DISSERTATION

LONG-TERM HEMATOPOIETIC RESPONSE IN LEUKOCYTE COUNTS AND DIFFERENTIALS FOR RHESUS MACAQUES (*MACACA MULATTA*) FROM ACUTE WHOLE-BODY RADIATION EXPOSURE

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ABSTRACT

LONG-TERM HEMATOPOIETIC RESPONSE
IN LEUKOCYTE COUNTS AND DIFFERENTIALS
FOR RHESUS MACAQUES (*MACACA MULATTA*)
FROM ACUTE WHOLE-BODY RADIATION EXPOSURE

Accumulating evidence from A-bomb survivors and radiation therapy patients suggest that survivors are at risk of developing delayed effects of acute radiation exposure (DEARE). In contrast to acute radiation syndrome (ARS), the underlying mechanisms of DEARE are largely unknown. Better understanding of DEARE is vital for improving estimates of risk and predictions of long-term health outcomes following a variety of radiation exposure scenarios, whether accidental or intentional, and including nuclear accidents, cancer treatment, and space travel. The hematopoietic system is highly sensitive to ionizing radiation (IR) exposure; leukocyte counts reach a nadir in days to several weeks post-acute exposure, followed by a recovery period from 4-8 weeks to a year. Accumulating evidence from the A-bomb survivor cohort and animal studies suggests residual damage in the hematopoietic system persists for a long time. Long-term effects in hematopoietic system are very likely the underlying cause of DEARE disease, although there is limited understanding of the process. In this study, archival leukocyte counts and differentials from the Non-Human Primate Radiation Late Effects Cohort (RLEC), were analyzed to evaluate long-term effects. The RLEC cohort consists of over 200 Rhesus Macaques (*Macaca mulatta*) previously exposed to acute whole body irradiation from 1.14 to 8.5 Gy and approximately 50 control animals. The dataset was created from blood sampling started approximately 1 year post-exposure and continued every 2-6 months. Linear mixed models were developed for total

leukocyte count and the differentials including neutrophil, lymphocyte, and monocyte counts and their percentages.

Preliminary analysis was conducted for animals with the same dose level, sex, and age at the time of exposure and age and sex matched control animals. The linear mixed models had statistically significant elevations in leukocyte and neutrophil counts and neutrophil% in irradiated animals compared to the controls. Lymphocyte% was significantly lower in irradiated animals. Longitudinal trends for both control and irradiated animals were consistent with expected trends of aging in hematopoiesis, which is skewed towards production of myeloid lineage cells such as neutrophils and monocytes rather than lymphoid cells. There was no statistical difference among the longitudinal trends of control and irradiated animals.

Next analysis was extended from the preliminaly analysis with a larger dataset including animals with different dose, sex, age at the time of exposure, as well as mitigator assignment. Longitudinal trends were estimated for different dose levels (control, <LD10; 1.14 to 5.5 Gy, LD10-LD50; 5.5 to 6.8 Gy, >LD50; 6.8 to 8.5 Gy), and adjusted for sex, age at the time of exposure, and status of mitigator use. All models suggested that dose levels were a statistically significant factor for the longitudinal trends of leukocytes and the differentials. Controls showed a slight decrease of total leukocyte count and monocyte skewed differentiation, consistent with changes estimated from aging in hematopoietic system. The <LD10 animals were very similar to the controls. However, estimates for LD10-50 animals demonstrated a statistically significant increase and decrease in percentages of neutrophils and lymphocytes, respectively. The increase in monocyte counts was larger for LD10-50 and >LD50 animals than controls and <LD10 animals.

Elevated neutrophil and monocyte counts are indicative of inflammation status and/or a skewing of the immune system from innate to more adaptive. Long-term impairment of the

immune system could contribute to DEARE disease, including cancer and cardiovascular disease, which were observed more frequently in irradiated animals. Extended analyses include developing and testing machine learning models to improve accuracy of predictions related to a number of radiation biomarkers and health outcomes, key steps toward improved understanding and risk assessment of DEARE.

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1 Introduction

Leukocytes are one of the circulating blood cells and function for innate and adaptive immune system (Hoffman et al., 2017). After ionizing radiation doses over 0.7 Gy, the hematopoietic system, especially leukocytes, demonstrates the greatest response in cell counts (Dainiak, 2002; Hall and Giacia, 2019). The leukocyte count in peripheral blood declines in a few hours to days after irradiation because of apoptosis due to radiation damage as well as relocation to organs and tissues to fight against radiation injury. The decreased number of leukocytes are typically replenished by accelerated maturation from progenitor cells in bone marrow. When the dose is higher than 3.5 Gy, the hematopoietic stem cells could be destroyed and the progenitor cell pool may not be sufficient to replenish the circulating cells, leading to immune dysfunction and other hematologic deficiencies, possibly causing death. If the radiation dose is below the significant hematopoietic stem cell ablation threshold, leukopenia conditions gradually recover over a month to a year. The acute hematopoietic response depends on not only the exposed dose and dose rate but also the species, age of the individual as well as exposure scenarios (Kaur et al., 2017, MacVittie et al., 2020, Farese et al., 2021, Patterson et al., 2022).

Survivors of acute radiation syndrome (ARS) often experience delayed effects of acute radiation exposure (DEARE). DEARE is suspected as a cause of high disease morbidity in exposed cohorts including A-bomb survivors, Chornobyl workers, radiotherapy patients, and nuclear workers (Cardis and Hatch 2011; ICRP 2012; Ozasa et al., 2012). Long-term followup studies on A-bomb survivors and radiotherapy patients provided information on high risks especially in neoplasm, cardiovascular disease, myelodysplastic syndrome, as well as pulmonary and thyroid dysfunction (Armstrong et al., 2010; Kamiya et al., 2015). Different from acute radiation syndromes, there are no countermeasures available so far, and the mechanistic understanding of

the etiology is limited (MacVittee, 2023; Wu and Orschell, 2023).

In vivo murine studies established DEARE on the hematopoietic system as residual bone marrow damage, inducing impaired self-renewal and decreased repopulation of hematopoietic stem cells (HSC) as well as myeloid skewed differentiation of HSCs (Wang et al., 2006, Chua et al., 2012, Shao et al., 2014, Chua et al., 2019). These studies also reported persistent depletion of leukocyte and differentiation counts, such as neutrophils and lymphocytes. Most studies on delayed and long-term effects on the hematopoietic system are limited to mice, while emerging evidence from non-human primate (NHP) studies also supports delayed effects on hematopoietic system. For example, Hale et al (2019) reported prolonged effects to hematopoietic and immune systems of NHPs, including increased WBC count, and decreased platelets and T-lymphocytes, 2-5 years after a single total body irradiation (6.5-8.4 Gy).

The delayed or persistent effects of radiation exposure on the hematopoietic system are also observed in human cohorts. Long-term follow up studies on the A-bomb survivor cohort reported elevation or depletion in counts of leukocyte and lymphocyte and the subpopulations in correlation with radiation dose after >40 years of exposure (Kusunoki et al., 1998; Neriishi et al., 2001; Hayashi et al., 2003). Chornobyl clean-up workers had no evidence of differences in leukocyte, lymphocyte (%) and monocyte (%) counts yet showed prolonged depletion of T-lymphocyte subset populations 10 years after exposure (Kurjane et al., 2001). Studies on Chornobyl residents (Stepanova et al., 2008), nuclear workers, astronauts, and patients undergoing radiotherapy also provided evidence of long-term impact on hematopoietic and immune systems (Chernyshov et al.; 1997, Watanabe et al. 1997; Senyuk et al., 2002; Crucian et al. 2015; Wersal et al., 2019; Luxton et al., 2020; Wang et al., 2022), while the effect varies with many factors including the exposure scenario, dose and dose rate, and the exposed population. Persistent effects on the hematopoietic

system may be associated with high morbidity and mortality in exposed individuals. Additionally, features, such as dose response, sex, and age at exposure, are often associated with disease risk as demonstrated in A-bomb survivor cohort studies (Douple et al., 2011) and are often missed in the investigations.

Longitudinal analysis is useful to understand changes in the response of systems over time as well as the causal relationship (Caruana et al., 2015). Longitudinal analysis is also advantageous for analysis of long-term effects on the hematopoietic system because hematopoietic systems change due to aging of animals (Snoeck 2013). Since residual bone marrow damage from radiation exposure is suspected to resemble the effect of aging in the hematopoietic system (Richardson, 2009; Al-Jumayli et al., 2022),a robust statistical method is required to investigate the long-term effect of ionizing radiation to the hematopoietic system.

Linear mixed effect models (LMMs) are one of the best methods for longitudinal analysis of a dataset with repeated measurements (Edwards 2000). LMMs have advantages over commonly used methods such as repeated measures ANOVA and generalized estimate equations especially when the dataset contains missing observations (Park 1993, Boisgontier and Cheval, 2016). Hsu et al. (2010) first applied LMMs to the leukocyte count and the differentials of the A-bomb survivor cohort. They reported elevated total leukocyte counts in the >2 Gy exposed group persisting over 50 years after exposure. Yoshida et al. (2019) also conducted longitudinal analysis with linear mixed models and reported increased monocyte percentage in the older population of the survivor cohort, indicating delayed effects from ionizing radiation damage to hematopoietic system. No other studies with linear mixed models exists for longitudinal analysis on A-bomb survivor cohort. The A-bomb survivor cohort does have limitations. Lifestyle factors such as BMI and smoking status, as well as medical history can affect the leukocyte count between individuals

(Nagasawa et al., 2004; Abel et al., 2005), and some information is not completely available. Additionally, the dataset of the A-bomb survivor cohort is from blood samples taken only once every few years and sometimes one person had only one sampling, which is not ideal for the longitudinal analysis of leukocyte counts because leukocyte counts change on a daily and hourly basis.

The Radiation late effect study cohort (RLEC) at Wake Forest University includes over 200 Rhesus macaques (*Macaca mulatta*). Some animals were exposed to whole body ionizing radiation prospectively, or served as controls under different research projects at different facilities, and moved to the university for long-term follow up study. NHP models have been used to analyze health effects and immune responses associated with ionizing radiation exposures (Farese et al., 2012; MacVittie et al., 2014) because they have hematopoietic and immune systems similar to humans and they have a relatively long-life span (~20 years) (Monroy et al., 1986). Advantages of utilizing NHPs for longitudinal analysis include an easily controlled lifestyle, and complete medical records are available. Moreover, accumulating studies of late effect disease and organs in the RLEC cohort, such as neoplasms, diabetes mellitus, heart, brain, and kidney are available (Debo et al., 2019; Bacarella et al., 2020; Andrews et al., 2020; Sills et al., 2022; Schaaf et al., 2023), which is an asset to understand the results of longitudinal analysis as an underlying cause of DEARE disease development.

In this study, longitudinal analysis was applied to an archival data set of leukocyte counts and differentials from an NHP cohort with acute radiation to understand the long-term effects of radiation exposure to the hematopoietic system. Confounding factors including different doses, sex, age at the time of exposure, and ARS mitigator use were studied to determine factors influencing long-term effects. The current study will provide valuable translatable information for

improving understanding of long-term responses of the hematopoietic system following radiation exposure. Unfortunately, the cohort did not have significant differences in dose rate, therefore dose rate could not be studied.

2 Aims and hypothesis

This study investigated the overall hypothesis that:

1. Long-term hematopoietic effects from acute radiation exposure occur in NHP cohorts.
2. High- dose exposed cohorts experience stronger effects than low dose cohorts (<LD50).
3. Sex, Age at the time of exposure, and mitigator use may also affect the long-term hematopoietic system response.

To test the hypothesis, the specific aims are to:

1. Investigate the influence of radiation exposure on long term effects on leukocyte counts and differentials from whole body irradiation (WBI)

   Hypothesis: Long-term effects on the hematopoietic system from WBI exist and are presented as a longitudinal trend of leukocyte counts and the differentials.

2. Investigate the influence of different doses, sex, age at exposure and use of mitigator for the long-term after WBI

   Hypothesis: Higher dose exposure (>LD10) causes more significant effects on the longitudinal trend of leukocytes and the differentials. Female animals, animals younger at the time of WBI exposure, and animals with mitigator assigned for ARS have more significant effects on the longitudinal trend.

The first aim is addressed in Chapter 4 as a preliminary analysis and the second aim is in Chapter 5.

