation of a research question as below:

Question: On a balance, does Low Dose Ionizing Radiation (LDIR) stimulate or suppress the immune system?

A thorough search of registers such as the Cochrane reviews and the international prospective register of systematic reviews (PROSPERO) were searched to avoid duplication of research. Other databases such as PubMed and Google Scholar were also searched to rule out existence of a similar systematic review. To the best of author's knowledge, no systematic review currently exists that investigated LDIR stimulatory or inhibitory effects on the immune system. Understanding these effects could lead to valuable knowledge where LDIR's potential can be utilized in various conditions including but not limited to inflammatory conditions, pneumonia, cancer etc. Moreover, such knowledge will be extremely useful in the formulation of radiation risk assessment guidelines as currently the risks evaluation in the low dose region are primarily drawn from back extrapolating evidence from high dose exposures. It was speculated that the research question can be answered in a straightforward manner by weighing the endpoints

on a balance to determine if it would shift more to stimulation or suppression of the immune system (Figure 5).

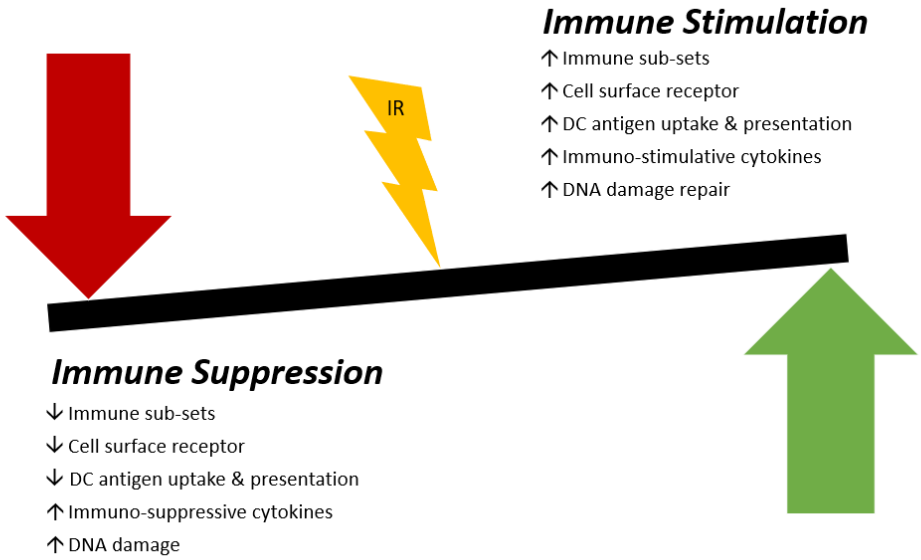

Figure 5: Mind map of a hypothetical balance to see if LDIR stimulate or suppress the immune system.

The literature has shown some conflict in the dose and dose range currently accepted as "low dose", hence for the purpose of this systematic review, LDIR was defined as x-ray or gamma-ray dose below 0.1 Gy (100 mGy). The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Guidelines were followed for the current systematic review (Moher et al. 2009, see appendix 1). The PRISMA guidelines were adhered to for the most part of the review, apart from the involvement of a second reviewer and registering this protocol in a register.

2.1. Data sources and study Selection

A literature search of MEDLINE (Appendix 2), EMBASE (Appendix 3) and, the Web of Science Portal (Appendix 4) was conducted from their inception through June 10, 2021, after formulating an appropriate search strategy (Appendix 5) with the assistance of an institutional librarian. RIS was used to export all articles from three databases and then imported into Covidence, a web-based software platform which allows better systematic review management. As the articles were being imported, Covidence automatically de-duplicated the retrieved records from the three databases.

2.2. Study's eligibility: inclusion and exclusion criteria

Following inclusion criteria for study selection were followed.

1) An original, peer reviewed journal article.
2) Experimental in-vivo or in-vitro studies.
3) Used an acute external low LET exposure of either x-ray or gamma ray <0.1 Gy (100 mGy).
4) Only studies reporting dose units in Gray were included.
5) Investigated an immune endpoint with relevance to immune system function.

Following exclusion criteria were followed to exclude studies to ensure feasible comparison and quantification.

1) Review articles, book chapters, editorials, opinion or commentary articles, conference papers.

2) Papers not available in English language as translation services were out of budget for this research.

3) The selected articles which could not be found due to indexing error or due to not having subscription to the journal after all possible attempts to obtain.

4) Mixed beam experiments where gamma or x-ray effect were mixed with a high LET, UV or particle radiation.

5) Studies that investigated a combined effect of gamma or x ray with another variable such as a radiomitigative dietary compound, freezing, hyperthermia, mutagens, toxins, and REDOX modifiers.

6) Occupational doses studies.

7) Plant studies.

8) Epidemiological studies investigating low dose chronic radiation exposure.

9) Radiotherapy focused clinical trials and high dose radiotherapy > 0.1 Gy delivered within the context of cancer treatment.

10) Low dose fractionated and protracted radiation exposures that were not delivered acutely or on the same day.

11) LDIR from diagnostic imaging such as CT scans, mammography and angiography to radiation workers and patients.

12) Only healthy subjects were considered for this study and studies that investigated conditions such as Ehlers-Danlos syndrome (EDS), Behcet's syndrome and severe combined immunodeficiency (SCIDs), E.Coli, arthritis and cancer stricken subjects were excluded.

13) Studies on non-immune cells such as keratinocytes, fibroblasts, epithelial cells, Chinese hamster ovary (CHO) cells, cancer and transformed cell lines etc. Few examples of such cell lines are listed below.

    a. K562 cells (human immortalized myelogenous leukemia cell line
    b. Mouse lymphoma cell line
    c. Human T-cell leukemia
    d. Walker-256 tumor model mice
    e. Mouse lymphoma (EL4) cells
    f. Human promyelocytic leukemia cells (HL-60)
    g. Cell line isolated from a papillary thyroid carcinoma TPC-1
    h. U937 lymphoma cell line.
    i. TK6 human lymphoblastoid cells
    j. Human B-lymphoblast IM-9 cells
    k. S180 is a murine sarcoma cancer cell line

14) Where immune system function and component was not the main goal of the study and topics such as biodosimetry assessment, comparison of cytogenetic analysis, comparison of immune cells' radiosensitivities and inter-individual variabilities manifested by immune cells in response to LDIR measured were excluded.

Additionally, an ancestry search, in which reference lists of eligible papers were hand searched in order to ensure that no relevant articles were missed.

2.3. Screening and data extraction

The first step was title and abstract screening to identify studies that met the pre-set criteria to be included in the full text review. Next, articles identified in the first step, were examined thoroughly to see if they met all component for the inclusion criteria and if they can be pushed to the next step of data extraction. A form for data extraction was prepared to include all the information with potential relevance to the research question. The data items extracted from each study were as follows:

1) Radiation delivery method (in-vivo or in-vitro)
2) Cell/ Tissue used
3) Model and Size of the subject.
4) Age and gender of the subject.
5) If prior stimulation with a mitogen was administered.
6) Dose employed.

7) Dose rate
8) Protocol used.
9) Immune endpoint investigated.
10) Time of analysis after LDIR.
11) Main study findings.
12) Remarks about the Immune System effect

2.4. Quality assessment

There is a lack of standardized tool for quality assessment of in-vitro studies, therefore a customized checklist formulated by Khan et al. (2014) and Rathish et al. (2019) from Methodological Index for In Vitro Studies was used. This checklist consists of 12 items where each item is scored from 0-2. Number 0 was assigned to the item not reported, 1 is when an item is reported but not adequately, and 2 meant that the item is sufficiently reported (Appendix Table 5). Thus, each study could attain a maximum of 24 and a minimum of 0 points.

For the in-vivo animal studies, the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) assessment tool (Hooijmans et al. 2014) was used to assess the quality of each in-vivo study. This checklist consists of 10 items to assess different types of biases such as the attrition bias, selection bias, detection bias, reporting bias, performance bias etc. In particular items 1, 3, 8, 9 and 10 correspond to the items in

the Cochrane RoB tool. The answer options were a yes, no, and unclear where these represented low, high, and unclear risk of bias, respectively.

2.5. Data synthesis

Due to great heterogeneities found in measured outcomes and variable experimental techniques, it was difficult to synthesize in a quantitative way, the study outcomes via meta-analysis. Therefore, a narrative synthesis approach was adopted for the data synthesis of included studies. Included studies were broadly categorized as in-vivo, invitro animal studies, and in-vitro human studies.

CHAPTER 3

3. Results

3.1. Study selection and characteristics

A total of 5471 citations were collected from the three databases of which 438 were removed at the de-duplication step leaving 5033 studies which were title and abstract screened leaving 213 potentially eligible studies for full text review. 24 articles met the complete eligibility criteria where 189 articles were excluded after the full-text review. PRISMA flowchart (Figure. 6) outlines the screening and step by step selection process.

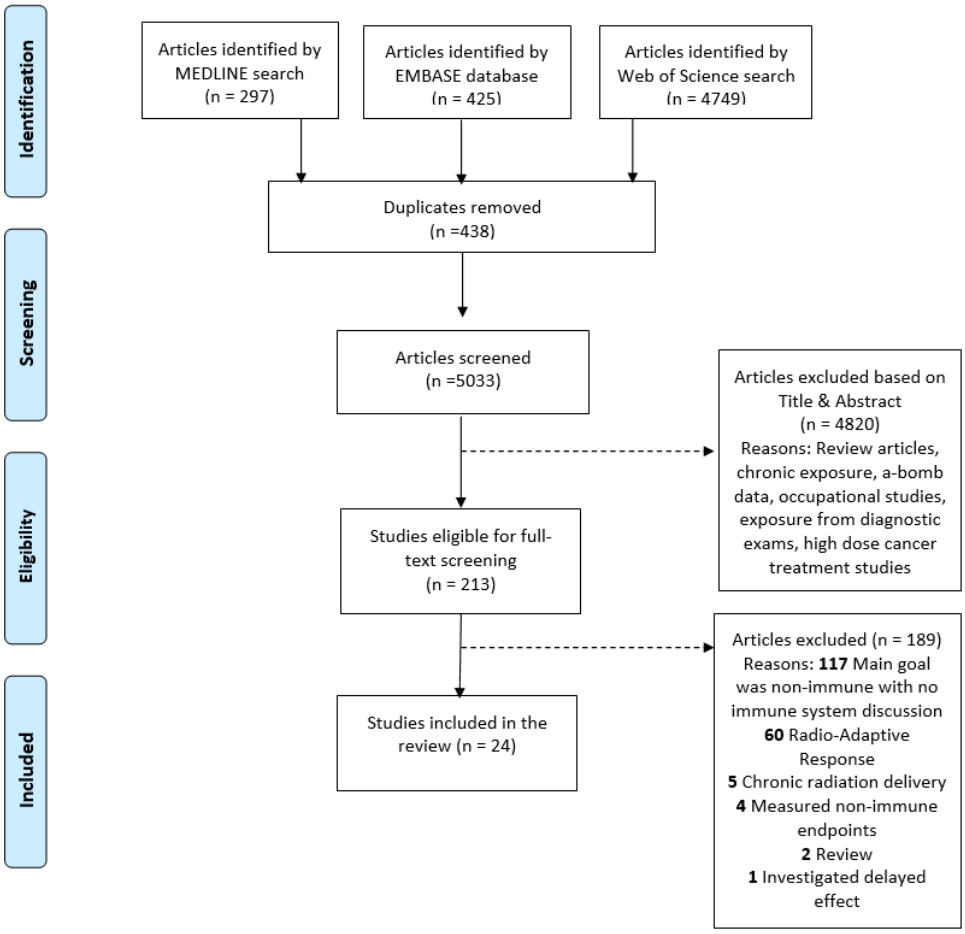

Fig 6. PRISMA Flowchart outlining the process of screening and selection of records.

The study characteristics of the included studies are summarized in Table 1. Of 24 included studies, 13 were in-vivo animal (mice and rats) studies, 4 in-vitro human and 7 in-vitro mice studies.

Table 1: Summary of included study characteristics

Ref	Cell/ Tissue	Model & Size	Age & Gender	Prior (+)	Dose (Gy)	Dose Rate	Protocol	Immune Endpoints	T post IR	Main Study Findings	Remarks about the Immune System
Healthy Human in vitro studies (cell-cultures irradiation)											
Chen et al. 2010	HPBL, T-cells & NK cells	7	29.8 ± 3.56 yrs	No	0.05	0.5 Gy/ min	Phenotype analysis, ELISA, RT-PCR, MTT assay	Effect on T and NK cells subsets, Cytokine production & cytotoxic activity of HPBL	24 hr	No change in HPBL subsets (T & NK cells), ↑mRNA expression (IFN-γ, IL-2 & TNF-α) in supernatant of HPBL ↑Cytotoxicity of HPBL.	LDIR can enhance immune response.
El-Saghire et al. 2013 a	Whole blood	10	NM	No	0.05 & 1	3 cGy/ min	RT-PCR, Microarray data & Pathway analysis, GSEA	Individual genes, pathway analysis, Immune response, signal transduction and growth signaling, apoptosis & damage response, Enrichment map analysis at 0.05 Gy	8 hr	↑Innate immune gene sets ↑Adaptive immune gene sets ↑MAPK genes (ERK, p38 &JNK), no effect on DNA damage response, a clear network of innate-related immune pathways connected with MAPK & NFκB gene sets; cytokine/ chemokine related gene sets showed innate-adaptive related responses.	LDIR induces a hybrid response as in addition to DNA damage, the innate & adaptive immune responses are activated via MAPK, NF-κB, chemokines, & cytokines.
El-Saghire et al. 2013 b	Moncytes from whole blood	8	NM	No	0.05, 0.1 & 1	30 mGy /min	RNA isolation, cDNA synthesis, qRT-PCR	TLR signaling axis, NF-κB signaling and MAPK activation	6 hr	↑TLR4 at 0.05 Gy & TLR9 at 0.1 Gy leading to increased HMGB1. ↑MyD88 (0.05 Gy) & IRAK1 ↑Phosphorylation of IκBα at (0.05 & 0.1 Gy) ↑ phosphorylated form of 3 MAPKs (p38, ERK and	LDIR is immune-stimulatory via TLR4 signaling axis involvement as seen by the activation of MAPK & NF-κB

										JNK) at 0.05 Gy while ↓at 0.1 Gy	positive regulative pathways suggesting pro-survival & pro-inflammatory responses
Yang et al. 2014	PMBC, NK cells	10	30–38 yrs; 6 M, 4 F	No	0.025, 0.075, 0.15, 0.5	12.5 mGy/min	Western Blot, ELISA, LDH assay	Expansion, cytotoxicity & cytokine levels of NK cells	24 hr	↑NK expansion observed at 0.075 Gy, ↑Cytotoxicity, ↑IFN-γ and TNF-α levels at 0.075 Gy	LDIR can induce expansion & cytotoxic function of NK cells mediated via P38-MAPK pathway.
In-vitro animal studies (cell-cultures irradiation)											
Ibuki and Goto 1999	Macrophages, spleno-lymphocytes	C57 BL/6 mice, NM	7 wks, Male	Con A	0.04 or 4	0.2 cGy/min	PCR assay, mRNA purification and RNA-PCR	Con A induced proliferation of spleno-lymphocytes, Effect on cytokine release from spleno-lymphocytes when co-cultured with irradiated macrophages	1 or 4 hr	No change in spenlo-lymphocytes proliferation via irradiated macrophage, ↑IL-1β and IFN-γ production from spleno-lymphocytes when co-cultured with irradiated (0.04 Gy) macrophages at 4 hr.	LDIR enhances accessory function of macrophages via release IL-1β stimulating lymphocytes, which also releases cytokines further activating more macrophages.
Hosoi et al. 2001	Peritoneal Macrophages	Inbred albino	8-10 wks	No	0.1-10 Gy	0.72 Gy/min	PCR	Cytokine (IL-1β and IL-6) production	2 hr	No effect on IL-1β, ↑IL-6 expression 2 hr (0.1 Gy)	LDIR augments the immune

		strain WHT/Ht mice, NM								which did not become normal until 4 hr	response.
Shigematsu et al. 2007	Splenic DCs	C57BL/6 mice, NM	8 wks, Male	No	0.02, 0.05, 0.1, 0.5, or 1	1.0 Gy/min	Flow cytometry, RT-PCR, ELISA	Proliferation of T cells by DCs, production of IL-10 and IL-12 & Th1 cytokines	48 hr	↑T-cell proliferation via DC activation (peak 0.05 Gy) ↑Levels of IL-10 & 12 in supernatants of irradiated DCs (0.05 Gy at 48 hr) ↑mRNA expression of Th1 cytokines (IL-2, IL-12 & IFN-γ) by DCs (0.05 Gy at 8 hr)	LDIR activates T cells via Th1 cytokines from DCs, resulting in the induction of Th1 cells from naïve T cells.
Frischholz et al. 2013	Peritoneal macrophages	Balb/c, C57BL/6 mice, NM	30-50 wks	LPS	0.01, 0.05, 0.1 0.3, 0.5, 0.7 & 1.0	NM	ELISA	IL-1β and TNF-α secretion	16 hr	No change in cytokine secretion	LDIR (0.5 or 0.7 Gy) induces activated Peritoneal to release significantly lower amounts of IL-1 β
Wunderlich et al. 2015	Peritoneal macrophages	BALB/c, NM	30-50 wks	LPS	0.01, 0.05, 0.1, 0·3, 0·5, 0·7, 1·0 or 2·0	NM	Alamar Blue assay, Transwell migration assay, µ-Slide, annexin V-fluorescein isothiocyanate (FITC) (AxV-FITC)/PI-	cell death, transmigration, chemotaxis, phagocytosis, inflammatory cytokine secretion and cell signaling	24 hr	No apoptosis & necrosis detected, ↓Transmigration (0.1 Gy) & discontinuous dose dependent response (0.01 Gy) ↑Chemotaxis (0.1 Gy) no impact on phagocytosis, ↓IL-1β (0·01 Gy) ↑TGF-β (0.1 Gy)	LDIR (0·5 Gy) contribute to the reduction of degenerative & inflammatory disease associated pain by downregulation of inflammation,

							staining, Chemotaxis assay,				reduced macrophages transmigration, increased chemotaxis to an inflamed site & anti-inflammatory process.
Cho et al. 2018	CD4+ cells	NM, 5	6 wks, Male	anti-CD3/CD28 Ab	0.01 or 0.05	204 mGy/min	RNA-Seq analysis, GSEA, qRT-PCR, ELISA, Flow cytometry	Gene expression profile, cytokine genes, Treg cell differentiation and Treg cytokine (TGFβ1) expression	24 hr	Altered mRNA expression of following radiation responsive genes in CD4+ cells, ↑IFNγ (0.01 & 0.05 Gy), IL-4 (0.05 Gy), ↓IL-17A (0.01 Gy), ↓TGFβ1 (0.05 Gy), ↓IL-10 (0.05 Gy), ↓FoxP3+ cells (from 3.2% to 2.3%) on day 3 (0.05 Gy)	LDIR promotes immune response via Th1/Th2 cytokines stimulation (IFN-γ, IL-4, IL-5), and suppresses immune response via Tregs cytokines suppression (TGF-β1, TGF-β3
Wunderlich et al. 2019	pMΦ, BMDC and allogeneic T-cells	BALB/c NM	30-35 wks	LPS	0.01, 0.05, 0.1, 0.3, 0.5, 0.7, 1.0 or 2.0 Gy	NM	Flow cytometry, CFSE staining, various culturing techniques	surface expression of MHCII on activated pMΦ, Activation of T-cells incubated with irradiated macrophages, Modulation of CD40 expression	24 hr	No effect on MHC-II, ↓CD25 expression after co-incubation of T cells + 0.01 Gy irradiated pMΦ, ↓CD40 expression after co-incubation with supernatant of 0.01 Gy irradiated pMΦ	Activated macrophages induce NTE in T-cells which alter the adaptive immunological response after single