3 Literature Review

3.1 Hematology

Blood circulates throughout the body via veins and vessels, delivering oxygen, nutrients, and signaling molecules to tissues and organs, as well as collecting wastes from tissues and organs (Schmaiser, 2019). The main components of the circulating blood are plasma, red blood cells (erythrocytes), white blood cells (leukocytes) and platelets (thrombocytes) (Figure 1). Blood plasma is the liquid component with 90% water and the rest contains nutrients, electrolytes, and proteins. Erythrocytes, leukocytes, and platelets are the cellular components of blood. Erythrocytes have no nuclei and carry oxygen to tissue and organs. Platelets also have no nuclei and are key to blood clotting. Leukocytes are composed of granulocytes, lymphocytes, and monocytes (Figure 1-3) and are the main constituents of the immune system. Leukocytes can move from the blood to tissue to fight against infection/damage. Granulocytes are the most abundant type of leukocytes and classified into neutrophils, basophils, and eosinophils. Neutrophils are the first responder of the innate immune system. Basophils hunt for parasites, Eosinophils for parasites and allergic reactions. Wright-Giemsa stained granulocyte cytoplasm has granules stained light blue (neutrophils), red (eosinophils), and dark blue (basophils). Lymphocytes are classified as T-lymphocytes, B-lymphocytes, and NK (natural killer) cells. T-lymphocytes can be classified as helper T-cells, which help activate other immunologic cells and reactions in adaptive immunity, and cytotoxic T-cells, which can destroy any cells with infections. B-lymphocytes turn into plasma cells upon activation, producing antibodies. NK cells are part of the innate immunity, and destroy cells infected by virus. Monocytes mature into macrophages and dendritic cells in tissues and organs and are an important modulator in both innate and adaptive immune response, presenting antigen to T-cells and secreting cytokines to activate other cells. Immune cell functions are

reviewed in Chapter 3.3 with immune system.

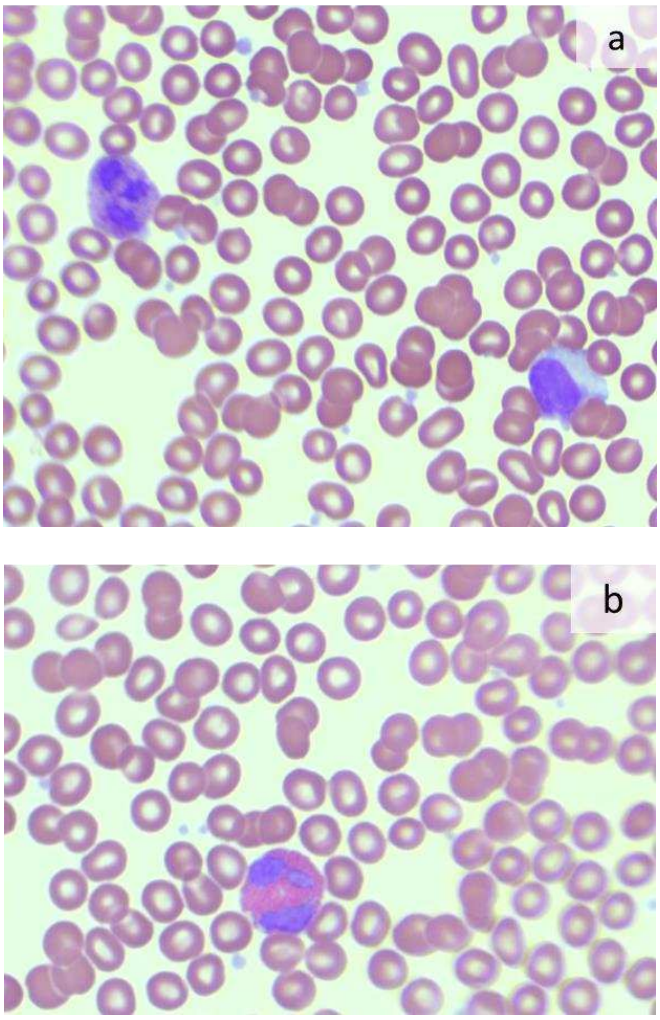

Figure 1: Picture of human peripheral blood smear.
Wright-Giemsa stained. (a) red blood cells and platelets with a neutrophil (on the left) and monocyte (on the right). (b) red blood cells and platelets with an eosinophil.

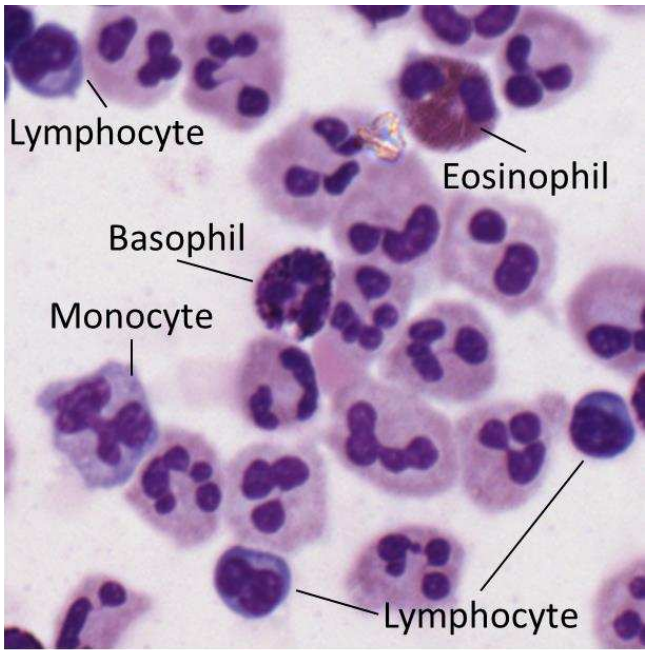

Figure 2: Human peripheral blood leukocytes.
Centrifuged, and leukocytes extracted, and Wright-Giemsa stained
All the other cells except lymphocyte, monocyte, eosinophil, and basophil are neutrophils.

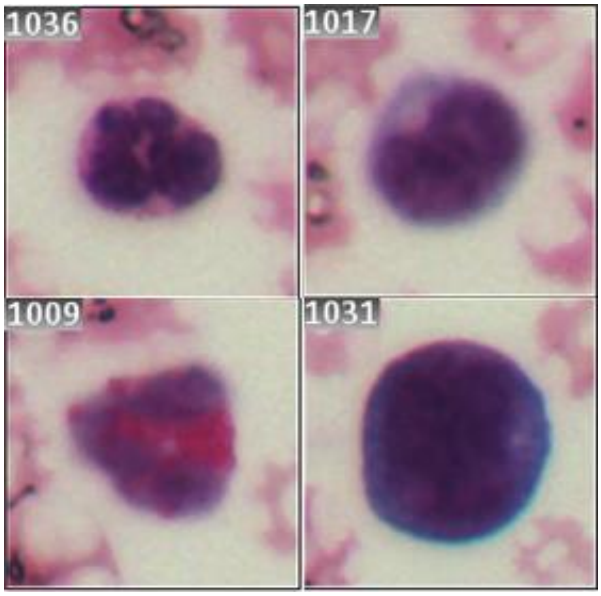

Figure 3: Rhesus Macaque's leukocytes.
Peripheral blood smear with Wright-Giemsa stain. Clockwise from the right-top corner, lymphocyte, monocyte, eosinophil, and neutrophil

Mammalian blood is composed of 55% blood plasma and 45% cellular elements. Among the cellular elements in circulating blood of adult humans, 95% are erythrocytes, 4.7% are platelets, and 0.3% are leukocytes. Leukocytes consist of neutrophils (40-60%), lymphocytes (20-

40%), monocytes (2-10%), eosinophils (1-6%), and basophils (<1-2%). (Table 1) (Bates et al., 2017). The count and ratio of each cellular element are similar between NHPs and humans (Koo et al., 2019). The number and ratio of the cells per unit circulating blood differs among individuals, being influenced by many factors including age, sex, and environmental conditions. For humans, factors such as smoking and body mass index (BMI) also influence blood cell counts (Nagasawa et al., 2004; Abel et al., 2005). Blood cell counts can vary for the same individual by as much as 100% at different blood draw times, reflecting physiological changes in the body from exercise, diet and medication. Leukocytes typically increase in number whenever the organism is fighting infection. Neutrophil and lymphocyte counts are inversely related; when neutrophil count increases, lymphocyte counts decreases and vice versa. Neutrophil-to-lymphocyte ratio (NLR) is widely used to assess the function of innate (neutrophils) and adaptive (lymphocytes) cellular immune response (Zahorec, 2021).

Table 1: Standard hematologic cell counts and percentages for normal human adults and nonhuman primates
(Data from Bates et al., 2017 and Koo et al., 2019)

Adult human

Cell type	Counts per 1L	Leukocyte cell type	Counts per 1L	%
Erythrocytes	$5.0 \pm 0.5 \times 10^{12}$			
Platelets	$280 \pm 180 \times 10^{9}$			
Leukocytes	$4.0–10.0 \times 10^{9}$	Neutrophils	$2.0–7.0 \times 10^{9}$	40-80
		Lymphocytes	$1.0–3.0 \times 10^{9}$	20-40
		Monocytes	$0.2–1.0 \times 10^{9}$	2-10
		Eosinophils	$0.02–0.5 \times 10^{9}$	1-6
		Basophils	$0.02–0.1 \times 10^{9}$	<1-2

Adult Rhesus Macaques (male)

Cell type	Counts per 1L	Leukocyte cell type	Counts per 1L	%
Erythrocytes	$5.4\pm 0.7 \times 10^{12}$			
Platelets	$320 \pm 100 \times 10^{9}$			
Leukocytes	$3.1–12.1 \times 10^{9}$	Neutrophils	$1.3–8.9 \times 10^{9}$	34-88
		Lymphocytes	$0.4–5.5 \times 10^{9}$	5.5-59
		Monocytes	$0.06–0.5 \times 10^{9}$	0.8-6.2
		Eosinophils	$0.09–0.7 \times 10^{9}$	1.5-8.6
		Basophils	$0.00–0.02 \times 10^{9}$	<0.3

3.2 Hematopoiesis

Hematopoiesis is the process where blood cells are differentiated from hematopoietic stem cells (HSCs) (Figure 4) (Ghiaur and Jones; 2019). Hematopoiesis begins at the early stage of development (mid-gestation) mainly in the liver, while this review focuses on adult hematopoiesis, which occurs in the red bone marrow of bones of the ribs, vertebrae, sternum, and pelvis, as well as lymphoid tissue including thymus and spleen (Jagannathan-Bogdan and Zon 2013). The hematopoietic processes are conserved among mammalian species (Orkin and Zon; 2008). The hematopoietic system maintains the homeostasis of circulating blood cells, which have a quite short life span and high turnover rate (Table 2), as well as to fight against emergencies of the organism, such as injury and infection.

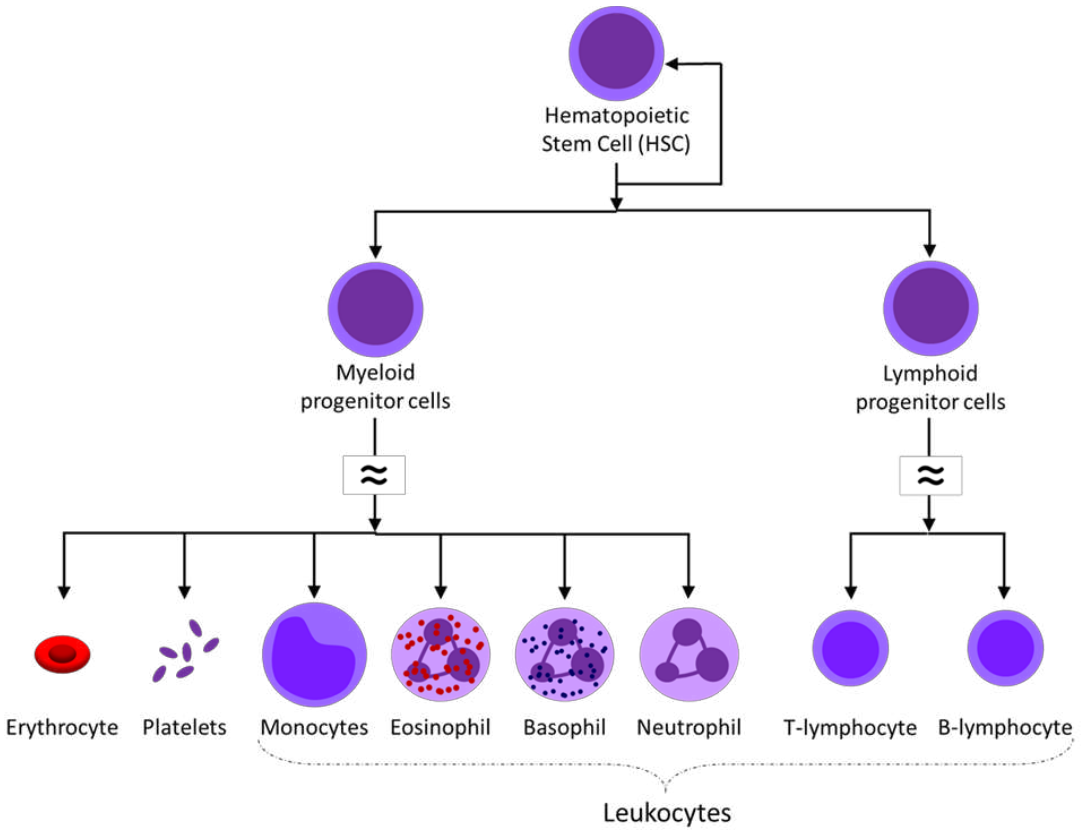

Figure 4: Hematopoiesis: Hierarchical differentiation of circulating blood cells from HSC (Modified from Ghiaur and Jones; 2019)

Table 2: Approximation of circulating blood cell kinetics under steady condition (Fliender et al., 2002; Tak et al., 2017)

Cell type	life span	turnover rate (cells per day)
Erythrocytes	108-127 days	200×10^9
Platelets	9-12 days	150×10^9
Granulocytes	24-30 hrs	120×10^9
Lymphocytes	4.4 years*	20×10^9
Monocytes	0.4-2.5 days	$1\text{-}8 \times 10^9$**

*on average, ** calculated from life span and number of cells in the circulating blood.