								on BMDC by supernatant of pMΦ			LDIR up to 2 Gy.
In vivo animal studies (whole body irradiation)											
Chen et al. 1999	Splenocytes	Kunming Mice; NM	NM, Male	No	0.075	**12.5 mGy/min**	SDS-PAGE, 2D-PAGE, Silver Staining	Protein expression in mouse splenocytes	4 hr	↑Upregulation of 4 proteins, 20 alternated proteins found	LDIR alters & redistributes protein expression which may be related to mechanisms of immune enhancement & adaptive response
Matsubara et al. 2000	Thymocytes	C57BL/6 and ICR mice; 3	5 wks, M	No	0.075, 0.5, 1	1.76 cGy/s	Apoptosis assay (Hoechst 33342), Anti-SRBC PFC Assay	Thymocyte apoptosis	6 hr	↑Apoptosis in thymus in both mice models	LDIR generates delayed radioresistance which may enhance immune system function as LDIR acts at the cellular or organismic level by facilitating interaction between various immune components.
Liu et al. 2001	Peritoneal macrophages,	Kunming mice; NM	6-7 wks	ConA	0.075	0.0125 Gy/min	Flow cytometry, ELISA	Role of APCs in the activation of T cells, Expression	24 hr	↑Proliferation of splenocytes in response to ConA after WBI with adherent APCs	LDIR upregulates CD28 with downregu

	splenic lymphocytes							of B7 molecules on APCs, Expression of CD28/CTLA-4 on lymphocytes, Secretion of IL-12p70 by peritoneal macrophages and synthesis of IL-10 in lymphocytes		↑B7-1 expression on APCs at 12 hr ↑B7-2 expression on APCs at 8 hr ↑CD28 expression on splenocytes at 4 hr with opposite change in CTLA-4 expression ↑CD28 expression on thymocytes at 72 hr with opposite change in CTLA-4 expression. ↑IL-12p70 secretion at 4 hr with rapid return to basal level	lation of CTLA-4 on the T cells which suppresses IL-10 production resulting in immune enhancement.
Liu et al. 2003	Splenocytes & peritoneal macrophages	Kunming mice; NM	5-6 wks, Male	No	0.075	0.0125 Gy/min	Northern blot, Flow cytometry & ELISA	mRNA levels of IL-10 & IL-12 p35+ p40 subunits, secretion of IL-12 p70, synthesis of IL-10	1-48 hr	↓IL-10 expression in splenocytes from 1 h till 48 hr ↑IL-12 (p35 & p40 subunit) expression in peritoneal macrophages at 1-2 hr ↑IL-12p70 expression at 4 h, rapid recovery to basal level (16–48 h) ↓IL-10 synthesis with no recovery until 48 hr	LDIR induced immune function stimulation & 0.075 Gy may be the most optimal dose for immune function up-regulation.
Li et al. 2004	BM cells in PB	Kunming mice, BALB/C mice;	NM; 60 Female & 50 male	No	0.01, 0.025, 0.050, 0.075, 0.1	75 mGy/min	Slot blot hybridization and Northern blot assays	Bone marrow HPC proliferation & peripheral blood mobilization	24 hr, 48 hr	↑Stimulation of CFU-GM (0.05) & BFU-E (0.075), ↑Mobilization of CFU-GM in PB circulation	LDIR can enhance body immunity & defense functions
Shan et al. 2007	Peritoneal macro	Kunming mice;	NM, Male	No	0.05, 0.075,	12.5 mGy/min	ELISA, EMSA, Flow	Cytokine secretion, changes in	4-48 hr	↑IL-12 and IL-18 (0.075 Gy) ↑Nuclear	LDIR can stimulate the

	phages	NM			0.1, 0.2, 0.5-6 Gy		cytometry	NF-κB & MyD88, changes in expression of surface molecules (CD14 and TLR4–MD2)		translocation of NF-κB & MyD88 expression (0.075 Gy) at 8 hr. ↑CD14 surface expression earlier than TLRL-MD2 expression at 16 hr.	secretion of IL-12 & IL-18 via activation of toll signaling pathway in the macrophages which may stimulate adaptive immune responses.
Bogdándi et al. 2010	Splenocytes	C57BL/6; 262	6-8 wks, Female	No	0.01, 0.05, 0.1, 0.5 & 2 Gy	0.034 Gy/min	Flow Cytometry, TUNEL assay, RT-PCR	Apoptosis in Spleen Lymphocytes, Alterations in the total & relative number of lymphocyte subpopulation in the spleens, Cytokine Expression Profile	4 hr, day 1, 3 & 7	↓Apoptosis in CD4+, CD8+, NK, B-cells & DCs, NS increase in total number of splenocytes on day 1 (0.01 Gy), ↓All cell types on day 3 (0.05 Gy), which did not change for 0.1 Gy, NS decrease in relative number of Tregs, NK & DCs (0.1 Gy), ↑CD44+ memory T cells within the CD8+ population increasing dose (non-linear). On Day 7, IR induced recovery was absent for most cells, except DCs, Variable patterns in cytokine expression (GM-CSF, IFN-γ, IL-4, IL-5, IL-6, IL-12, TNF-α, TGF-β) at all doses observed at 4 hr, day 1 &	LDIR significantly effects the quantitative and crucial immune system functional parameters of murine splenocytes where this effect is less clear and shows interindividual variability compared to high dose.

										day 3 ↓IFN-γ, IL-2 expression at 4 hr on day 1 & 3. ↓IL-12 expression at 4 hr & normalized by day 3. ↓IL-4 expression at 4 h ↑IL-6 at 4 hr then decrease at day 1 & normalized by day 3.	
Li et al. 2015	T-Cells	C57 BL/6 Mice	10-12 wks, Male	anti-CD3/ CD28 Ab	0.1, 0.5, or 3 Gy	1.7 Gy/ min	qRT-PCR, Metabolomics profiling by UPLC-QTOF	T-cell proliferation & cytokine production, TCR activated T-cell metabolomic changes	4 hr, 1 wk, 2 wk	No effect in proliferation of and cytokine secretion by CD4+ & CD8+, Mild Elevation in 2 metabolites	LDIR modulates metabolic reprogramming of activated T-cell with implications in T-cell function via alterations in cell metabolism, in addition to traditional cellular immune endpoints. LDIR compromises T-cell metabolism.
Song et al. 2015	Splenocytes	C57 BL/6 Mice	6 wks, Female	No	0.001, 0.01, 0.1	0.1 cGy/ min	Flow cytometry, qRT-PCR, Western blot	Alteration in splenocyte subpopulations, Activation markers,	2,7,14 days	↑CD4+ temporarily (0.001 & 0.1 Gy), ↓DC (all doses) & macrophages (0.01 & 0.1 Gy) at day 2,	LDIR significantly impacts the quantitati

							analysis	Cytokine expression profiles, Induction of ROS-related molecules		↓ CD8+ (0.01 & 0.1 Gy) at day 7 until day 14. ↓CD86+ activation marker on DC on day 2 at all doses (0.001, 0.01 and 0.1 Gy) with recovery at day 14, ↓CD28+ (T cells) on day 7 until day 14 at dose 0.01 & 0.1 Gy, ↓CD69+ cells (NK cells) on day 2 at doses 0.001 and 0.01 Gy. ↑IFN-γ, IL-4, and IL-5 in a dose-dependent manner at day 2, ↑ IFN-γ and IL-12 at day 7, ↑ Nrf2 and HO-1 expression at day 7, ↑iNOS expression (0.1 & 1 cGy) until day 7, ↑NF-κB activity	ve and functional immune parameters of murine splenocytes.
Szatmári et al. 2017	BM Cells & splenocytes	C57/BL6 mice 12-15	9-14 wks, Male	No	0.1, 0.25 & 2	NM	γ-H2AX Assay, TUNEL assay, RNA isolation & miRNA profiling with PCR	Effect of EVs from IR mice to the Spleen of non-IR mice, Chromosomal aberrations, Quantitative change in BM, Apoptosis	24 hr	↑DNA damage in IR mice & frequency of average foci/cell & γ-H2AX + cells in non-IR bystander mice spleen exposed to EV from IR mice BM ↑ Chromosomal aberration in directly IR mice & a moderate increase in non-IR bystander mice. ↓BM-HSCs in directly IR mice, no change in apoptosis in both	Radiation is associated with inflammatory and immune responses.

										groups (IR + Bystander)	
Gao et al. 2018	Thymus	ICR Mice; 16	6-8 wks, Half Male /Half female	No	0.075	12.5 mGy/min	Gene profiling microarray, ELISA	Th1-Th2-Th3 cytokine gene expression profiles, Th1-Th2-Th3-related gene functional analysis	24 hr	↑Expression of 8 and ↓Expression of 5 Th1-Th2-Th3-related genes. ***Functional analysis:*** ↑Th1 cell–related genes Stat4 and Socs1 ↓Th2 and Th3/Tr type cell–associated genes Il-4ra, Cebpb, Gata3 and Tgfb3 ↑Th1-type immune response gene Sftpd. ↓Th1-type immune response gene Tnf	LDIR induces the Th1 immune response increasing immune function.
Persa et al. 2018	Splenocytes & DCs	C57 BL/6 Mice, NM	8-13 wks, NM	No	0.1, 0.25, & 2	NM	Flow cytometry, qRT-PCR	Antigen uptake & Presentation by splenic DCs, Cytokine expression	24 hr	↓Antigen presentation but no effect on antigen uptake by splenic DCs at 24 hr (0.1 Gy), IL-1β, IL-6 & IL-10 expression mildly increased	LDIR enhances DC-antigen uptake but alters DC-antigen presentation suggesting a a less mature DC phenotype with reduced immune stimulation.
Liu et al. 2020	Splenocytes & thymocytes	BALB/C; NM	6-8 wks, Male	No	0.01, 0.05, 0.2, 0.5 or 1	3.93 cGy/min	Flow cytometry, RT-PCR, Western	Effect on peripheral blood cell populations, Effect on	24 hr	No significant change: in blood cell population, apoptotic rate, in the total number of	LDIR inhibits the immune system

							blot analysis	immune organs (apoptosis, quantity, activation marker expression)		thymic T cells, mature T cells (CD4+ T cells/CD8+ T cells), and T lymphocyte activation markers after LDIR ↓DCs number & corresponding marker (CD86) in spleen (0.01 Gy) ↓Macrophage number & corresponding marker (CD68) in spleen (0.01 Gy)	function.

Symbols (+) indicates stimulation, (↑) indicates significantly increased response, (↓) indicates significantly decreased response.

M= Male; F= Female; IR= Irradiated; PMBC= Peripheral blood mono-nuclear cells; HPBL= human peripheral blood lymphocytes; NK= Natural Killer; ELISA= Enzyme-linked immunosorbent assay; LDH= Lactate dehydrogenase; NM= Not mentioned; GSEA= Gene Set Enrichment Analysis; ERK= extracellular signal-regulated kinases; p38; JNK= c-Jun N- terminal kinases; NFκB= Nuclear factor kappa-light-chain-enhancer of activated B cells; qRT-PCR= quantitative real-time reverse transcriptase polymerase chain reaction; HMGB1= high mobility group B1; IRAK1= Interleukin-1 receptor-associated kinase 1; HPC= hematopoietic progenitor cell; BM= Bone marrow; PB= Peripheral Blood; Ab= Antibody; LPS= lipopolysaccharide; pMΦ= peritoneal macrophages; Con A= Concanavalin A; PCR= Polymerase chain reaction; HSC= hematopoietic stem cells; LDIR= Low Dose Ionizing Radiation; CFU-GM= Colony forming unit- granulocyte macrophage; BFU-E=Burst-forming unit-erythroid; CPM= counts per million; NS= Not-significant

3.2. In-vitro human studies

4 in-vitro human studies included in this systematic review investigated immune cells such as T-cells, monocytes and natural killer (NK) cells derived from human whole

blood, peripheral blood human peripheral blood lymphocytes (HPBL), Peripheral blood mono-nuclear cells (PBMC), (Chen et al. 2010; El-Saghire et al. 2013a; El-Saghire et al. 2013b; Yang et al. 2014). See Table 1 for their summary.

Cytokine production, quantitative changes, and cytotoxicity

2 studies (Chen et al. 2010 and Yang et al. 2014) investigated cytokine production, quantitative changes, and increased cytotoxicity in HPBL, T-cells and NK cells respectively. Chen et al. (2010) reported an increase in IFN-γ and TNF-α mRNA expression levels in the supernatants of HPBLs and increased cytotoxicity post irradiation (0.05Gy) at 24 hours. Whereas no significant change was found in percentage of T and NK subsets of HPBL and CD4+: CD8+ T lymphocytes ratio at same radiation parameters. Yang et al. (2014) reported significant increase in the expansion and absolute number of NK cells, the cytotoxicity of the expanded NK cells and IFN-γ and TNF-α secretion was also increased post irradiation (0.075 Gy) at 24 hours.

Immune associated gene expression

2 studies by a same research group (El-Saghire et al. 2013a and El-Saghire 2013b) examined immune system associated genes. El-Saghire et al. (2013a) observed an increased expression of 3 genes (PF4, GNG11 and CCR4), top ranking of innate immune gene sets (Toll-like receptors [TLR], RIG-I like receptors, NOD-like receptors, and cytosolic DNA sensing) and adaptive immune gene sets (natural killer [NK] cell signaling, B-cell receptor [BCR] signaling, and T-cell receptor [TCR] signaling) 8 hours post irradiation (0.05 Gy). Additionally, MAPK gene sets (extracellular signal-regulated

kinases (ERK), p38, and c-Jun N terminal kinases (JNK) signaling) also scored top rank at 0.05 Gy with no effect on DNA damage response. Lastly, a clear network of innate-related immune pathways connected with MAPK & NFκB gene sets, as well as cytokine and chemokine related gene sets showed an association with innate-adaptive responses at 0.05 Gy.

El-Saghire (2013b) found an increase in TLR4 signaling at 0.05 Gy and TLR9 signaling at 0.1 Gy in monocytes leading to an increased secretion of HMGB1. Moreover, a significant increase in MyD88 (an adapter molecule) typically involved in the signal transduction of the activated TLRs, was observed at 0.05 Gy. IRAK1, which interacts with MyD88 further facilitating TLR signaling, leads to the activation of NF-κB and MAPKs. This study also found an increase in the IRAK1 levels at 0.05 and 0.1 Gy. Following NF-κB activation, increased phosphorylation of IκBα at 0.05 and 0.1 Gy was observed. An increase in phosphorylated form of 3 MAPKs (p38, ERK and JNK) at 0.05 Gy while a decrease at 0.1 Gy was also reported. All the endpoints investigated in this study were analyzed at 6 hours.

Quality assessment of in-vitro human studies

Quality assessment was performed on the Methodological Index (MI) for in-vitro human studies which is displayed in Table 2. All studies (100%) explicitly stated purpose of the study (MI Item # 1), had adequate control groups (MI Item # 2), well described the tissue/cell type investigated (MI Item # 3), organism identity (MI Item # 6), culture methods used (MI Item # 8) with an unbiased outcome assessment (MI Item # 12). None

of the studies (100%) mentioned priori power analysis (MI Item # 4), infection risk (MI Item # 7), cleansing agent (MI Item # 10) and method (MI Item # 11) used. The appropriate statistical analysis (MI Item # 5) was reported adequately for 3 studies (75%) while 1 study (25%) reported it inadequately. Culture time (MI Item # 9) was reported inadequately in 2 studies (50 %) since most researchers cultured their cells for less than 6 days, a criterion that was set to give a score of 1 to such studies (Khan et al. 2014), where 2 study (50%) reported it adequately as their culture time was more than 7 days. 3 studies (75 %) scored 15, where 1 study (25%) scored 16 out of the total score of 24.

Table 2: Quality assessment score for in-vitro human studies.

Crietria	Study Purpose	Control Groups	Tissue Type	Priori Power Analysis	Statistical Analysis	Outcome Assessment	Organism Identity	Infection Risk	Culture Method	Culture Time	Cleansing Agent	Cleansing Method	Total Score
Chen et al. 2010	RA	RA	RA	NR	RA	RA	RA	NR	RA	RI	NR	NR	15
El-Saghire et al. 2013a	RA	RA	RA	NR	RI	RA	RA	NR	RA	RA	NR	NR	15
El-Saghire et al. 2013b	RA	RA	RA	NR	RA	RA	RA	NR	RA	RI	NR	NR	15
Yang et al. 2014	RA	RA	RA	NR	RA	RA	RA	NR	RA	RA	NR	NR	16

RA= Reported Adequately (1); RI= Reported Inadequately; NR= Not Reported (0)

3.3. In-vitro animal studies

7 in-vitro mice studies included in this systematic review investigated murine immune cells such as CD4+ T-cells, macrophages, bone marrow derived dendritic cells (BMDC), spleno-lymphocytes and splenic dendritic cells (Ibuki and Goto 1999; Hosoi Y et al.

2001; Shigematsu et al. 2007; Frischholz et al. 2013; Wunderlich et al. 2015; Cho et al. 2018; Wunderlich et al. 2019). See Table 1 for their summary.

Mitogenic stimulation

As opposed to in-vitro human studies where no prior stimulation with mitogen occurred, the in-vitro animal studies used variable compounds for stimulation of immune cells under investigation where 3 studies (Frischholz et al. 2013; Wunderlich et al. 2015; Wunderlich et al. 2019) used lipopolysaccharide (LPS), 1 study (Ibuki and Goto 1999) used Con A and 1 study (Cho et al. 2018) used anti-CD3/CD28 Ab. 2 studies (Shigematsu et al. 2007; Hosoi Y et al. 2001) did not stimulate immune cells in their experiments.

Non-targeted effects

2 studies examined the non-targeted effects, Wunderlich et al. (2019) study demonstrated decreased CD25 and CD40 expression after co-incubation with supernatant of 0.01 Gy irradiated peritoneal macrophages at 24 hours and Ibuki and Goto (1999) showed increased cytokine expression (IL-1β and IFN-γ) from spleno-lymphocytes when co-cultured with irradiated (0.04 Gy) macrophages at 4 hours.

Cytokine secretion

5 studies (Ibuki and Goto 1999; Hosoi et al. 2001; Shigematsu et al. 2007; Frischholz et al. 2013; Wunderlich et al. 2015) investigated cytokine secretion as at least one endpoint in their study. Ibuki and Goto (1999) study reported an NTE effect as mentioned earlier where Shigematsu et al. (2007) reported an increased IL-10 and 12 levels in supernatant of irradiated (0.05 Gy) DCs at 48 hours. Hosoi et al. (2011) reported a significant increase

in IL-6 expression 2 hr after 0.1 Gy which did not become normal until 4 hr where no effect was observed on IL-1β levels. Frischholz et al. (2013) reported no change in cytokine secretion in the supernatants of peritoneal macrophages at 0.01, 0.05, 0.1 at 6 hours. Wunderlich (2015) reported a significantly reduced level of IL-1β at dose 0·01 Gy while TGF-β (inflammatory cytokine) significant increased at 0.1 Gy in peritoneal macrophages when analyzed after 24 hours of irradiation.

Gene expression

2 studies investigated mRNA expression levels of cytokines. (Cho et al. 2018) reported significantly increased IFN-γ mRNA (signature Th1 cytokine) post irradiation (0.01 and 0.05 Gy) and IL-4 mRNA (Th2 signature cytokine) post irradiation (0.05 Gy) at 24 hours in CD4+ cells. While IL-17A mRNA (inflammatory cytokine) at 0.01 Gy significantly decreased, TGFβ1 (Tregs cells cytokine) significantly decreased while IL-10 mRNA significantly increased at 0.05 Gy. Shigematsu et al. (2007) reported an increased mRNA expression of Th1 cytokines (IL-2, IL-12, IFN- γ) by DCs at 8 hours when irradiated to 0.05 Gy.

Cell surface expression

1 study (Wunderlich et al. 2019) investigated cell surface molecules and reported an NTE as mentioned earlier where expression of CD25 and CD40 was decreased after co-incubation with supernatant of 0.01 Gy irradiated peritoneal macrophages at 24 hours.

Macrophage transmigration, chemotaxis, and phagocytosis

1 study (Wunderlich 2015) reported decreased transmigration of peritoneal macrophages at 0.1 Gy while a discontinuous dose dependent response was observed at 0.01 Gy after 24 hours. Additionally, the same study reported an increased chemotaxis at 0.1 Gy while no effect was seen on phagocytosis of macrophages.

Quantitative changes

Ibuki and Goto (1999) reported no significant change in spleno-lymphocytes when cultured with irradiated (0.04 Gy) macrophages 1 and 4 hours post irradiation. Shigematsu et al. (2007) observed an increased T-cell proliferation via DC activation which peaked at 0.05 Gy when analyzed after 2 days. Cho et al. (2018) reported downregulation of FoxP3+ regulatory T-cells (Tregs) were on day 3 after 0.05 Gy.

Apoptosis and necrosis

Only 1 study (Wunderlich et al. 2015) assessed apoptosis and necrosis in peritoneal macrophages. No significant changes in subG1 DNA content and rupture of plasma membrane integrity was detected, as these were the biomarkers for apoptotic and necrotic activity respectively assessed at various doses (0.01, 0.05, 0.1 Gy) 24 hours post-irradiation.