HSCs are the beginning of hematopoiesis (Ghiaur and Jones; 2019). HSCs mainly reside in the bone marrow as a rare population and 95% of them are in quiescent phase (G_0). HSCs are often referred as LSK ($Lin^-Sca^-1^+c\text{-}Kit^+$) cells based on their presenting membrane molecules. LSK cells are composed of long-term HSC (LT-HSC) and short-term HSC (ST-HSC), and multipotent progenitor cells (MPP) (Seita and Weissman, 2010). LT- HSCs are the top of the hierarchy of the differentiation with full self-renewal potential. LT-HSCs are generally quiescent, highly tolerant against RT and rarely self-renew in a steady state hematopoiesis. ST-HSCs divide more frequently and are responsible for maintaining hematopoietic homeostasis (Wilson et al., 2009). The self-renewal capacity of ST-HSC is limited in terms of the time and condition. Cytokines including interferon-α (IFN- α), IFN-γ, and granulocyte colony-stimulating factor (G-CSF) activate HSCs to start self-renewal. Multipotent progenitor cells (MPPs) have no self-renewal potential yet can differentiate into any blood cells. Many different intrinsic and extrinsic factors regulate the self-renewal and differentiation of the LSK cells to maintain the homeostasis of circulating blood at the same time as keep the HSC pool (see 3.2.3). MPPs give rise to either common myeloid progenitor cells (CMP) or common lymphoid progenitor cells (CLP). These HSCs and progenitor cells are often referred as hematopoietic stem and progenitor cell (HSPC).

CMPs differentiate into megakaryocyte-erythrocyte progenitor (MEP) with IL-3, SCF, TPO, and TLR ligands or granulocyte-monocyte progenitor (GMP) with GM-CSF and IL-3 (Akashi et al., 2000; Fiedler and Brunner 2012; Ghiaur and Jones; 2019). With erythropoietin (EPO), MEP differentiate into erythroblasts and then erythrocytes by acquiring hemoglobin and losing their nucleus. MEP with thrombopoietin turns into a megakaryoblast and then megakaryocyte, which is multi-nucleated cells and produces platelets by shedding their cytoplasm. GMP will mature into a myeloblast. Myeloblast with IL-3, IL-5, and AG-CSF will become a monoblast, which differentiates into monocytes. Myeloblasts with IL-3, IL-5, and G-CSF will be myelocytes, metamyelocytes, and turn into granulocytes such as neutrophils, eosinophils, and basophils. Immature granulocytes have a band-shaped nucleus, while fully matured granulocytes have a segmented nucleus, indicating no active function of the chromatin, often referred as polymorphonucleated cells. A neutrophil takes about 4-5 days to be transformed to a postmitotic state from the stem cell and additional 5 days to mature in the bone marrow.

CLPs with IL-7 mature into T cell NK cell progenitor (TNK) or B cell progenitor (BCP) (Fiedler and Brunner 2012). TNK with IL-7 and IL-15 will be NK progenitor cells and turn into NK cells with SCF (stem cell factor), IL-2 and IL-7 in bone marrow (Gollamudi et al., 2019). TNK cells with IL-7 and IL-2 move from bone marrow to thymus, which are referred as thymocytes. Thymocytes are educated and become naïve T cells. Thymocytes that have too much activation with self-antigen will be put to acute apoptosis (negative selection), and then thymocytes with appropriate signaling through T cell receptor (TCR) will move to the maturation (positive selection). Survived T cells with TCRs that binds to MHC I (Major histocompatibility complex class one, see 3.3.1) will be naïve cytotoxic T cells. T cells with TCR which recognize MHC II (Major histocompatibility complex class two, see 3.3.1) become naïve helper T cells. The naïve T

cells are released to the peripheral blood, exposed to pathogen at lymph nodes and activated as effector T cells. Helper T cells then divide into one of the 5 types of cells, Th-1, Th-2, Th-17, T_{reg}, and T_{fh}. BCP experience similar training as T cell progenitors at bone marrow. then moves to spleen and lymph nodes to mature. After released to the blood as naïve B cell and activated by helper T cells, they turn into plasma cells produce large number of antibodies. Detailed function of these cells is reviewed later in 3.3.1.

It has been a standard to assume the hematopoietic system is a hierarchical process with clear lineage differentiation. However, accumulating evidence suggests the system has less lineage differentiation, and is more like a landscape (Ceredig et al., 2009; Grootens et al., 2018; Brown and, 2019). HSCs may be heterogenous, and their linearge commitment may be determined baed on the localization in the bone marrow niche and genetic and epigenetic heterogeneity (Haas et al., 2018), while one cell type with similar physiology and functions can be derived from different lineage such as monocyte derived DCs and lymphoid lineage DCs (Wu and Liu., 2007).

3.3 Immune system

3.3.1 Cells in immune response

The main function of circulating leukocytes is in the immune response. Neutrophils, Macrophages, Dendritic cells, Natural Killer (NK) cells are the main participants in innate immune response, and lymphocytes are mainly for adaptive immune response (Table 3). Among the innate immune cells, neutrophils, macrophages, and DCs are phagocytic cells, which eat pathogens, destroy, or process. In the following, the functions of each immune cells are described (Kumar et al., 2014; Murphy and Weaver, 2016).

Table 3 Summary of immune cells and the functions

	Subtypes			Main function	System
Neutrophil				phagocytosis, stimulate inflammatory cells	Innate
Monocyte				divide into macrophages or DCs at tissue	
	Macrophage			phagocytosis, antigen presentation to T and B cells	
	Dendritic cell			phagocytosis, antigen presentation to T and B cells	
Lymphocyte	NK cell			destroy virally infected cells	
	T	helper	naïve	recognize antigen, be activated, and differentiate into effector cells	Adaptive
			Th1	stimulate inflammatory cells	
			Th2	activate B cells to plasma cells	
			Th17	stimulate inflammatory cells	
			Treg	anti-inflammation, suppress T cell mediated	
			Tfh	regulate memory cell formation	
		cytotoxic	naïve	recognize antigen, be activated, and become effector	
			effector	initiate apoptosis of infected cells	
	B	naïve		phagocytic, antigen presentation to T cells	
		plasma		production of antibody	

Macrophages and DCs are differentiated from Monocytes in tissue. They infiltrate? tissues looking for pathogens, infection, and injury. They recognize Pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs). PAMPs are small molecules conserved in microorganism species. DAMPs are fragments of proteins and nucleic acids derived from tissue and cells in response to trauma, ischemia, and tissue damage, with or without pathogenic infection. PAMPs are recognized through pattern recognition receptors (PRR), on the cell membrane (e.g., Toll-like receptor) and cytoplasm (e.g., Nod-like receptor) of macrophages and DCs. Macrophages and DCs which have recognized pathogens initiate phagocytosis. Phagocytosis takes pathogens inside of the cells by forming phagosome around the pathogens (engulfment), and then produce reactive oxygen species (ROS) and recruit lysosome containing digestive enzymes, and destroy the pathogen (digestion). Macrophages then recruit Neutrophils to the site of injury and infection by secreting chemokines such as IL-8 (CXCL8). Macrophages also secrete proinflammatory cytokines such as IL-1β, TNF-α, and IL-6, which can trigger systemic inflammation including fever and more production of inflammatory

cytokines. IL-12 and other IFNs from macrophages activate NK cells and induces CD4 T cell differentiation to Th-1 cells (see the following paragraph about T lymphocytes). Macrophages also clean the site of infection and inflammation via phagocytosis after the response is over. DCs present antigens to CD4 T cells at lymph nodes, spleen, and tonsils and activate naïve T cells through major histocompatibility complex (MHC) molecules. MHC molecules are cell surface proteins presenting part of antigens or self-antigens (epitope) to initiate or inhibit immune response. MHC molecules are largely classified as class 1 and 2. Class 1 molecules exist in any cell membrane except for erythrocytes, while MHC class 2 only exists on antigen presenting cells such as DCs, macrophages, some endothelial cells and epithelial cells, as well as B lymphocytes.

Neutrophils are attracted to injury and infection sites by cytokines and chemokines from macrophages. IL-1β and TNF-α activates endothelial cells of the blood vessel to present endothelial selectins, which attaches to leukocyte selectins and make neutrophils roll the endothelial wall. With interactions to other molecules such as integrins and CAMs, neutrophils are strongly attached to the endothelial cell wall and start squeezing into the tissue through the gap between endothelial cells (diapedesis). Neutrophils entering the tissue moves toward the cytokine gradient by macrophage and engage them to react against injury and infection. The entire process involving circulating neutrophils entering the tissue is referred as neutrophil extravasation. Neutrophils destroy pathogens vis phagocytes in tissue with lysosome and granules containing digestive enzymes and peroxidase. Phagocytosis by neutrophils is destructive, which leads to apoptosis. Neutrophils sometimes go through a suicidal process, which is referred to as a neutrophil extracellular trap (NETs). NETs are composed of chromatin fibers from neutrophils, released to the extracellular matrix. NETs provide a high local concentration of antimicrobial substance and destroy without phagocytosis. NETs may also provide a physical barrier to avoid

the further spread of pathogens. NETs are then cleaned by macrophages.

NK cells are only 15-20 % of circulating lymphocytes and one of the innate immune cells fighting viral infection. NK cells recognize infected cells (mostly viral infection) with and without presentation by MHC class 1 molecular and destroy the infected cells through inducing apoptosis. NK cells also produce cytokines such as IFN-γ to attract macrophages and DCs.

T and B lymphocytes are the major contributors to the adaptive immune system. T lymphocytes are 70-80 % of the circulating T-cells. T lymphocytes are classified as CD4 (Helper) T-cells and CD8 (Cytotoxic) T-cells based on their cluster of differentiation (CD). CDs cell surface protein is widely used to classify the subtypes of immune cells. CDs also have their own functions. For example, CD4 and CD8 are co-receptors of T cell receptors (TCR) of CD4 and CD8 T-cells, respectively. T-cells have T-cell receptors (TCR) on the surface. TCR has both a variable and constant region. Through rearrangement of DNA in the thymus, the variable regions of TCRs are different depending on individual cells, while the constant region is conserved in different cells. Since the variation of TCRs is enormous, T-cells can recognize almost any antigen. Naïve T-cells in lymph nodes are activated when they recognize an antigen through their TCR. Naïve T-cells which recognized MHC class 1 differentiate into CD8 T cells. Naïve T-cells which recognized MHC class 2 differentiate into CD4 T-cells. Activated CD8 T-cells lead the infected cells to apoptosis by secreting perforin, which is a pore-forming protein letting pro-apoptotic protein of granzyme to enter the cell. The other mechanisms of inducing apoptosis in the infected cell is to activate Fas on the cell surface by the Fas ligand on CD8 T-cells. These mechanisms are consistent with the cell-killing process of NK cells, while one CD8 T-cells only work for one specific type of antigen.

Activated naïve CD4 T-cells differentiate into different types of CD4 T-cells, such as Th1,

Th2, Th17, Tfh, or Treg, based on the cytokine signaling by DCs. When there is microbial infection, DCs secretes IFN-γ and IL-12, which induce differentiation to Th1 cells. Th1 cells activate macrophage, NK cells, and CD8 T cells to destroy the infected cells. When microbial signaling is low and IL-4 signaling exists, naïve CD4 cells will mature into Th-2 cells. Th2 cells produce IL-4, IL-5, and IL-13 which activate B cells to produce antibodies. Th2 also induce some portion of the activated B cells to be memory cells. With TGF-β, IL-6, and IL-23, naïve CD4 cells turn into Th17. Th17 cells activate neutrophils, macrophages, and DCs with IL-17 and IL-22, inducing inflammatory response. IL-21 and IL-6 lead the differentiation to Tfh cells, which activates B-cells with IL-21, IL-4, and IFN-γ. With IL-2 and TGF-β, the naïve cells will be Treg cells. Treg cells are immunosuppressive and anti-inflammatory cells, suppressing or downregulating the function and population of other effector T-cells through secreting TGF-β and IL-10.

Naïve B lymphocytes also have receptors (BCR), which experience rearrangement of DNA in bone marrow. BCR have heavy and light chain, and both has constant and variable regions. Variable regions recognize antigens, and there are enormous number of different naïve B-cells with different BCRs because of the rearrangement. When naïve B-cells recognize antigens with BCRs at lymph nodes, they present it to CD4 T-cells. B cells are phagocytic just like other antigen presenting cells such as macrophages and DCs. When naïve B cells present antigens to CD4 T-cells (Th2 cells) which recognize the same antigen by antigen presentation of DCs, Th2 cells activate the naïve B cells through cytokine signaling such as IL-4, which mechanism is referred as linked recognition. The activated B cells change the class of BCRs they produce. B-cells further activated by Tfh cells, starts proliferating and somatic hypermutation. Somatic hypermutation induce producing different BCRs based on the BCRs which originally recognized the antigen in

order to produce BCRs with much more affinity to the antigen. Cells with BCR of great affinity will move to bone marrow and become plasma cells. Plasma cells produce more BCRs and secrete them as antibody. Cells that no longer bind to the antigen will be destroyed. Some of the activated B-cells will be memory cells.