Quality assessment of in-vitro animal studies

Quality assessment was performed on the Methodological Index (MI) for in-vitro animal studies which is displayed in Table 3. All studies (100%) explicitly stated purpose of the study (MI Item # 1), had adequate control groups (MI Item # 2), well described the

tissue/cell type investigated (MI Item # 3), culture methods used (MI Item # 8) with appropriate statistical analysis (MI Item # 5) and unbiased outcome assessment (MI Item # 12). None of the studies (100%) mentioned priori power analysis (MI Item # 4), infection risk (MI Item # 7), cleansing agent (MI Item # 10) and method (MI Item # 11) used. The organism identity (MI Item # 6) was reported properly in 1 study (14%) with organism type and quantity while the remaining 6 studies (86 %) only reported organism type but failed to report its quantity. Culture time (MI Item # 9) was reported inadequately in 6 studies (86 %) since most researchers cultured their cells for less than < 6 days, a criterion that was set to give a score of 1 to such studies (Khan et al. 2014), where 1 study (14%) did not mention the culture time. 5 studies (71 %) scored 13, where 1 study (14%) scored 12 and 1 study (14%) scored 12 out of the total score of 24.

Table 3: Quality assessment score for in-vitro animal studies.

Criteria	Study Purpose	Control Groups	Tissue Type	Priori Power Analysis	Statistical Analysis	Outcome Assessment	Organism Identity	Infection Risk	Culture Method	Culture Time	Cleansing Agent	Cleansing Method	Total Score
Ibuki and Goto 1999	RA	RA	RA	NR	RA	RA	RI	NR	RA	RI	NR	NR	**14**
Hosoi et al. 2001	RA	RA	RA	NR	RA	RA	RI	NR	RA	RI	NR	NR	**14**
Shigematsu et al. 2007	RA	RA	RA	NR	RA	RA	RI	NR	RA	NR	NR	NR	**13**
Frischholz et al. 2013	RA	RA	RA	NR	RA	RA	RI	NR	RA	RI	NR	NR	**14**
Wunderlich et al. 2015	RA	RA	RA	NR	RA	RA	RI	NR	RA	RI	NR	NR	**14**
Cho et al. 2018	RA	RA	RA	NR	RA	RA	RA	NR	RA	RI	NR	NR	**15**
Wunderlich et al. 2019	RA	RA	RA	NR	RA	RA	RI	NR	RA	RI	NR	NR	**14**

RA= Reported Adequately (1); RI= Reported Inadequately; NR= Not Reported (0)

3.4. In-vivo animal studies

13 in-vivo studies included in this systematic review is as follows of which 2 studies: Chen et al. 1999; Matsubara et al. 2000; Liu et al. 2001; Liu et al. 2003; Li et al. 2004; Shan et al. 2007; Bogdándi et al. 2010; Song et al. 2015; Li et al. 2015; Szatmári et al. 2017; Liu et al. 2020; Persa et al. 2018 and Gao et al. 2018. See Table 1 for their summary.

Mitogenic Stimulation

Only 2 studies (Liu et al. 2001; Li et al. 2015) stimulated T-cells via Con A and Anti-CD/CD28 antibody respectively while the remaining 11 studies did not stimulate immune cells prior to irradiation.

Non-Targeted effects

1 study (Szatmári et al. 2017) reported an increase in DNA damage in C57/BL6 mice when their non-irradiated spleen was exposed to exosomes from irradiated (0.1 Gy) mice BM cells at 24 hours suggesting a bystander effect. Chromosomal aberrations and increase in BM-HSCs number was increased in directly irradiated mice, where a moderate increase in bystander mice was observed.

Apoptosis

4 studies assessed apoptosis rate in immune cells as at least one endpoint as their outcome. Of these 4, Matsubara et al. (2000) investigated acute apoptotic rate in immune cells as a primary goal of their study. Their study concluded that a dose of 0.075 Gy did not affect splenocytes but induced significant apoptosis in thymocytes at 6 hours.

Bogdándi et al. (2010) reported that dose ranging from 0.01- 0.1 Gy induced a reduction in apoptotic response in CD4+, CD8+, NK, B-cells and DCs at 4 hours. Szatmári et al. (2017) found no change in apoptosis in directly or indirectly irradiated (0.1 Gy) cells at 24 hours while Liu et al. (2020) found no significant change in apoptosis in BM and thymus after doses of 0.01 and 0.05 Gy at 24 hours.

Metabolic reprogramming

Only one study (Li et al. 2015) investigated and observed mild elevation in two metabolites such as uridine monophosphate (UMP) and glutathione (GSH) in activated T-cells where one metabolite glutathione disulfide (GS-SG) was significantly reduced after a dose of 0.1 Gy at 72 hours. This study was unique as it measured non-traditional endpoint such as LDIR induced metabolic reprogramming of TCR activated T-cells which impacts T-cell metabolic pathways and hence alters its functional capacity. Although the commonly investigated traditional endpoints such CD4+ and CD8+ proliferation and cytokine secretion were also examined but no significant effect was observed.

Cytokine expression

6 studies (Liu et al. 2001; Liu et al. 2003; Shan et al. 2004; Li et al. 2015; Bogdándi et al. 2010; Song et al. 2018; Persa et al. 2018) looked at cytokine expression as at least one endpoint in their experiments. 2 studies (Liu et al. 2001; Persa et al. 2018) used a dose 0.075 and 0.1 Gy respectively where cytokine analysis was done by both at 24 hours. 2 studies (Liu et al. 2003; Shan et al. 2007) used a similar dose of 0.075 Gy with almost

similar cytokine analysis time between 1- 48 hours. Liu et al. (2003) reported decreased IL-10 expression in splenocytes from 1 h till 48 hours, increased IL-12 (p35 & p40 subunit) expression in peritoneal macrophages at 1-2 hours, increased IL-12p70 expression at 4 hours with rapid recovery to basal level (16–48 h) and decreased IL-10 synthesis with no recovery until 48 hr. Shan et al. (2007) reported a sustained increase over 48 hours in two cytokines i.e., IL-12 and IL-18 at 0.075 Gy. 1 study (Bogdándi et al. 2010) observed variable cytokine expression patterns at all doses (0.01, 0.05, 0.1,) analyzed at 4-hour, day 1 and day 3. 1 study (Li et al. 2015) assessed cytokine secretion at 4 hour, 1 and 2 weeks after a dose of 0.1 Gy with no change in their expression.

Quantitative changes

6 studies (Liu et al. 2001; Li et al. 2004; Bogdándi et al. 2010; Li et al. 2015; Song et al. 2018; Liu et al. 2020) investigated radiation induced quantitative changes and proliferation in immune cells. Of these, 3 analyzed this endpoint at 24 hours (Li et al. 2001; Liu et al. 2020 and Li et al. 2004), 1 analyzed it on day 1 and 3 (Bogdándi et al. 2010) while 2 studies analyzed it on week 1 and 2 (Li et al. 2015; Song et al. 2015).

At 24 hours post irradiation, Liu et al. (2001) reported an increase in proliferation in stimulated splenocytes at 0.075 Gy and Liu et al. (2020) found no significant change in mature thymocytes (CD4+ and CD8+) at 0.01 and 0.05 Gy but a significant change was observed in DCs and macrophages number at this dose. Li et al. (2004) reported stimulation of bone marrow progenitors such as CFU-GM at 0.05 Gy and BFU-E at 0.075 Gy at 24 hours. Additionally, increased mobilization of CFU-GM was found in peripheral

blood circulation 48 hours later since mobilization from bone marrow into peripheral blood is a relatively long process. Bogdándi et al. (2010) did not observe a statistically significant increase in splenocytes (CD4+, CD8+, NK, B-cells & DCs) on day 1 at 0.01 Gy while all cell types significantly decreased on day 3 at 0.05 Gy. There was a significant increase in CD44+ memory T cells on day 3 at 0.1 Gy. Li et al. (2015) observed no effect in CD4+ and CD8+ after 1 or 2 weeks of irradiation at 0.1 Gy. Song et al. (2015) reported a temporary increase in CD4+ at 0.001 & 0.1 Gy while DC number decreased at all doses used in this study (0.001, 0.01 and 0.1 Gy), macrophages number decreased at 0.01 & 0.1 Gy at day 2 and a decrease in CD8+ at 0.01 & 0.1 Gy at day 7 which lasted until day 14.

Gene and protein expression

2 studies (Chen et al. 1999 and Gao et al. 2018) assessed immune system relevant gene and protein expression in murine splenocytes and thymocytes respectively at 0.075 Gy. Chen et al. (1999) reported an increased upregulation of 4 proteins where 20 proteins were alternated after 4 hours of irradiation. Gao et al. (2018) reported an increase in 8 cytokine related genes (Stat4, Junb, Socs1, Sftpd, IL7, Nfatc2ip, Il2rα, Hprt), and decrease in 5 cytokine related genes (Tgfb3, Il4rα, Tnf, Cebpb, Gata3) related to Th1-Th2-Th3 responses 24 hours post-irradiation. Additionally, this study also performed functional analysis where upregulation of Th1 cell–related genes (Stat4 and Socs1), downregulation of Th2 and Th3/Tr type cell–associated genes (Il-4ra, Cebpb, Gata3 and Tgfb3), upregulation of Th1-type immune response gene (Sftpd) and downregulated of

Th1-type immune response gene (Tnf) was reported (See Table 1 in Gao et al. 2018 for information on gene's characteristics).

Cell surface expression

4 studies (Liu et al. 2001; Shan et al. 2007; Song et al. 2015; Liu et al. 2020) reported alteration in cell surface expression molecules in splenocytes and macrophages. Of these, 1 (Song et al. 2015) reported a decrease in CD86+ (DC cell surface marker) on day 2 which recovered on day 14 at all doses used in this study i.e., 0.001, 0.01 and 0.1 Gy, a decrease in CD28+ (T cell surface marker) on day 7 until day 14 at doses 0.01 and 0.1 Gy, and a decrease in CD69+ (NK cell surface marker) on day 2 after a dose of 0.001 and 0.01 Gy. 1 study (Liu et al. 2020) reported a decrease in CD86+ (DCs) and CD68+ (macrophages) where no significant change was found in T-cells expression marker at a dose of 0.01 Gy when analyzed at 24 hours. 1 study (Shan et al. 2007) reported an increase in CD14 and TLRL-MD2 expression at 16 hours post irradiation (0.05 Gy). The last study in this set (Liu et al. 2001) reported an increase in B7-1 (CD80) and B7-2 (CD86) expression on APCs at 12 and 8 hours respectively post irradiation (0.075 Gy). Moreover, upregulation of CD28 expression on splenocytes at 4 hours and on thymocytes at 75 hours with an opposite effect for CTLA-4 expression was also reported at 0.075 Gy.