3.3.2 Innate immune response and inflammation

Innate immune response is not specific to antigens but starts within minutes and hours. Macrophages and DCs residing in tissues, organs, and skin detect infection or injury by PRR and recruit neutrophils to the site. Macrophages, DCs, and neutrophils phagocyte the pathogens and destroy or process them. DCs present the epitope of the pathogen to the CD4 T-cells at lymph nodes and to initiate adaptive response. NK cells also detect the infected cells and lead them to apoptosis. Macrophages, DCs, and neutrophils also produce inflammatory cytokines, such as IL-1, IL-6, IFN-γ, and TNF-α which recruit and activate more immune cells to the infection site.

Inflammatory cytokines allow communication between immune cells, resulting in inflammation. Inflammation is a tissue response to infection or injury by recruiting and coordinating immune cells and molecules in the circulating system. Initial inflammation in response to infection and damage is referred as acute inflammation, which lasts only for several hours to a few days. Acute inflammation is composed of a vascular reaction and cellular response coordinated by different mediators. The vascular reaction involves dilation of blood vessels (vasodilation) as well as increased permeability of microvasculature. Vasodilation is induced by histamines and increases and slows down the blood flow. Vascular permeability is increased by histamine and kinin, which enhances plasma proteins and induces immune cells to leave circulation. Innate immune cells, predominantly neutrophils, are recruited to the infection or injury site due to chemotaxis and leaves the blood flow by extravasation (see 3.3.1), which is also

enhanced by the vascular reactions. Neutrophils and macrophages eliminate pathogens by phagocytosis, which involves creations of phagolysosome and destruction the pathogen by free radicals, reactive oxygen species (ROS) and nitric oxide (NO), from lysosomes. Acute inflammation is terminated by anti-inflammatory cytokines such as IL-10 and TGF-β when the elimination of pathogen is achieved, followed by the initiation of tissue repair. Acute inflammation will result in either resolution (complete repair), scarring or fibrosis (healing by being replaced with connective tissue) with loss of the function, or chronic inflammation. The four signs of acute inflammation are redness (*rubor*), warmth (*calor*), swelling (*tumor*), and pain (*polor*). Redness, warmth, and swelling is mainly due to the vascular reaction, while pain is from mediators such as prostaglandins and bradykinin released from neutrophils and mast cells.

Chronic inflammation is the prolonged inflammatory status over weeks, months, and years, often associated with tissue injury and repair at the same time. Chronic inflammation can be caused by incomplete acute inflammation, persistent infections and exposure to toxic agents, as well as hypersensitivity disease such as autoimmune and allergic disease. While acute inflammation is predominant with enhanced infiltration of neutrophils, chronic inflammation is manifested by infiltration of mononuclear cells such as macrophages, lymphocytes, and plasma cells. The macrophage is the main contributor to chronic inflammation, presenting antigens to CD4 T-cells and activating them as well as producing inflammatory cytokines. CD4 T-cells, especially Th1 and Th17 cells, can activate macrophages by IFN-γ, producing other inflammatory mediators. The communication between macrophages and CD4 T-cells can accelerate and prolong chronic inflammation. Plasma cells are also found in chronic inflammatory sites, possibly producing antibodies for the persistent antigen. Neutrophils are also elevated, induced either by persistent pathogens or inflammatory cytokines from macrophages and CD4 T-cells. Chronic inflammation

is known to be a cause of many diseases including Arthritis, Asthma, Atherosclerosis, and Pulmonary fibrosis.

3.3.3 Adaptive immune response

Adaptive immunity is specific to an antigen and effective compared to the innate immunity, while it takes more than a few days to initiate. Adaptive immunity starts with antigen presenting of DCs at lymph nodes to naïve T lymphocytes. When CD8 T-cells are activated, cellular immunity initiates and destroys the infected cells. When CD4 T-cells are activated, humoral immunity starts with communication between CD4 T-cells and B-cells. Because of the rearrangement of DNA in naïve T lymphocytes and B lymphocytes, they have wide variety of receptors, allowing highly specific binding to antigens. B-cells further go through somatic hypermutations to find receptors with higher affinity, which will be released as antibody. Antibodies are Immunoglobulin proteins. There are 5 types of antibodies: IgM, IgG, IgA, IgE, and IgD, and have different features and functions.

IgMs are produced by Naïve B cells, and work as pentamer, which increase their possibility to bind to antigens. IgMs enhance clumping of antigens (agglutination), which become good targets of phagocytic cells. IgMs also bind to compliment component C1, leading to enhanced phagocytosis (opsonization) as well as cytolysis, which is a part of necrosis, bursting of cell cytoplasm induced by osmotic imbalance, through classical compliment pathway.

IgGs are the most abundant and 75% of the antibodies in the circulation, produced by plasma B-cells. IgGs have high affinity to bacteria and their pathogens. IgGs enhance agglutination. IgGs also induce cytolysis through the classical compliment pathway. IgGs are smaller than IgMs and transported faster to the infection site or antigen. IgGs also bind to antigens and neutralize by blocking them from interacting with host cells (neutralization).

IgAs are a secreted antibody, frequently found in mucous membranes such as respiratory and digestive tracts. IgA interacts with inhaled and ingested antigens, neutralizing and agglutinating them, but not inducing compliment pathway and the cytolysis.

IgEs are only 0.05% of the antibodies and produced for parasite infections and allergens. They binds to the Fc receptors found on the surface of mast cells and basophils. IgDs also occupies very small percentages in the serum immunoglobulins, and the function is not clearly identified.

Important feature of adaptive immunity is memory cells. While most of the lymphocytes have limited life span, part of the activated (effector) T cells and B cells will remain as memory cells. While memory B cells stay in circulation in quiescence memory T cells self-renew and reside in surface tissues of organs such as intestine, skins, lungs. Due to these memory cells, the immune response to the same antigen will be more robust, producing more antigens quicker, initiating within a few hours.

3.4 Biological effect of IR

3.4.1 Immune and hematopoietic system after IR

The Hematopoietic system is one of the most sensitive systems to ionizing radiation exposure (Dainiak 2002; Paganetti 2023). The number of circulating hematologic cells typically decrease in response to ionizing radiation exposure through reduced bone marrow production, as well as the redistribution and apoptosis of mature blood cells. Lymphocytes are the most radiosensitive cells with a D_0 of 0.2-0.3 Gy for small lymphocytes. D_0 is the dose that reduces the number of surviving cells to 37%. After acute whole-body radiation exposure, Lymphopenia starts first, followed by granulopenia, thrombopenia, and anemia (Figure 5). For radiation doses as high as 4-6 Gy, the number of granulocytes could increase (temporal granulocytosis) for the first few

days to a week to compensate the loss of the number of leukocytes, which is achieved by release of cells from the maturing-only pool. The number of granulocytes decreases rapidly to reach a minimum value on day 18-20. Granulocyte recovery starts after about a week of aplasia, almost the same time for all the cell types. The hematopoietic system will be fully recovered approximately one month after exposure. The speed of decline and recovery depends on the radiation dose as well as the level of injury to the stem cell pool (Fliedner et al., 2002). The depletion of circulating blood cells also enhances release of immature cells from bone marrow. For much higher doses, the decline of lymphocytes and granulocytes is earlier with a longer aplastic condition, resulting in a fatal condition such as hemorrhage and infection to the organism, which is referred to as acute hematopoietic syndrome and can lead to death within 30 days to 60 days after irradiation.

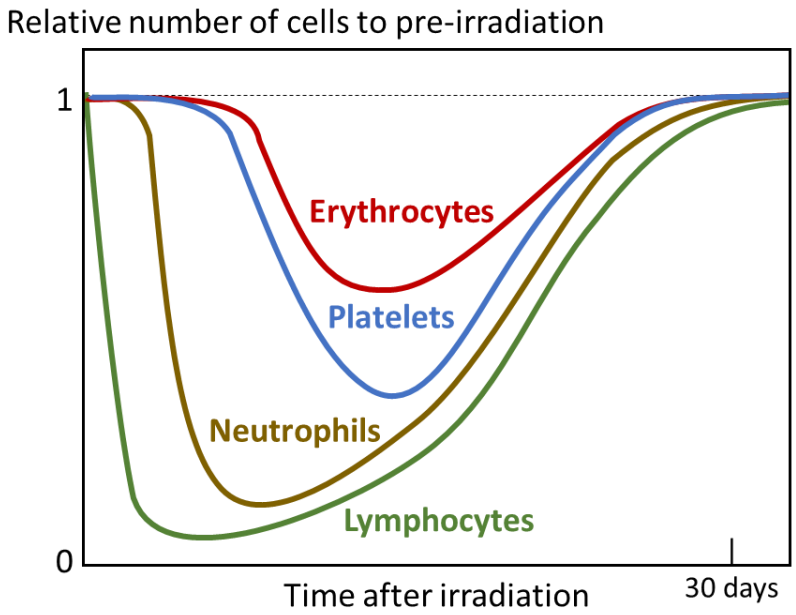

Figure 5: Conceptual depletion and recovery curve of the main peripheral blood components following an intermediate dose of total body irradiation (Modified from Hall and Giacia, 2019)

Hematopoietic response to partial body irradiation (PBI) resembles whole-body irradiation response. The response depends on the volume of the body irradiated, as well as the level of injury

to bone marrow and stem cell pool. When bone marrow is severely injured, medullary hematopoiesis, which is hematopoiesis outside of bone marrow such as the long bones, spleen, and liver, occurs to make up bone marrow functions.

In the case of chronic low-dose-rate radiation exposure, hematopoietic response may depend on the dose rate and the total dose received. Previous research is mainly focused on the risk of leukemia, and the hematopoietic response mechanisms have not been thoroughly investigated. International research for nuclear workers of French, American, and British population (INWORKS) revealed a correlation between chronic low radiation dose (1.1 mGy per year on average) to excess risk of leukemia mortality (Leurand et al., 2015), while several other studies provided conflicting results regarding the association between chronic low dose-rate radiation exposure and leukemia mortality (Luxin et al., 1990; Parkin et al., 1993).

Studies on astronauts provide evidence of damage to the human hematopoietic system likely resulting from chronic exposure to the low dose/low dose-rate space radiation environment during long-duration spaceflight aboard the International Space Station; WBC counts were typically increased during spaceflight, with a dose-dependent decrease post-spaceflight (Crucian et al., 2015; Bailey et al., 2022). Elevated WBC counts during spaceflight could be due to exposure to space radiation or the hostile environment, including microgravity, confinement, and limited nutrition inducing leukopenia. However, Luxton et al. (2020) demonstrated a negative linear correlation between WBC counts and frequency of inversions (as a proxy for dose) in blood lymphocytes, a relationship that was confirmed only for post-spaceflight. Since inversions are a type of chromosomal aberration induced by ionizing radiation, the finding indicates leukopenia was possibly caused by damage to hematopoietic stem or progenitor cells.

No significant correlation was found between radiation dose and white blood cell (WBC)

counts, lymphocyte, and neutrophil counts for populations receiving 5-25 mSv in total after three months to a year since Fukushima Daiichi Nuclear Power Plant Accident (Sakai et al., 2015). On the other hand, several studies on Japanese macaques in Fukushima provided evidence for possible hematopoietic damage by low-dose radiation exposure (Ochiai et al., 2014; Urushihara et al., 2018). The threshold of radiation dose and dose rate to induce hematopoietic response has not been determined for environmentally exposed populations but could be higher than expected considering no significant chromosomal aberrations were found for the macaques in Fukushima (unpublished data).

The impact of ionizing radiation on the immune system depends on the dose and dose-rate, the level of damage to hematopoietic system, and the number of surviving cells (Hall et al., 2019). Generally, high dose ionizing radiation is immune suppressive, which is utilized to inhibit the immune system before organ transplantation. High doses typically induce inflammatory response and possibly can persistently damage immune function. Alternatively, low dose ionizing radiation can modulate immune function as well as provide anti-inflammatory features, while low doses are also reported to cause slight immune function alterations for a long time (Lumniczky., 2020).

Studies on the effects of ionizing radiation to the immune system are well summarized in UNSCEAR's report in 2006; "Effects of Ionizing Radiation vol.2 Annex D." For low dose and low-dose rate ionizing radiation exposures (<0.2 Gy to whole body or <0.1 Gy/hr), the disturbance of innate and adaptive immune system is reported for both animal and human studies. The disturbance to the adaptive immune system is possibly caused by the high-radiosensitivity of lymphocytes and lymphoid tissue as well as restricted progenitor cell pool. Animal studies demonstrated suppression in adaptive immunity by the decline of lymphocyte and the alteration of cytokine production for an extent of time after irradiation. For instance, Seed et al (2002) reported

decreased leukocytes including granulocytes, monocytes, and lymphocytes in blood of canines irradiated with a dose rate as low as 3 mGy/d. Studies on mice with total dose of 75 mGy proved decreased and increased cytokines of IL-10 and IL-12 respectively (Liu et al., 2002). These altered count of cells, cytokines, and activities were typically recovered in several months.

Human studies are very limited, and results are controversial. Chang et al (1999) suggested chronic low-dose rate ionizing radiation caused a decrease of cellular immunity to residents exposed to 169 mSv on average (in the range of 8-1,662 mSv) from a building containing Co-60 contaminated material. Some studies of nuclear workers exposed to similar dose proposed the opposite result using the same method comparing the ratio of CD4+ T-lymphocytes to CD8+ T-lymphocytes (CD4+/CD8+) (Ress et al., 2004). CD4+/CD8+ is a standard method to evaluate cellular immunity. Activation of the immune system by low-dose rate radiation is reported in some animal studies, while the result is highly dependent on the dose rate and the condition, suggesting a need for more investigation.

3.4.2 Cancer and non-cancer disease after radiation

The main delayed and long-term health effects of ionizing radiation exposure are categorized into cancer and non-cancer disease. Cancer incidence and increased mortality rate have been investigated as the main delayed risk of ionizing radiation exposure. Epidemiological studies on atomic bomb survivors, nuclear workers, and radiotherapy patients have provided evidence of increased cancer mortality (UNSCEAR, 2006). More information on low dose exposures is accumulating over time. However, low dose exposure studies are typically associated with low statistical power, and possible biases concerning dose-response.

Models to estimate the effect of radiation exposure on cancer mortality have been developed. The estimated risk is primarily dependent on dose, age at exposure, sex, and the radiation exposed

population. The cancer mortality estimation for low dose or chronic low dose radiation exposure needs additional investigation because of limited data and possibility of bias.

Circulatory disease has been studied as the most common non-cancer disease after radiation exposure along with respiratory, digestive, and infectious disease. Several studies on A-bomb survivor cohorts and medically and occupationally exposed populations provide evidence of a positive correlation with radiation dose and disease incidence. The Adult Health Study, which is an investigation of A-bomb survivors, showed a significant quadratic dose response for myocardial infarction with a relative risk of 1.25 (95% confidence interval of 1.00 and 1.69) for 10,339 people exposed 0->3Sv (Yamada et al., 2004). Early radiation therapies (~1970s) increased survival from lymphoma and breast cancer, but also caused cardiac disease over time, especially because of the high dose and treated volume (Haybittle et al., 1989; Hancock et al., 1993). With the development of science and technology, the incidence of CVDs after medical radiation treatment decreased, while the knowledge of the impact from lower dose radiotherapy is limited. Also, the risk of CVD became harder to assess due to the incorporation of cardiotoxic drugs in treatment (Bovelli et al., 2010). Several studies on nuclear workers showed significant dose-response relationship on CVDs (Cardis et al., 1996; Gilbert, 2001), but the data is limited and could be misinterpreted especially for low-dose exposures because of lifestyle and socio-economic factors leading to CVDs. Overall, the impact on incidence and mortality of CVDs after radiation exposure requires more studies, as well as investigations on the mechanisms.

3.4.3 Long-term radiation impact on hematopoietic and immune system

Prolonged deficiencies in hematopoietic and immune system have been investigated in various exposure cohorts including A-bomb survivors, Chornobyl clean-up workers and resident children, nuclear workers, and radiation therapy patients. Table 4 summarizes long-term follow-

up studies of ionization exposure for hematological and immune cell and molecular parameters. "Long-term" for this proposal refers to animals that have survived a minimum of year after radiation exposure events. Long-term studies are classified into cross-sectional and longitudinal analyses. Cross-sectional studies, where the target parameters were obtained from one or several time points after irradiation, are typically meant to provide dose-response at the time of the investigation. On the other hand, longitudinal studies utilize data from a continuous sampling in an extended amount of time to investigate the temporal trend.

The A-bomb survivor cohort is the most extensively analyzed for long-term effects of ionizing radiation on the hematopoietic and immune system. These studies typically have a large number of samples sufficient to provide statistically reliable results. Most studies are cross-sectional, and findings summarized in UNSCEAR (2006);

(i) T lymphocytes are most sensitive, and the subset population and proportion are highly affected by ionizing radiation. Functional deficiencies of T lymphocytes were also detected. Recovery from radiation exposure varies depending on the cell type, naïve cells were typically slower to recover than memory cells.

(ii) B-cell population increased significantly as well as some types of immunoglobulins.

(iii) Erythrocytes showed the functional deficiency for the highly exposed group.

In addition to these cellular parameters, increased inflammatory markers such as C-reactive protein (CRP) and interleukin 6 (IL-6) were observed (Hayashi et al., 2003). All these abnormalities were detected up to several decades after the exposure and are suggested to cause chronic inflammation, CVDs, and accelerated aging on both hematopoietic and immune system.

Longitudinal analysis on A-bomb survivors has not been conducted until recently. Hsu et al. (2010) was the first to conduct a longitudinal analysis of white blood cell and the differentials for

19-59 years after exposure. A persistent elevated WBC count in the >2 Gy exposed group was found for the study period. Yoshida et al. (2019) also suggested the >2 Gy exposed group had a higher monocyte percentage at older ages, indicating a delayed effect on hematopoietic system after radiation exposure. Considering the evidence of a prolonged effect on both hematopoietic and immune system, more longitudinal studies are required to understand the long-term effect of ionizing radiation.

Studies on clean-up workers and residents exposed by the Chornobyl nuclear power plant accident were generally consistent with A-bomb survivor studies, associated with different lymphocyte subset population and proportion as well as functional deficiencies. However, heavy metal contamination, mainly from lead, could exaggerate health conditions of the clean-up workers. Longitudinal studies are very limited, and Stepanova et al. (2008) first reported six-year trend of WBC, red blood cell (RBC), platelet counts and hemoglobin in children's peripheral blood. While RBC, platelet count and hemoglobin increased during the study period, WBC count remained constant with relatively smaller count for cohorts from highly contaminated areas (no p-values provided). Considering many studies reported prolonged damage in hematopoietic and immune system, longitudinal studies are required to understand the temporal changes for the exposed population.

The Techa river resident cohort is another group of people exposed to high dose radiation (estimated bone marrow dose 0.5-2.3 Gy) for an extended of time. Kossenko et al. (1998) investigated 940 people with chronic radiation syndrome for 1-30 years since the exposure started. The range and mean of leukocyte and neutrophil count were below the 90% CI of normal values for the whole study period (no p-value available), and stab (band) neutrophils were above the 90% CI of normal values for the study period. While statistical significance was not provided, the

linearly increasing trend of WBC, neutrophil, and thrombocytes after the end of the exposure indicated gradual recovery of hematopoietic system.

Studies of residents in Fukushima who received 5-25 mSv in total provided no evidence of dose response to white blood cell, lymphocyte, and neutrophil count after three months to a year since the Fukushima Daiichi nuclear power plant accident (Sakai et al., 2015). There was only a limited follow-up after the exposure, but it is unlikely the population will have any prolonged hematopoietic or immune deficiencies due to the relatively small radiation dose. However, 2-2.5 year follow up studies on wild macaques in Fukushima proved the WBC count and platelets in peripheral blood declined with internal dose-rate (7.6 μGy/day on average, Urushihara et al. (2018)). Ochiai et al. (2014) also provided evidence of a negative correlation with WBC counts and muscle radioactive cesium concentration for macaques captured 1-2 years after the accident. It may be important to keep monitoring wildlife hematologic parameters, considering they could provide evidence of the impact of chronic low-dose radiation exposure.

Follow-up studies on cancer patients after radiation therapy also provide evidence of long-term effects on hematopoietic and immune system. Several studies comparing long-term follow up of leukocytes and the differentials for breast cancer patients after surgery with and without radiation therapy suggested depleted count of lymphocytes and the subpopulations remained for as long as 5 years after irradiation (Baral at al., 1977; Toivanen et al., 1984; Shukla et al., 1986). The depletion and recovery trend depend on the lymphocyte subpopulations. Watanabe et al. (1997) investigated the depletion and recovery in CD4+ and CD8+ of naïve and memory T lymphocytes. Decline of both CD4+ and CD8+ naïve cells persisted up to 30 years. The total count of CD8+ cellsrecovered over the 5 years post irradiation however the composition of CD8+ cells shifted to increased memory cells, and decreased naïve cells. Treatment regimen also influences

long-term damage and recovery in hematopoietic and immune system. Wersal et al (2019) compared breast cancer patients with intraoperative radiation therapy of accelerated partial breast irradiation with and without additional whole breast radiotherapy (IORT/WBRT and IORT/APBI, respectively). IORT/WBRT showed decreased WBC and RBC counts, hemoglobin, and platelet counts as well as increased lymphocyte (%) compared to preoperative values ($p<0.005$). These conditions persisted up to 5 years after the completion of treatment. The prolonged effect of RT should be investigated carefully due to the confounding factors from the patients' disease.

These immune deficiencies are also found in NHPs exposed to total body gamma irradiation (TBI). DeBo et al., (2016) reported NHPs exposed 6.5-8.4 Gy TBI had significantly higher pro-inflammatory protein markers as well as increased incidence of myocardial injury 5.6-9.7 years after irradiation. Elevated CRP and IL-6 was also suggested, although it was not statistically significant. Studies on NHPs exposed to 4 Gy TBI provided little evidence of the impact of radiation on the adaptive immune system but indicated late immune deficiency regarding to immunoglobulin G binding to several polysaccharide antigens (Macintyre et al., 2021).

As reviewed above, an increasing number of studies provide cumulative evidence of long-term effects on the hematopoietic and immune systems following ionizing radiation in many exposure scenarios including, accidental, environmental, medical, and experimental. While most of studies reviewed here are high dose exposures, follow up studies of low-dose exposure using international nuclear worker cohorts are also emerging (Laurier 2017). Yet, most of the investigations are cross-sectional, only providing the impact at one or several time points, and longitudinal studies are required to reveal the temporal trend of the impact as well as to predict response for longer periods. In addition, further investigations are required to clarify the relationship to these conditions on CVDs or other diseases as well as the mechanisms.

Table 4: Overview of studies for long-term effects on leukocytes and the differentials after ionizing radiation exposure

study cohort	n	follow-up period (yrs after exposure)	dose/dose rate	Type of study	Parameter	Trend of change	Citation
A-bomb survivor	411	43-47	≥1.5 Gy (mean: 2.4 Gy)	cross-sectional	T lymphocyte subset (%):		(Kusunoki et al., 1998)
					CD4+	decreased (p<0.01) for irradiated group	
					CD8+	no significant difference	
					NK cell	no significant difference	
					B lymphocyte subset (%):		
					CD5+	increased (p<0.01) for irradiated group	
					CD23+	increased (p<0.01) for irradiated group	
	6304	43-47	0.01->2.0 Gy	cross-sectional	leukocyte	positive correlation with radiaiton dose (p<0.05)	(Neriishi et al., 2001)
					neutrophil	positive correlation but not statistically significant (p>0.1)	
	453	50-52	0.005->1.5 Gy	cross-sectional	T lymphocyte subset (%):		(Hayashi et al., 2008)
					CD3+	decreased with radiation dose (p<0.05)	
					CD3+CD4+	decreased with radiation dose (p<0.05)	
					CD3+CD8+	no significant difference	
	7562	19-59	0.005->2 Gy	longitudinal	leukocyte	High dose exposure (>2Gy) increased (p<0.05)	(Hsu et al., 2010)
					neutrophil	High dose exposure (>2Gy) increased (p<0.05)	
					lymphocyte	High dose exposure (>2Gy) increased(p<0.05)	
					monocyte	High dose exposure (>2Gy) increased(p<0.05)	
	229	66-68	0.005-≥0.5 Gy	cross-sectional	total dendritic cell (DC)	no significant dose response	(Kajimura et al., 2018)
					conventional DC	no significant dose response	
					plasmacytoid DC	negative dose response (p = 0.035) for female	
						no significant dose response for male	
	14000	13-65	0.005->2 Gy	longitudinal	lymphocyte (%)	no significant difference	(Yoshida et al., 2019)
					neutrophil(%)	no significant difference	
					monocyte (%)	increased with radiation dose (p<0.005)	
					monocyte	increased with radiation dose (p<0.005)	
					lymphoid to myeloid ratio	no significant difference	
Chernobyl	432	10-14	0.01-0.5 Gy	cross-sectional	leukocyte	no significant difference	(Kurjane et al., 2001)
					lymphocyte (%)	no significant difference	
					monocyte (%)	no significant difference	
					T lymphocyte subset (%):		
					CD3+	decreased in exposed group (p<0.05)	
					CD4+	decreased in exposed group (p<0.05)	
					CD8+	decreased in exposed group (p<0.05)	
					NK cell	decreased in exposed group (p<0.05)	
					B lymphocyte (%)	no significant difference	
	207	7-8 (1993-1994)	0.57-3.09 Sv	cross-sectional	T lymphocyte subset (%):		(Chernyshov et al., 1997)
					CD3+	non-RRDC[*1]: no significant difference	
						RRDC: lower than control (p<0.05) for exposure group	
					CD3+CD4+	non-RRDC: no significant difference	
						RRDC: lower than control (p<0.005) for ASD[*2] >1.0mSv	
					CD3+CD8+	no significant difference	
					CD3+CD4+/CD3+CD8+	no significant difference	
					NK cell (%)	no significant difference	
					B lymphocyte(%)	no significant difference	
	1251	7-12 (1993-1998)	No dose range available	longitudinal	leukocyte	decreased with area Cs-137 contamination (p<0.05)	(Stepanova et al., 2008)
					platelet	decreased with area Cs-137 contamination (p<0.05)	
						increased over the study period (no p-values)	