NF-κB & MyD88 expression

Examination of nuclear translocation of NF-κB (p65/p50 heterodimer and p50/p50 homodimer) and the cytoplasmic adaptor protein MyD88 were immune endpoints that did not appear as commonly investigated in records screened for this systematic review. Only

one study (Shan et al. 2007) reported increased nuclear translocation of NF-κB & MyD88 expression at 16 hours post irradiation (0.075 Gy).

Oxidative stress

Although a fairly common stress signaling endpoint, only 1 study (Song et al. 2015) investigated oxidative stress in this systematic review. The study reported elevated levels of ROS-related molecules and Nrf2 and HO-1 expression which led to increased expression of iNOS and NF-κB activity after irradiation (0.001 and 0.01 Gy) on day 7.

Antigen presentation and uptake by DCs

Another not commonly encountered endpoint was antigen presentation and uptake by antigen presenting cells (APCs) such the dendritic cells (DCs). Only 1 study (Persa et al. 2018) reported reduced antigen presentation but no effect on antigen uptake by splenic DCs at 24 hr post irradiation (0.1 Gy).

Quality assessment of in-vivo animal studies

Risk of bias score on the SYRCLE RoB tool is displayed in Table 4. The blinding of caregivers, researchers, and outcome assessors (SYRCLE tool item # 5 and 6) remained unclear for all the in-vivo animal studies included in this review. Random outcome assessment (SYRCLE tool item # 7) measures were not performed in 1 study (8 %) while in the remaining 12 studies (92%) it was not clear if randomization techniques at the time of outcome assessment was followed. It was noticed that 3 studies (23%) clearly stated some type of randomization approach in animal housing (SYRCLE tool item # 4), while 10 studies (77 %) remained unclear if they housed the animals randomly. It was possible

to determine the incomplete outcome data reporting (SYRCLE tool item # 8) in 1 study (8%) only, while it remained largely unclear for the remaining 12 studies (92%). The baseline characteristics (SYRCLE tool item # 2) remained same for the experimental and control group for all studies (100%). The selective outcome free reporting (SYRCLE tool item # 9) was mentioned appropriately for 11 studies (91%) while it was unclear for 1 study (7 %). The researchers did not describe a random component in the sequence generation process (SYRCLE tool item # 1) in 11 studies (85%) where 2 studies (15 %) did well on the sequence generation technique. No study (100 %) adequately ensured allocation concealment to different groups (SYRCLE tool item # 3) or mentioned any other sources of bias (SYRCLE tool item # 10) in their study methods or approach.

Table 4: SYRCLE risk of bias assessment for in-vivo animal studies.

Criteria	Sequence generation	Baseline characteristics	Allocation Concealment	Random Housing	Blinding (Caregiver/ Researcher)	Blinding (Outcome Assessor)	Random Outcome Assessment	Incomplete Outcome Free Reporting	Selective Outcome	Other Sources of Bias
Chen 1999	×	✓	×	?	?	?	?	?	✓	?
Matsubara et al. 2000	×	✓	×	?	?	?	?	?	✓	?
Liu et al. 2001	×	✓	×	?	?	?	?	?	?	?
Liu et al. 2003	×	✓	×	?	?	?	?	?	✓	?
Li et al. 2004	×	✓	×	?	?	?	?	?	✓	?
Shan et al. 2007	×	✓	×	?	?	?	?	?	✓	?
Bogdandi et al. 2010	×	✓	×	✓	?	?	?	?	✓	?
Song et al. 2015	×	✓	×	?	?	?	?	?	✓	?
Li et al. 2015	×	✓	×	?	?	?	?	?	✓	?
Szatmari et al. 2017	✓	✓	×	✓	?	?	?	?	✓	?
Gao et al. 2018	✓	✓	×	✓	?	?	?	?	✓	?
Persa et al. 2018	×	✓	×	?	?	?	×	✓	✓	?
Liu et al. 2020	×	✓	×	?	?	?	?	?	✓	?

CHAPTER 4

4. Discussion

Radiation induced immune modulation may qualify to be a fitting example of Gestalt theory where the whole is something different than sum of its individual parts (Upton et al. 2014). It was realized that radiation induced immune suppression or enhancement is not a solitary event, the context is key, for instance, in some cases immune suppression may well be needed by tissue to curtail an out-of-control inflammatory response. This thesis brought to light the paradox related to the LDIR induced immune system modulations. The systematic review highlighted some of the oddities in research methods where the "like" was not compared to "like" in order to draw inferences about LDIR induced immune alterations. To that effect one more question stems from the original question with which this thesis project was initiated:

Is it possible to determine in a scientifically sound manner whether LDIR stimulates or suppresses the immune system?

The data related to LDIR induced immune alterations were gathered and synthesized in a systematic way. Immune endpoints which were investigated in the low dose region included cytokine secretion, gene expression, cell surface expression and quantitative changes in the immune cells when directly irradiated. Although the studies looking at indirect or non-targeted effects (NTEs) were fewer, a definitive response can be seen in the immune system after exposure to ionizing radiation in the low dose range via both direct and indirect means. Macrophage’s transmigration, chemotaxis and

phagocytosis, apoptotic and necrotic activity of the immune cells, metabolic reprogramming, antigen uptake and antigen presentation by DCs, crucial immune system pathways involving NF-κB and oxidative stress remained some of the less commonly investigated immune endpoints at low doses.

Surprisingly, only three studies investigated LDIR induced immune endpoints with a focus on NTEs despite their well-established role in the low dose range as reviewed in great detail in the introduction section. This is a potential area of concern, as NTE may greatly impact the final outcome of biological system as complex as the immune system. Endpoints like increased DNA damage in non-irradiated spleen when exposed to exosomes from irradiated BM cells, chromosomal damage in the bystander organism (Szatmari et al. 2017), increased cytokines production (IL-1β and IFN-γ) from spleno-lymphocytes when co-cultured with irradiated macrophages (Ibuki and Goto 1999) and decreased cell surface receptor expression (CD25 and CD40) in T cells were co-incubated with irradiated macrophages (Wunderlich et al. 2015) were the three studies in this systematic review associated with RIBE. Two of these studies (Ibuki and Goto 1999, and Wunderlich et al. 2015) made conclusive remarks about LDIR definitive role in the immune enhancement and activation respectively by way of an NTE mechanism, where one study (Szatmari et al. 2017) maintained a neutral stance towards radiation induced immune effects and no firm statement was made about it being beneficial or detrimental to the immune system. A noteworthy point is that the two studies with firm conclusions on the immune system were conducted in-vitro as opposed to the study that maintained a neutral stance which was conducted in-vivo. Ibuki and Goto (1999) reported

no significant change in spleno-lymphocytes when co-cultured with irradiated macrophages in their study, but significant changes were observed when an earlier study (Ibuki and Goto 1994) by the same research group that performed similar experiments with in-vivo design suggesting an in-direct mechanism at play which did not occur in-vitro. There is a need for research preferably in-vivo as it includes both direct and in-direct systemic effects as opposed to only in-vitro which predominantly allows for direct LDIR effects only (Jiang et al. 2008). Additionally, in-vitro experiments which make up the bulk of radiobiological literature provides a two-dimensional monolayer cell culture information only, which is more prone to missing the complexity of three-dimensional structures seen in-vivo (Tesei et al. 2013). The in-vivo proof will be more representative of the many immune processes triggered by LDIR at the systemic level in biological organisms (Liu et al. 2004; Mitchel 2006). There is a lack of standardized tools for the quality assessment of in-vitro and in-vivo studies, hence customized tools were used in this systematic review which revealed some surprising findings where all the included in-vitro studies conducted did not indicate important aspects of infection risk, cleansing agent and cleansing methods utilized.

Even though appreciation of the difference in biological response in the low dose region compared to high dose has increased, the number of studies that examine immune system effects in the low dose below 0.1 Gy remain small. This prevents the exploration of novel findings and may limit the full understanding of already identified processes with clinical implications. For instance, if there were well-researched randomized controlled human data on the therapeutic potential of LDIR, we may have been able to

reduce the death toll caused by the COVID-19 pandemic. In Germany, low-dose radiation therapy (LD-RT) is an approved and commonly utilized option in the treatment of degenerative inflammatory conditions and non-malignant disorders. (Seegenschmiedt et al. 2004; Schröder et al. 2019).

Immune system relevant genes and protein expression was investigated by 2 in-vivo studies (Chen et al. 1999 and Gao et al. 2018) and 2 in-vitro studies (Shigematsu et al. 2007; Cho et al. 2018). Chen et al. (1999) reported an increased upregulation of 4 proteins where 20 proteins were alternated suggesting LDIR's ability to alters and redistribute protein expression which may enhance immune responses. Gao et al. (2018) reported an increase in 8 cytokines related genes and decrease in 5 cytokine related genes related to Th1-Th2-Th3 responses where authors suggested that LDIR have immune increasing function via Th1 response induction. The variable gene expression shown in this study may be associated with the extraction of immune cells from different immune organs i.e., thymus and spleen as reported elsewhere that low dose γ-ray stimulated the thymocytes' maturation and inhibited splenocytes' DNA synthesis (Meng et al. 2005). A similar study that employed relatively higher dose (10 Gy) also reported differential expression of p53 protein dependent apoptosis in thymocytes and spleen cells (Chiang et al. 2012). Shigematsu et al. (2007) reported increased mRNA expression of Th1 cytokines (IL-2, IL-12 & IFN-γ) by DCs stating that LDIR activates T cells via Th1 cytokines, while Cho et al. (2018) reported an increase in IFNγ and IL-4 mRNA expression in CD4+ cells but a decrease in IL-17A, TGFβ1 and IL-10 where LDIR was considered immune enhancing via Th1/Th2 cytokines stimulation (IFN-γ, IL-4, IL-5), and

immune suppressive via Tregs cytokines suppression (TGF-β1, TGF-β3). While same doses were used in these studies to assess gene expression, but the dose rate was quite different as one study used 0.2 Gy/min while the other study used 2 Gy/min leading to variabilities related to dose rate. Furthermore, cytokine expression shift to and fro between pro-inflammatory and anti-inflammatory states after irradiation and these changes persist until immune mechanisms have dealt with challenges to host integrity (Schaue et al. 2012).