Table 4 (continued)

study cohort	n	follow-up period (yrs after exposure)	dose/dose rate	Type of study	Parameter	Trend of change	Citation
Techa river resident	940 (with CRS[*3])	1-30 (1950-1970)	0.5-2.3 Gy (RBM dose)	cross-sectional	leukocyte	range and meanwere below reference for the study period	(Kossenko et al., 1998)
						linearly increasing trend for the study period	
					neutrophil	range and mean were below reference for the study period	
						band neutrophils were above reference for the study period	
						linearly increasing trend for the study period	
					platelet	linearly increasing trend for the study period	
Fukushima (human)	45278	0.2-1	≤25 mSv	cross-sectional	leukocyte	no significant difference	(Sakai et al., 2015)
					neutrophil	no significant difference	
					lymphocyte	no significant difference	
Fukushima (NHPs)	65	2-2.5	7.6 μGy/day (external dose) 13.9 μGy/day (internal) in average	cross-sectional	peripheral blood:		(Urushihara et al., 2018)
					leukocyte	negative correlation with internal dose-rate (p<0.05) for adults (≥5 yrs old)	
					platelet	negative correlation with internal dose-rate (p<0.05) for adults (≥5 yrs old)	
					bone marrow:		
					myeloid cell	negative correlation with internal dose rate (p<0.05) for adults (≥5 yrs old)	
					megakaryocyte	negative correlation with internal dose rate (p<0.05) for adults (≥5 yrs old)	
					hematopoietic cell	negative correlation with internal dose rate (p<0.001) for adults (≥5 yrs old)	
Nuclear worker	375	1-5	0.3-7.0 mSv (cumulative)	cross-sectional	leukocyte	lower than control (p<0.001)	(Wang et al., 2022)
					neutrophil(%)	higher than control (p<0.05)	
					eosinophil (%)	lower than control (p<0.001)	
					basophil(%)	no significant difference	
					monocyte (%)	no significant difference	
					lymphocyte (%)	no significant difference	
					platelet	no significant difference	
Radiotherapy patient	110	2	45 Gy[*4]	cross-sectional	lymphocyte	RT group: lower than pretreatment (p<0.001)	(Baral et al., 1977)
						non-RT group: no significant difference	
	10	3	40-43 Gy (all) + 30 Gy (n=?)[*5]	cross-sectional	leukocyte	lower than pretretment (p < 0.0005)	(Toivanen et al., 1984)
					lymphocyte	lower than pretretment (p < 0.0025)	
					monocyte	no significant difference	
					T lymphocyte	lower than pretretment (p<0.005)	
					helper cells	lower than pretretment (p<0.01)	
					surppressor cells	lower than pretretment (p<0.005)	
					thymocytes	lower than pretretment (no p-value)	
	56	0.3-32	25-44 Gy (n=21)vs 25-44 + 26-51 Gy (n=35)	cross-sectional	leukocyte	higher than control for >5yr follow-up group (p<0.05)	(Watanabe et al., 1997)
						no significant difference to pretreatment	
					neutrophil	higher than control for >5yr follow-up group (p<0.05)	
						no significant difference to pretreatment	
					B lymphocyte	higer than control and pretreatment (p<0.05) for all follow-up period	
					T lymphocyte subset:		
					NK cell	higher than control and pretreatment (p<0.05) for >5yr follow-up group	
					naïve CD4+	lower than control and pre treatment (p<0.005) for all follow-up period	
					memory CD4+	lower than control and pretreatment (p<0.005) ≤5yr follow-up group	
					naïve CD8+	lower than control (p<0.005) for all study period	
					memory CD8+	higher than control and pre-treatment (p<0.005) for >5yr follow-up	

Table 4 (continued)

study cohort	n	follow-up period (yrs after exposure)	dose/dose rate	Type of study	Parameter	Trend of change	Citation
Radiotherapy patient	256	1-5	12-20 Gy (IORT) vs 12-20 + 45-56 Gy (WBRT) *6	cross-sectional	leukocyte	lower than pretreatment (p<0.001) for the study period	(Wersal et al., 2019)
					lymphocyte (%)	higher than pretretment (p<0.005) for the study period	
					platelet	lower than pretreatment (p<0.001) for the study period	
NHPs	43	3.7-5.8	6.5-8.4 Gy	cross-sectional	leukocyte	increased with dose (p=0.01)	(Hale et al., 2019)
					neutrophil(%)	no significant dose response	
					eosinophil (%)	no significant dose response	
					monocyte (%)	no significant dose response	
					platelet	decreased with dose (p=0.05)	
					lymphocyte	no significant dose response	
					lymphocyte (%)	no significant dose response	
					T lymphocyte (%)	decreased with dose (p=0.0004)	
	16	0-2.7	4 Gy	longitudinal	leukocyte	decreased with time (p=0.02) with interaction of irradiation and time (p<0.0001)	(Macintyle et al., 2021)
					neutrophil	decreased with time (p=0.02) with interaction of irradiation and time (p<0.0001)	
					lymphocyte	decreased with time (p<0.0001) with interaction of irradiation and time (p<0.0001)	
					eosinophil	decreased with time (p=0.045) without significant interaction of time and irradiation	
					monocyte	decreased with time (p=0.012) with interaction of irradiation and time (p<0.0001)	
		0.3-1.75			PBMC cells *7 (%);		
					myeloid DC	decreased with time (p=0.0117) without significant interaction of time and irradiation	
					plasmacytoid DC	decreased with time (p=0.0008) without significant interaction of time and irradiation	
					B lymphocyte	decreased with time (p<0.0001), no effect of irradiation	
					CD80high B cells	decreased with time (p=0.0039), no effect of irradiation	
					CD27+ B cells	decreased with time (p<0.0001), no effect of irradiation	
					T lymphocyte	increased with time (p<0.001), no effect of irradiation	
					CD4/CD8 T cell ratio	increased with time (p<0.0001), no effect of irradiation	
					CD4+CD8+ T cell	no difference between control vs irradiated for all the study period	
					Naïve CD4 T cell	increased with time (p<0.0036) with significant effect of radiation exposure (p=0.0099) with interaction of time and irradiation (p<0.0001)	
					Naive CD8 T cell	increased with time (p<0.0001) with significant effect of radiation exposure (p=0.0241)	

*1 RRDC: recurrent respiratory disease children
*2 ASD: average summary (internal and external) doses (ASD) of ^{137}Cs and ^{90}Sr
*3 CRS: chronic radiation syndrome
*4 patients with pre- or post- operative radiation treatment (45 Gy) vs only surgery
*5 the number of patients with additional RT was not available
*6 WBRT: whole breast radiation treatment
*7 PBMC: peripheral blood mononuclear cell

3.5 Factor of age for hematologic system after ionizing radiation exposure

3.5.1 Aging in hematopoietic system

Hematopoiesis changes with maturation and aging. Juvenile hematopoiesis after birth is lymphoid biased to develop the adaptive immune system (MacKinney et al., 1987). In the steady

state of matured individuals, lymphoid and myeloid biased hematopoietic stem cells are balanced, providing both lymphoid and myeloid cells equally for daily self-renewal of blood cells as well as hematopoietic challenges such as infection and inflammation (Kovtonyuk et al., 2016). With aging, the hematopoietic system becomes myeloid biased with less self-renewal and regenerative potential of the stem cells (de Haan and Lazare, 2018). Even though the overall function of hematopoiesis is maintained, myeloid biased hematopoiesis causes functional deficiency in both the adaptive and innate immune system, leading to age-related conditions such as higher susceptibility to infectious disease, lower efficacy to vaccines, and higher probability of hematologic and autoimmune disease (Kovtonyuk et al., 2016). Some studies also proved a difference in blood cell counts depending on age or age group (Xia et al., 2009; Xie et al., 2013). However, age-dependent differences in leukocyte counts were not statistically confirmed or investigated thoroughly for either human or NHPs (Smucny et al., 2001; Kun et al., 2020).

3.5.2 Factor of age for risks of ionizing radiation exposure

Age is an indispensable factor to assess the impact and long-term consequence of ionizing radiation exposure since the system responding to ionizing radiation changes with age. In addition, long-term assessment of radiation effects should take the factor of age into account. Health effects possibly caused by ionizing radiation are long term, such as cancer and cardiovascular disease, which naturally increase with advancing age. Generally, radiation sensitivity is the highest at development and early ages of organisms and the lowest after maturation, but increases again with aging (Tong and Hei, 2020).

Epidemiological studies for A-bomb survivors and patients who experienced radiation therapy provided evidence for age-related cancer risk after high dose or high dose-rate ionizing radiation. For most types and sites of cancer, excess relative risk decreases with age at exposure

(Little 1993; Little 2003). Little et al. (1998) also proposed that solid cancer risks for population exposed at an earlier age decreases with time, while those risks are consistent over time for adult exposures. Leukemia risks from radiation exposure generally increases for a short period, is conserved for a limited time, and then decreases with time (Preston et al., 1994). Models fitted for excess relative risk for leukemia suggested time after exposure is a more important factor than age at exposure for leukemia risk estimation. Both age and time after exposure are important factors for solid cancers (Preston et al., 2004). Long-term consequences of ionizing radiation exposure in terms of cancer risk changes with time after exposure, age at exposure, and cancer type, as well as dose and dose-rate.

Compared to cancer risks, the factor of age as a risk for cardiovascular disease and non-cancer disease has been less focused. This is because the excess risk itself is much smaller than cancer, and the background risks of non-cancer disease are higher than those of cancer (UNSCEAR, 2006). Preston et al (2004) analyzed epidemiological data from A-bomb survivors, suggesting lifetime risk of non-cancer disease for population exposed in their childhood was half or less of that for solid cancer risk, while the risks were equal for population exposed at older age. In contrast, several studies found that age at exposure is not significantly related to the risk of non-cancer disease (Little 2004; Zhang et al., 2005).

Experimental studies on the NHP cohort have proposed that there is an effect of aging and age at time of irradiation for irradiated animals (Little et al., 2022). The effect of age at time of exposure proved to have much less significance on delayed health effects than expected from epidemiologic studies of human cohorts (which ones?). However, attained age had a very significant impact on many delayed radiation health effects, such as cardiac disease, hypertension, and cancer ($p<0.005$), exceeding the effects of radiation exposure. Overall, both aging and age at

exposure are important factors to understand long-term consequences from ionizing radiation exposure, however these relationships are not fully understood due to a limited number of studies. The factor of age must be considered carefully because some of the health outcomes are also age-related.

4 Preliminary analysis

4.1 Methods

4.1.1 Rhesus Macaques Dataset

A remarkable dataset of exposures, doses, and complete blood cell counts (CBC) from non-human primates (NHPs) in the Wake Forest School of Medicine Radiation Late Effects Cohort (WFSM RLEC) were analyzed. NHPs in this study were male Chinese-origin macaques (Macaca mulatta). Twenty-one animals with long term observation (8-14.6 years) of leukocyte counts and the differentials were chosen for control and irradiated cohorts for longitudinal analysis. Animals were housed socially in indoor-outdoor pens whenever possible, or in group cages, if necessary, for safe handling or medical care. Care was taken to ensure that animals in the pens were socially compatible. Twelve animals were controls, having no radiation exposure and obtained from the University of Illinois and Primate Products Company. Nine animals were exposed to one-time whole-body irradiation with no mitigator assigned after exposure. The radiation dose delivered varied from 7.2-8.4 Gy, a potentially lethal dose, considering that the LD50/30 and LD90/30 for Rhesus Macaques are approximately 6.7 and 8 Gy (MacVittie et al., 2015). NHP radiation exposures were performed at different facilities around the country prior to relocation to WFSM; the University of Maryland for 5 animals and University of Illinois for 4 animals. The irradiations were conducted under Institutional Animal Care and Use Committee (IACUC) oversight with a protocol of linear accelerator-derived photons at a nominal mean energy of 2 MeV, dose rate at 80 cGy/min as a split dose in half anterior-posterior and half posterior-anterior. NHP age at irradiation varied from 38 to 52 months (3.2-4.4 years). Animals received no mitigator after IR exposure except for one animal who received full clinical support, which included blood transfusions,

intravenous and subcutaneous fluids, antibiotics and nutritional support. Following irradiation, the animals were transferred to WFSM for long-term observation. They were monitored twice a day by trained veterinary technical staff for health and welfare. WFSM is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. All the procedures at WFSM were conducted in compliance with IACUC of Wake Forest University. NHP's developed numerous co-morbidities during the observation period including periodontitis, neoplasia, type II diabetes, cardiovascular and heart disease and GI, kidney, and lung disease. Half of the control animals were deceased, either from euthanasia or natural death at 17.1±1.0 (mean±SD) years of age. All irradiated animals except for one were deceased at 16.5±0.9 (mean±SD) years. Detailed demographic data for individual animals is listed in table 5.