A relatively high dose rate of 1.0 Gy/min (Shigematsu et al. 2007) and 1.7 Gy/min (Li et al. 2015) was employed by two studies compared to other studies included in this systematic review. In particular, the appropriateness of this dose rate for the low doses (0.02, 0.05 and 0.1) used and how it could impact the final immune outcome needs further validation. In addition to these, 17 studies (see table 1 for details) used a single dose rate for a range of doses uses. It is proposed that a mention of dose rate for each dose category should have been done as seen in the study by Puukila et al. (2019), where dose rate was specified for each dose such as 2 mGy (20 mGy/min) 20 (15.6 mGy/min) 200 (15.3 mGy/min) and 4 Gy (2.2 Gy/min). This makes the dose rate employed for each dose very clear and is an important factor to consider when planning future experiments. Lumniczky et al. (2021) reviewed how initial inflammatory status of the irradiated tissue, radiation dose and dose quality applied in variable dose rate triggers discontinuous modulations in the immune system where low/intermediate dose typically shifts the immune response from a pro-inflammatory (Th1) towards an anti-inflammatory (Th2) state. The inconsistencies related to inter-laboratory variations in experimental set up,

radiation dose, dose rate, and time of analysis of immune endpoints post irradiation warrants standardizations in the field of radio-immune biology to better elucidate effects and mechanisms trigged by LDIR in the immune system (Cui et al. 2017).

A less investigated non-traditional endpoint was metabolic reprogramming where one study (Li et al. 2015) observed LDIR induced mild elevation in two metabolites and a significant reduction in one metabolite in activated T-cells while no change was seen in the typically investigated endpoints. The lack of response in typical endpoints was surmised to be due to compromised metabolic reprogramming induced by LDIR making T-cells more sensitive to associated damage responses. Hence, metabolic impairment, if any, led to lack of cellular responses such as proliferation and cytokine production but no firm conclusions about LDIR being immune-suppressive was made in this study. LDIR can induce a metabolic shift from oxidative phosphorylation to aerobic glycolysis, which is a relatively unrecognized response showing increased radioresistance in in-vitro and in-vivo experiments (Lall et al. 2014). Exploring these metabolic pathways may add valuable insights to cancer therapy and other disease management knowledge base.

Cytotoxicity was measured by two studies where Chen et al. (2010) used MTT and Yang et al. (2014) used the LDH assay for cytotoxicity measurement and both reported an increase in this response induced at a dose of 0.05 and 0.075 Gy respectively. The use of different assays may lead to different sensitivities in detection of cytotoxicity. A study compared the two assays and found that MTT assay was a more sensitive assay for the cytotoxicity detection than the LDH assay (Fotakis and Timbrell 2006). It was also

suggested that the use of more than one assay to investigate cell viability in vitro may increase the reliability of the study results.

As for the selection of immune cells to study radiation effects, human peripheral blood lymphocytes (HPBL) and human peripheral blood mononuclear cells (PBMCs) seems like a good choice as T-cells are a major fraction of these. They are highly radiosensitive nondividing cells with a low activity of protein, RNA, and DNA synthesis making them an appropriate cell population for radiobiological studies compared to their counterpart radioresistant nondividing cells (McWilliams et al. 1983). These cells are also widely used for detecting LDIR induced chromosome aberrations and for bio dosimetry assessments where a majority of studies that were excluded were conducted on HPBLs. Paraswani et al. (2018) states that investigation on unstimulated and non-dividing PBMCs, isolated from freshly drawn venous blood allows to capture early cellular events eliminating biases associated with transformed cell lines allowing for altered repair mechanisms and cell-cycle progression. Some of the older studies (Ibuki and Goto 1999; Chen et al. 1999; Matsubara et al. 2000; Liu et al. 2001; Liu et al. 2003) included in this review did not mention which lymphocytes' sub-populations were investigated, whereas newer studies did specify the exact population under consideration. This specification is important as CD4+ T cells are generally considered to play significant roles in immune system via cytokine release and cell-surface expression which activates other immune cells (Cho et al. 2018). Gruel et al. (2018) reports high sensitivity of CD4+ T-cells as alterations were observed 3 h post irradiation (0.05 Gy) whereas B-cells are typically seen

in low number in the circulating blood making them a poor choice for radiation induced immune studies.

As seen in the results section, some studies were stimulated with mitogens where some studies did not stimulate immune cells prior to irradiation. An unstimulated cell is primarily in the Go phase of the cell cycle (Shankar and Sainis 2005), it is not very clear if LDIR may have led to different findings in 17 studies (see Table1) that were not stimulated before radiation exposure. It is reported that relaxed chromatin which is less fragmented than heterochromatin repairs and misrepairs faster from LDIR (3 Gy) induced DNA damage compared to compact chromatin (Mosesso et al. 2010). Additionally, this study also reported how different chromosomes demonstrated differential distribution of DNA break in the human chromosomes 18 and 19 in both G0 and G1 phases of cell cycle. Although a higher dose was used in this study, future experiments will be needed to verify this effect in the low dose region. Fachin et al. (2007) emphasizes on the importance of investigating radiation induced effects in proliferating lymphocytes since these effects may persist for a longer time, especially in cells undergoing one or two cell divisions. There is also data reporting that prior stimulation of lymphocytes with a mitogenic compound before irradiation triggers DNA damage response such as phosphorylation of the histone H2AX and ATM activation (Moreno-Villanueva et al. 2018). Moreover, there is also HRS response which depends on cell cycle phase as cells are more radiosensitive at doses below 0.2 Gy (Piotrowski et al. 2017), another factor worth considering while designing radiation induced immune experiments. 3 in-vitro studies (Wunderlich et al. 2015; Wunderlich et al. 2018; Frischolz et al. 2013) utilized

LPS stimulation for macrophages which is primarily a B-cell mitogen and hence this may have led to less responsiveness to LPS leading to a reduced cytokine secretion that facilitate lymphocyte activation (Leshchinsky and Klasing 2001). Two studies (Ibuki and Goto 1999, and Liu et al. 2001) used Concanavalin A (Con A) which seems like a good mitogen choice as it causes a higher proliferative response when compared to other typically used mitogens such as PHA (Joling et al. 1993). It is reported that CD3/CD28 stimulation is better adapted at LDIR induced damage repair compared to non-stimulated counterparts via DNA crosslink repair efficiency (Heylmann et al. 2018). An old study reports similar findings where CD4+ T-cells became radioresistant with prior stimulation with mitogen (Stewart et al. 1988). In this systematic review, one study (Cho et al. 2018) stimulated CD4+ cells with CD3/CD28 antibodies manifesting both immune-stimulating and suppressive responses. A limitation of potential radioresistance which may be acquired by CD4+ was not addressed by this study. Contrastingly, prior stimulation of immune cells before irradiation may lead to an enhanced CD4+ and CD8+ T-cell activity suggesting a facilitative role in the overall response which may have implications in virus-specific or auto-immune T-cells. (Spary et al. 2014).

Time lapse studies over a long course is also an important consideration where a response at a particular time point may reveal opposite findings at a later time mainly with LDIR experiments where primary non-lethal damage may lead to secondary biological processes such as genomic instability and transcriptional changes (Fachin et al. 2007). For instance, two studies (Li et al. 2003 and Shan et al. 2007) were interesting with respect to time lapse as it tracked changes over a long course compared to investigating

outcomes at one time point or two time points, as was the case with most studies included in this review (See Table 1 for details).

By and large, three strains of mice (BALB/c, C57BL/6, and Kunming) were consistently used in the in-vitro and in vivo animal studies included in this review but two studies (Hosoi et al.2001; Gao et al. 2018) used different strains such as inbred albino strain (WHT/Ht) and ICR mice respectively while one study (Cho et al. 2018) did not specify the strain of animal used. Shankar et al. (1999) reports alteration in the immune response when LDIR (a total dose of 0.2 Gy) was delivered to two different strains of mice i.e., C57BL/6 and BALB/c. Bhilwade et al. (2004) found a high magnitude of variability in LDIR induced DNA damage among seven strains of mouse at different doses. Hence, this is an important factor to pay attention to when interpreting the results from the animal data presented in this systematic review.

Of in-vivo human data, only 1 study (6 males and 4 females) mentioned the gender of the samples. 3 studies in the in-vitro and 11 studies in the in-vivo animal studies mentioned the gender in their methods section (See Table 1). While all the in-vitro animal studies had cells extracted from male population, the in-vivo animal studies comprised of 7 studies that recruited male population, 2 studies with females and 2 study included a mix of both genders. Gender-specific differences in radiation responses are currently understudied in the field of radiobiology despite evidence of it being a significant confounding variable in biological studies (Jones et al. 2019) and variable modulations in the immune system induced after an ionizing radiation exposure (Kusunoki 1994; Hall and Garcia 2012). As for sample size, Bogdándi et al. (2010) was

the only study that recruited a large sample of 262 mice making it a good study in terms of capturing the inter-individual variability. It is very common to imagine that a large sample size is needed for a good power analysis of a research study, but Karp (2018) presents an alternative view where valid and reproducible pre-clinical research is possible without a large sample size if the animal experiments are appropriately designed with robust and reproducible methods. Festing (2018) reports of $28 billion going to waste per annum in the United States because of the pre-clinical animals' research producing results that are irreproducible and therefore there is a need to establish tools to determine appropriate sample size for laboratory animals. Additionally, interesting statistics were presented where 271 papers were scrutinized for their methods and showed that 87% did not report random allocation of experimental subjects to the treatments and 86% did not report blinding when measuring these outcomes. Moreover, these studies did not give any justification for their choice of sample size, and a substantial number of papers failed to state the sex, age or weight of the animals leading to a high false-positive rate. The author also expressed concern of how too many animal experiments are under-powered which leads to a high false-negative rate in their results.

This agrees with the quality assessment (risk of bias) results of the studies included in this systematic review. Not a single in-vitro study provided justification of sample size for either experimental or control groups which is required to determine statistical significance. Only 6 animal studies reported animals' weight and none of the human in-vitro study mentioned weight of their included samples introducing biases

associated with variable weight, for instance over-weight study samples receive a greater effective dose compared to normal weight samples (Cornacchia et al. 2020).