Table 5: Summary of demographic information of Rhesus macaques in the preliminary analysis

Control						
Source	Birth Date	Death				
		Date	Age (yr)			
UNIVERSITY OF ILLINOIS	04/24/03					
UNIVERSITY OF ILLINOIS	05/08/04	09/25/19	15.3			
UNIVERSITY OF ILLINOIS	01/31/06					
PRIMATE PRODUCTS	06/08/01	03/25/21	19.8			
PRIMATE PRODUCTS	09/06/01					
PRIMATE PRODUCTS	05/03/02	11/18/20	18.5			
PRIMATE PRODUCTS	05/03/02					
PRIMATE PRODUCTS	03/17/03	11/04/20	17.6			
PRIMATE PRODUCTS	05/03/03					
PRIMATE PRODUCTS	01/25/04					
PRIMATE PRODUCTS	05/03/04	01/23/20	15.7			
PRIMATE PRODUCTS	01/26/05	09/24/20	15.6			
Irradiated						
Source	Birth Date	Death		Radiation Dose (Gy)	Irradiation	
		Date	Age (yr)		Date	Age (yr)
UNIVERSITY OF MARYLAND	03/18/02	12/19/19	17.8	7.55	08/13/06	4.3
UNIVERSITY OF MARYLAND	06/04/02			8.4	10/29/06	4.3
UNIVERSITY OF MARYLAND	05/02/03	08/14/18	15.3	7.2	08/13/06	3.3
UNIVERSITY OF MARYLAND	05/12/03	01/27/21	17.7	7.85	09/17/06	3.3
UNIVERSITY OF MARYLAND	07/15/03	01/17/19	15.5	7.55	08/13/06	3.0
UNIVERSITY OF ILLINOIS	03/03/03	10/02/19	16.5	7.55	08/25/07	4.4
UNIVERSITY OF ILLINOIS	06/01/03	05/14/19	15.9	7.55	04/14/07	3.8
UNIVERSITY OF ILLINOIS	03/17/04	03/11/21	16.9	8.05	07/07/07	3.3
UNIVERSITY OF ILLINOIS	05/03/04	03/18/21	16.8	7.2	07/07/07	3.2

Blood sampling was conducted as part of routine clinical examination at intervals that varied between several weeks to 6 months. The animals were sedated with intramuscular ketamine and blood was collected from the femoral vein with EDTA-vacutainers. The CBC dataset included white blood cell counts, red blood cell counts, hematocrit, lymphocyte % and absolute lymphocyte count, neutrophil % and absolute neutrophil count, monocyte absolute and %, basophil %, eosinophil %, band neutrophil % and absolute band neutrophil %. The CBC was counted by flow cytometry and random samples were reviewed with light microscopy by board-certified veterinary pathologists for irregularities.

The CBC dataset from observations at the age of 8-17 years old were analyzed for age-matched longitudinal analysis. Observations from the first four years post exposure were not included in this study to ensure that all acute phase effects were resolved, as well as for age matching purposes. Observations within 6 months prior to the time of death at WFSM were also excluded from the analysis. Any observations of blood parameters below or above 1% percentile of the dataset were excluded from the analysis as potential outliers. Each animal had 21-46 data observations of each blood parameter during the study period. In total, 409-413 and 334-339 observations of each blood parameter were analyzed for control and irradiated animals, respectively. Different longitudinal trends based on irradiation status were estimated from simple linear regressions of the data (Figure 6). The mean and standard deviation of the counts and percentages were calculated, suggesting the difference between unirradiated and control animals (Table 6).

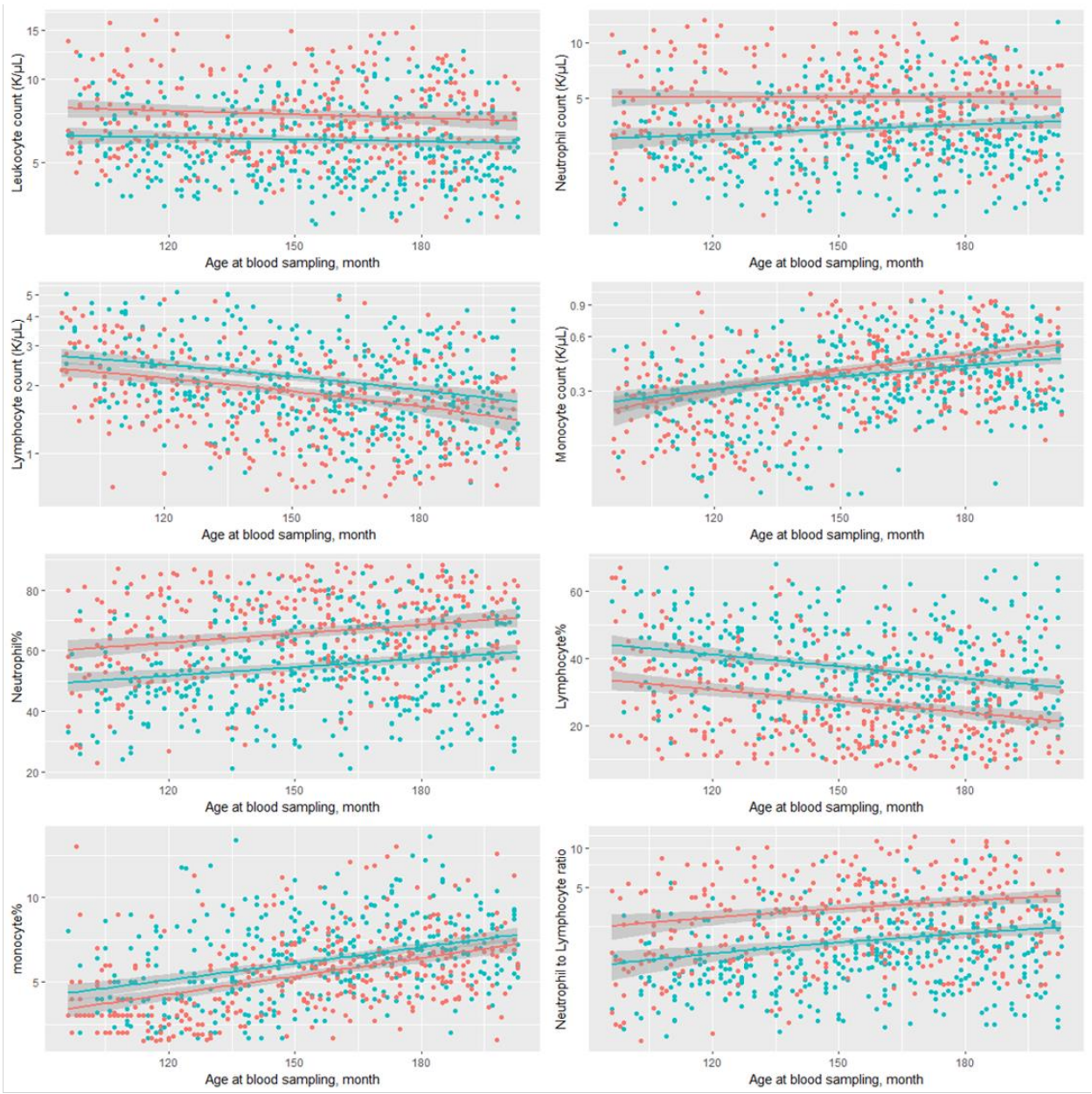

Figure 6: Plot of leukocyte and the differential counts with simple linear regression. Red is the irradiated and blue is the control group.

Table 6: Summary of Leukocytes and the differential counts and percentages

	Count (K/μL)		Percentage (%)	
	control	irradiated	control	irradiated
Leukocyte	5.99 ± 2.09	7.66 ± 3.01		
Neutrophil	3.35 ± 1.82	5.25 ± 2.96	54.48 ± 15.00	65.98 ± 15.51
Lymphocyte	2.16 ± 1.00	1.88 ± 0.93	37.33 ± 13.74	26.96 ± 14.08
Monocyte	0.37 ± 0.18	0.41 ± 0.25	6.33 ± 2.91	5.52 ± 2.86
NLR			1.92 ± 1.47	3.72 ± 3.11

4.1.2 Statistical Analysis

Linear mixed models (LMM) were used for longitudinal analysis of leukocyte counts and WBC differentials. LMM assigns random intercepts and/or slopes for each animal in order to account for the correlation between repeated measurements from the same animals over the study period. LMM is assumed to be the best method for the dataset and study goal since the dataset contains repeated measurement and leukocyte counts have individual differences (Capitanio et al., 1999) and fluctuate in a large variance even within a single day (Henriette et al., 2011). LMM have been identified as the best statistical model to utilize in longitudinal studies of biomedical parameters (Edwards et al., 2000; Liu et al., 2010; Garcia et al., 2017). Previous longitudinal studies of WBCs and differentials for atomic bomb survivors also used LMM to take the effect of repeated measures into account (Hsu et al., 2010; Yoshida et al., 2019). LMM assumes individual effects are constant over the study period. A response variable for ith(?) individual is described as

$$y_i = X_i \alpha + Z_i \beta_i + \varepsilon_i \qquad (1)$$

where ε_i is the residual term distributed as $N(0, R_i)$ (normal distribution with mean 0 and covariance matrix Ri) and assumed to be independent. α denotes a fixed effect and constant for the population and X_i is the predictor variable for the response variable, y_i. The $Z_i\beta_i$ term represents the random effect for the individual, assigning different intercepts and slopes for each animal (Laird and Ware; 1982).

A linear mixed model was fit to each response variable separately (leukocyte, absolute neutrophil, absolute lymphocyte, or absolute monocyte count, as well as neutrophil%, lymphocyte%, monocyte%, and neutrophil to lymphocyte ratio (NLR). Irradiation (yes or no), time in months (scaled with the mean and standard deviation), and the interaction of the two variables were included in the model as fixed effects. A random intercept and slope were allowed for each

animal to account for the correlation between samplings from the same animals. Residual diagnostic plots were used to confirm model assumptions. A log-transformation was used for leukocyte, absolute lymphocyte, neutrophil, and monocyte count, and NLR to satisfy model assumptions. Estimated marginal means (EMMs) and EMMs of linear trends were calculated based on the models with the confidence level of 0.95. EMMs are estimated values of the blood parameter based on the models at the average age at sampling. EMMs of linear trends are the average slope calculated for control and irradiated animals respectively. EMMs and EMMs of linear trends were utilized to compare the values and trends between control and irradiated animals. All analyses were conducted using R version 4.2.3 with lme4 and emmeans packages (Bates et al., 2015; Lenth, 2023).

4.2 Results

The categorical factor of irradiation status (irr) was statistically significant for all models ($p<0.0001$). The significance of age at time of blood sampling was also confirmed for all blood parameters ($p<0.05$) except for total leukocyte counts and neutrophil counts, indicating these counts did not have any age-related linear trends over time in the study period. Absolute lymphocyte count and lymphocyte% statistically decreased with age at the blood sampling and neutrophil%, monocyte count, monocyte%, and neutrophil to lymphocyte ratio statistically increased, which agreed with the trend estimated with the simple linear regression (Figure 6). The interaction of irradiation status and age at the time of blood sampling did not show any statistical significance in any models. Residual plots of the models had equally distributed residuals, indicating the adequate fit of the models to the dataset. Correlations between random slopes and random intercept calculated for the models were small (0.45- -0.2 in range) enough to assure the significance of random slopes and intercepts to represent the data.

Contrasts of control and irradiated animals using EMMs demonstrated long-term differences in leukocyte counts, neutrophil counts, neutrophil%, lymphocyte%, and NLR (Table 7). For example, the result of the analysis for leukocyte counts indicates, at the scaled parameter of age at blood sampling -0.115, the estimated marginal mean of the log of leukocyte count for control animals is 1.75 and that for irradiated animals is 1.95. The contrast between these estimated marginal means is statistically significant ($p = 0.0310$), suggesting that irradiated monkeys have a statistically higher leukocyte count than control monkeys. In the same way, the results indicating that irradiated animals had statistically larger values of absolute neutrophil counts ($p=0.0006$), neutrophil% ($p=0.0012$), and NLR ($p=0.0007$) and smaller values of lymphocyte% ($p=0.0004$) compared to the controls. No significant effects on monocyte numbers.

Statistically significant longitudinal trends ($p<0.05$) were estimated for absolute lymphocyte counts, neutrophil% lymphocyte%, and monocyte% from both irradiated and control animals. In contrast to the EMMs, estimated linear trends did not show any statistically significant difference between control and irradiated animals.