Factors that inevitably affect host immune responses is the omnipresence of environmental stressors constantly putting strains on their defense system such as noise, heat, infection, light etc. (Robinson and MacDonell 2004). This leads to a mixed type of effect during radiation studies, where some unnoticeable stressors which often appear insignificant, will cause significant effects when mixed with LDIR. In order to better report radiation and immune interactions, the dose-response relationship at the level of individual exposure down to the cellular level and from there back up to the organismic level as a whole with the consideration of complex physiological processes potentially interfering with the final response must be fully understood (Robinson and MacDonell 2004).

Systematic review and meta-analysis are still a novel idea in pre-clinical research (Sena et al. 2014). Too many preclinical experiments, although provides empirical evidence, lack methodological rigor leading to inflated treatment effects (Sena et al. 2014). Systematic reviews and meta-analyses sit at the top of hierarchy of evidence-based medicine, a concept first introduced by David Sackett in 1996 (Sackett et al. 1996). There is substantial amount of literature that considers this research design as the highest quality of evidence possible in answering a research question with clinical implications (Borgerson 2009). Although, biases are inherent in any systematic review, but the transparency of the methods used are designed to reduce bias where the main principle is that an independent researcher should be able to perform the same review and yield the

same data. It was the intention of the author to statistically synthesize findings from the individual studies via meta-analysis. However, great many differences in the experimental design of the included studies were noticed and was the reason that made it rather impossible to quantitively synthesize studies via meta-analysis. Factors related to experimental techniques such as timing of analysis after irradiation, range of doses used, dose rate employed, sample size, age, gender, and protocol used for analysis were the main source of differences. Haidich (2010) states that systematic reviews may not contain a meta-analysis and there are situations when it is not appropriate or possible.

Altogether, the data presented above does not clearly answer our questions due to the inherent heterogeneities in the experimental designs where lack of standardized experimental techniques made it impossible to answer both our initially laid out question and the question posed in the beginning of the discussion in a scientifically sound manner. A linear dose response relationship of immunological parameters does not exist in the low dose range, let alone the variations in researcher's experimental techniques and methodologies. However, this systematic review did show that LDIR (<0.1 Gy) have different effects at different doses and dose rates where some immune responses manifest discontinuous characteristics due to many molecular mechanisms at play initiated at different threshold doses following variable kinetics (Rodel et al. 2012). Another factor that complicates this enigma is the growing evidence on the role of NTEs in the immune system modulations induced by LDIR.

4.1. Strengths and limitations of the systematic review

The current systematic review has several strengths. This is the first review to explore immune system modulations induced by LDIR of less than 0.1 Gy. Twenty-four studies met the pre-set inclusion criteria, where most studies investigated immune endpoints including immune cells' subpopulation, cytokine secretion, immune relevant gene expression and cell surface molecules expression while some of infrequently investigated endpoints included apoptotic activity, metabolic reprograming and chromosomal aberrations. This systematic review followed the PRISMA guidelines except the involvement of a second reviewer and protocol registration.

The methods related limitation of this review is that the screening stages of title, abstract and full-text screening was performed by one reviewer (A.D) only. Recent literature suggests that a single-reviewer screening in a systematic review may miss substantial number of eligible studies when compared to dual-reviewer approach (Gartlehner et al. 2020; Waffenschmidt et al. 2019). Although an acceptable accelerated approach due to resources, time, and budget constraints, it must be noted that single-reviewer systematic reviews do not yield results of a high methodological standards that are typically expected from a systematic review.

Content wise, this review did not include the long time-course epidemiological studies with large population size exposed to acute and chronic low dose radiation from accidental and natural sources. A great bulk of such studies reported low dose radiation induced genomic instability (RIGI) which was considered detrimental, while in some cases RIGI is hypothesized to be beneficial when mutations allow for fitter population or

better phenotypes in un-irradiated progeny from irradiated parents. Additionally, radio adaptive response (RAR) studies were excluded, which may have missed significant beneficial effects of LDIR. RIGI and RAR may serve to be incredibly interesting topics for future systematic reviews with a possibility of meta-analysing the data, provided standardized synthesis protocols are created for pre-clinical radiobiological research. Lastly, since all the cancer and transformed cell lines were excluded from this systematic review, valuable insights on radiotherapeutic potential of LDIR in the field of cancer care may have been missed.

4.2. Future directions and conclusion

It became clear with the writing of this review that there has been a great shift in our understanding of the radiation induced immune effects over time and that this effect is multifarious and cannot be investigated in isolation. Throughout the literature search to gather data for this review, terms such as dualism, biphasic, dichotomous, duplicitous and many more synonymous terms for the "double-edged sword" type of modulation demonstrated by immune components when exposed to radiation, were encountered. Although, some cells such as the T-cell (specific subsets only), macrophages, dendritic cells have been established as elements that are definitely stimulated or suppressed to perform their role when stresses are encountered, involvement of other cells such as the B-cells, neutrophils, Tregs and Bregs remain to be fully understood. This thesis project sparked great interest to untie many complexities inherent in the framework of immune functions and how it intersects with radiobiological intricacies.

The systematic review highlighted some loopholes in the current literature surrounding radiobiological experimental research which is not rigorous and standardized. Therefore, the work of this thesis project may be used as a starting point to streamline some of the erroneous methodologies in LDIR induced immune studies. Some interesting facets that were found in the literature but could not be explored further were radiation triggered immune effects from occupational exposure, natural background sources, large-scale epidemiological studies and radio adaptive response unraveling interesting beneficial and detrimental effects to the immune system. As mentioned in the methods sections, a lot of the excluded studies from this systematic review (data available upon request) investigated LDIR effects on lymphocytes which is a heavily researched immune system component due to high radiosensitivity and convenience of being in large number in the peripheral blood. These studies seem to have been conducted with a non-immune goal without any mention of what their results meant for the radiation induced immune system modulations. Although many researchers are reviewing immune system mechanisms triggered by LDIR in a narrative way and great many reviews are written on this topic (see introduction), experimental work with LDIR focusing on immune responses is still scarce.

Appendices

Appendix 1 Prisma guidelines

PRISMA-2009-Checklist-MS-Word.pdf

Appendix 2 MEDLINE

Medline Appendix.pdf

Appendix 3 EMBASE

Embase appendix.pdf

Appendix 4 Web of Science

Advanced Search - Web of Science Core

Appendix 5. Search Strategy, ran on <June 10, 2019>

Concept 1 (Radiation)	Concept 2 (Immune System)	Concept 3 (Effect)
Low Dose Radiation	Immune system	Immune-enhanc*
Low Dose Ionizing Radiation	Immunity	Immune-stimulat*
Low Dose Irradiation	Antigen-presenting cells	Immune response
Low Dose X-irradiation	T-lymphocytes	Hormesis Effect
Low Dose Gamma-irradiation	T-cell population	Antigen Presentation
Low dose X-rays	T-Lymphocyte Subsets	Lymphocyte Activation
Low dose Gamma Rays	T-Lymphocytes, Regulatory	Macrophage Activation
Low dose radiotherapy	Lymphocytes	Radio-adaptive Response
Low dose Occupational Exposure	CD4-Positive T-Lymphocytes	Anti-inflammatory effects
Local irradiation	CD8-Positive T-Lymphocytes	Oxidative Stress
Targeted radiation	Cytotoxic T cells	Immune-Suppress*
	Leucocytes	Mutation
	T-Lymphocytes, Helper-Inducer	Chromosomal Instability
	B-Lymphocytes	Chromosome Aberrations
	Macrophages	Sister Chromatid Exchange
	Dendritic cells	DNA Damage
	Monocytes	DNA Repair
	Natural Killer Cells	DNA Break
	Cytokines	Micronucle*
	Hematopoietic Stem Cells	Micronucleus Tests
	Hematopoietic System	Radiation Induced Bystander Effect
	Innate immun*	Radiation Induced Genomic Instability
	Adaptive immun*	Non-targeted radiation effects
		Non-targeted effects of radiation
		Abscopal effect
		Macrophage Polarization
		Apoptosis
		Cell cycle progression
		Radiation injuries/im [immunology]
		Gene expression
		Cell proliferation

Appendix 6: Methodological Index for In Vitro Studies

1. Clearly stated purpose: The question addressed in the study is explicitly stated and testable by statistical means
2. Adequate control groups: Because graft contamination can occur without dropping, 2 control groups exist for in vitro studies
   - 0: control groups not adequately described
   - 1: control group of non-contaminated graft OR non-sterilized graft
   - 2: control groups of non-contaminated graft AND non-sterilized graft
3. Graft type: Description of graft material (tissue type, autograft/allograft, and devitalized/viable)
4. A priori power analysis: Justification of sample size for both experimental and control groups needed to determine statistical significance
5. Appropriate statistical analysis: Description and implementation of statistical tests appropriate to dataset with reported *P* values
6. Unbiased assessment of outcome: Objectivity of methodology/evaluator used to determine successful sterilization
   - 0: no description of outcome criteria AND evaluator
   - 1: qualitative/subjective methodology (turbidity, culture plates) with unblinded evaluator
   - 2: qualitative/subjective methodology with blinded evaluator (must be explicitly stated as blinded) OR quantitative methodology (RT-PCR, photospectrometric assays, automated counting)
7. Contaminant identity: The organism type and quantity used for inoculation are described
   - 0: not described
   - 1: organism type OR organism quantity (if graft dropped on floor, organism type may be determined by swab of operating room floor and reported in results)
   - 2: organism type AND organism quantity
8. Infection risk: Intraoperative graft contamination is most commonly due to dropping, but in vitro studies may expose grafts to inoculum concentrations exceeding those encountered in clinical scenario
   - 0: not described
   - 1: graft dropped on floor OR inoculated with flora at same concentration as that found on operating room floor ($<10^5$ CFU/organism)
   - 2: graft inoculated with concentrated bacterial load that exceeds that found on operating room floor ($\geq 10^5$ CFU/organism)
9. Culture method: Description of how contaminated grafts were cultured in sufficient detail to repeat (or detailed methodology is referenced)
10. Culture time: Because some infections may develop slowly, longer intervals of culture may be needed to ensure successful sterilization
    - 0: not reported
    - 1: culture of $\leq$6 d
    - 2: culture of $\geq$7 d
11. Cleansing agent: Description of agent used for sterilization, including identity, concentration, and volume
12. Cleansing method: Description of physical or mechanical conditions in which the graft was sterilized, including cleansing time

Note: References in steric were included in the systematic review.

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