Table 7: Estimate marginal means and the 95% confidential interval for each blood parameter and the longitudinal trend with p-values.
Contrasts are for Control vs Irradiated.

Model	EMMs			EMMs of longitudinal trends	
	Control	Irradiated	contrast	Control	Irradiated
Log (leukocyte (K/μL))	1.75 (1.64, 1.87)	1.95 (1.81, 2.08)	*	-0.019 (-0.068, 0.030)	-0.031 (-0.085, 0.024)
Log (absolute neutrophil (K/μL))	1.11 (0.98, 1.24)	1.49 (1.34, 1.64)	**	0.0358 (-0.0370, 0.1086)	0.0118 (-0.0684, 0.0921)
Log (absolute lymphocyte (K/μL))	0.671 (0.492, 0.850)	0.506 (0.300, 0.713)		**-0.112 (-0.174, -0.051)**	**-0.140 (-0.209, -0.071)**
Log (absolute monocyte (K/μL))	-1.13 (-1.29, -0.97)	-1.05 (-1.23, -0.87)		**0.195 (0.100, 0.289)**	**0.251 (0.144, 0.357)**
Log (NLR)	0.435 (0.248, 0.623)	0.986 (0.770, 1.201)	**	0.150 (0.047, 0.252)	0.166 (0.053, 0.279)
Neutrophil%	55.0 (51.1, 58.9)	65.9 (61.3, 70.4)	**	**2.45 (0.33, 4.57)**	**2.73 (0.40, 5.06)**
Lymphocyte%	3.54 (3.42, 3.66)	3.17 (3.03, 3.30)	**	**-0.098 (-0.161, -0.036)**	**-0.117 (-0.186, -0.048)**
Monocyte%	6.20 (5.48, 6.91)	5.61 (4.79, 6.43)		**1.07 (0.66, 1.49)**	**1.17 (0.71, 1.64)**

Note: trends with statistical significance ($p<0.05$) were expressed in bold. *$p<0.05$, **$p<0.005$.

5 Analysis of Factors in Longitudinal Trend

Based on results from our preliminary analysis, we conducted a more comprehensive analysis with more animals with different radiation dose, sex, age at the time of exposure, and mitigator assignment.

5.1 Method

5.1.1 Dataset

A dataset of complete blood cell counts (CBC) from non-human primates (NHPs) was provided from Wake Forest School of Medicine Radiation Late Effects Cohort (WFSM RLEC). NHPs in this study were either Indian, Chinese, or unknown origin rhesus macaques (Macaca mulatta). The control and irradiated animals used for this study were obtained from Wake Forest University, University of Maryland, University of Illinois, Armed Forces Radiobiology Research Institute, Lovelace Respiratory Research Institute, Citox Labs, and Primate Products. Animals were housed socially in indoor-outdoor pens whenever possible, or in group cages, if necessary, for safe handling or medical care. Care was taken to ensure the animals in the groups were socially compatible. Forty eight animals were controls with no ionizing radiation exposure. Two hundred and twenty (220) animals were exposed to one-time whole-body radiation which varied from 1.14-8.5 Gy. Note that the LD50/30 and LD90/30 for Rhesus Macaques are approximately 6.7 and 8 Gy (MacVittie et al., 2015).

NHP radiation exposures were performed at different facilities prior to relocation to WFSM. The irradiation was conducted under Institutional Animal Care and Use Committee (IACUC) oversight with a protocol of either (1) linear accelerator-derived photons at a nominal mean energy of 2 MeV, dose rate at 80 cGy/min as a split dose in half anterior-posterior and half posterior-

anterior or (2) Cobalt 60-derived gamma irradiation delivered simultaneously, bilaterally at 60 cGy/min. NHP age at irradiation varied from 38 to 52 months (3.2-4.4 years). Some animals received mitigators immediately after IR exposure. The mitigators included white blood cell and platelet growth factors, antibiotics, and other agents that aid in recovery of hematopoietic damage. Following irradiation, the animals were transferred to WFSM for long-term observation. They were monitored twice daily by trained veterinary technical staff for health and welfare. WFSM is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. All procedures at WFSM were conducted in compliance with IACUC of Wake Forest University.

NHP's developed numerous co-morbidities during the observation period including periodontitis, neoplasia, type II diabetes, cardiovascular and disease (CVD), as well as gastrointestinal (GI), kidney, and lung disease. Some of the animal data used for control (30.4 %) and irradiated animals (35.5%) were deceased, either from euthanasia or natural death. Table 8 summarizes sex, the use of mitigator, and age at the time of exposure distribution within dose levels.

Blood sampling was conducted as a part of routine clinical exams at an interval that varied between several weeks and 6 months. The animals were sedated via intramuscular ketamine injection and blood was collected from the femoral vein with EDTA-vacutainers. The CBC dataset included white blood cell counts (WBCs), red blood cell counts (RBCs), hematocrit, lymphocyte % and absolute lymphocyte count, neutrophil % and absolute neutrophil count, monocyte absolute and %, basophil %, eosinophil %, band neutrophil % and absolute band neutrophil %. The CBC was counted by flow cytometry and some samples were reviewed with light microscopy by board-certified veterinary pathologists for irregularities. All the CBC data available until November 18th,

2022 were incorporated in the longitudinal analysis. Two animals were excluded from the analysis because they developed hematopoietic diseases (lymphoma), and three animals were removed due to no irradiation date recorded. Each animal had 5-58 data observations for each blood parameter during the study period. In total, 1079 control samples (from 47 animals) and 3964 irradiated samples (from 216 animals) were analyzed (Table 8). Table 9 is the summary table of parameters in different dose groups. Different longitudinal trends based on dose levels were estimated from simple linear regressions of the data (Figure 7).

Table 8: Demographic of the data in the analysis of factors

	dose level			
	unirradiated	<LD10	LD10-LD50	>LD50
dose range (Gy)	0	1.14-5	5.5-6.8	7-8.5
animals, n	47	59	109	48
initial examination (m) mean ±sd	111±62	74±18	70±36	73±21
final examination (m) mean ±sd	176±58	113±31	119±44	148±37
age at exposure (m) mean ±sd		61±13	54±24	50±10
female, n (%)	5 (11)	23 (39)	36 (33)	10 (21)
mitigator used, n (%)	0 (0)	33 (56)	62 (57)	4 (8)

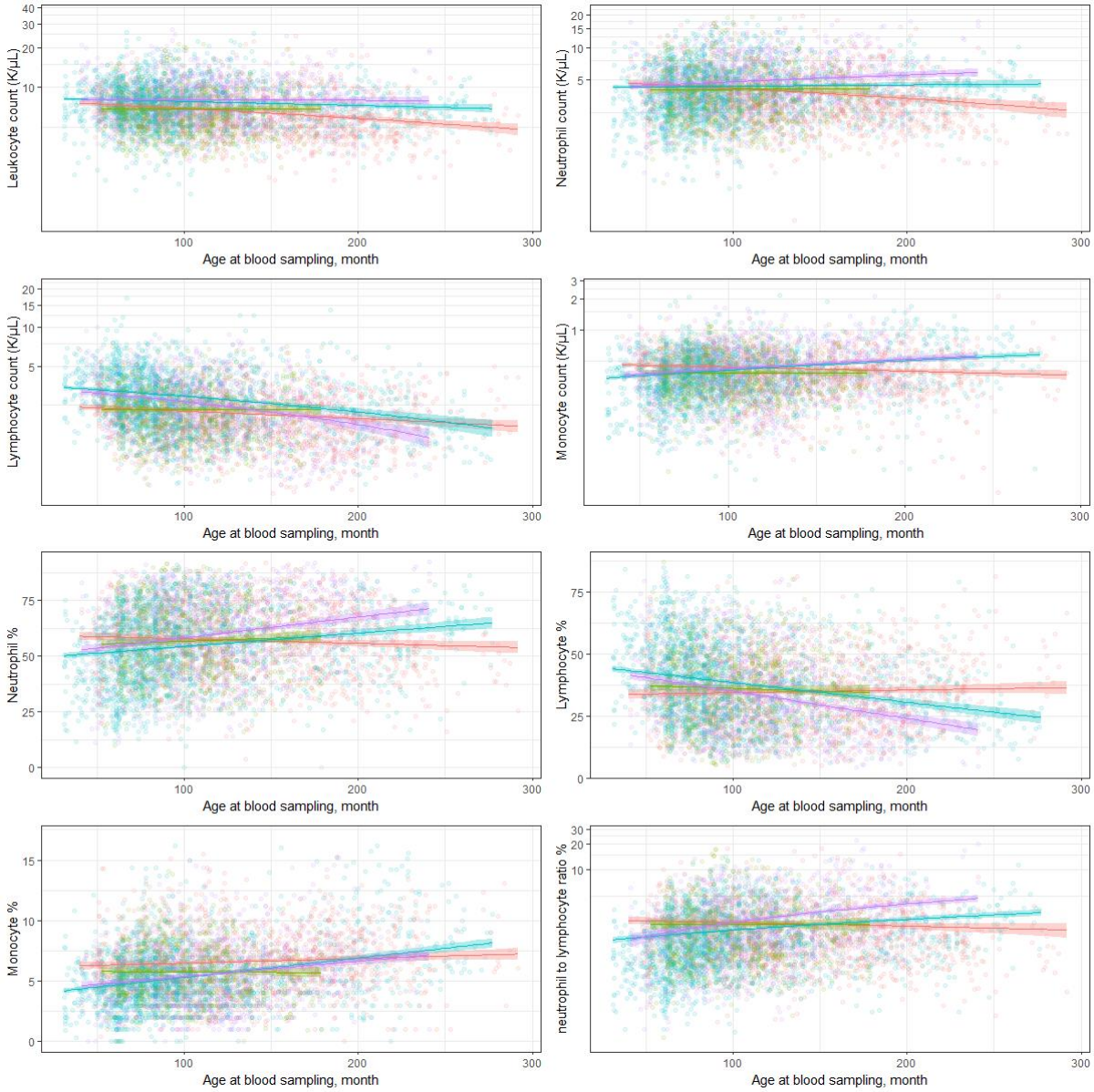

Figure 7: Plot of leukocyte and the differential counts with simple linear regression. Red is unirradiated, green is <LD10, blues is LD10-50 and purple is >LD50.

Table 9: Summary of Leukocytes and the differential counts and percentages

		control	<LD10	LD10-50	>LD50
Leukocyte	count (K/μL)	6.50±2.68	6.89±2.82	7.83±2.96	8.05±3.13
Neutrophil	count (K/μL)	3.87±2.48	4.11±2.62	4.40±2.51	5.00±2.94
	%	56.99±15.59	56.88±15.97	54.70±16.49	59.89±16.67
Lymphocyte	count (K/μL)	2.12±1.02	2.31±1.12	2.90±1.59	2.50±1.31
	%	34.78±14.50	35.97±14.94	38.10±15.70	33.1±15.44
Monocyte	count (K/μL)	0.42±0.21	0.38±0.19	0.41±0.23	0.44±0.25
	%	6.72±2.88	5.76±2.22	5.40±2.60	5.60±2.57
NLR	%	2.35±2.13	2.34±2.29	2.02±1.73	2.71±2.46

5.1.2 Statistical Analysis

Linear mixed models (LMM) were developed to evaluate longitudinal trends of the hematologic endpoints. Detailed descriptions of the models are presented in Chapter 4.1.2. For this analysis, a categorical variable for dose level was assigned based on the exposed dose of each animal and the LD10/30 and LD50/30 for rhesus macaques. The interaction of age in months at time of blood sampling and dose of exposure was also included to test the influence of different doses to the longitudinal outcome of the leukocyte counts and the differentials. The significance of interaction was tested by Chi-squared test in comparison with models without the interaction. Sex, administration of mitigator, and age at the time of exposure were included as covariates. sex and use of mitigators were categorical variables for male or female and yes or no of assignment of any mitigators after exposure. Age at the time of exposure is a continuous variable in month. The month of initial blood sampling was used for this variable of control animals. Models were

diagnosed with residual vs fitted plots. All models had equally spread residuals, indicating good fit of the model to the dataset.

5.2 Results

All estimates of coefficients and associated p-values are provided in Table 10, and graphical presentations are in Figures 8 and 9. Age at time of blood sampling was a significant factor for leukocyte, neutrophil, lymphocyte, and monocyte counts ($p<0.05$). While leukocyte counts decreased with time alongside with neutrophils and lymphocytes, monocyte counts increased. Percentages did not change with the age at time of blood sampling except for monocyte %, which increased with age. Dose level was suggested as an important factor for the longitudinal trends of leukocyte counts and the differentials, which was supported by Chi-square test ($p<0.05$) for all the models. Control animal leukocyte counts, lymphocyte and neutrophil counts decreased significantly over time, but radiation exposed animals had increased or unchanged counts over time (see Table 10 for the estimates and p-values). Monocyte counts and neutrophil to lymphocyte ratio increased more significantly in animals with LD10-50 and >LD50 dose compared to the controls and <LD10 exposed animals. Neutrophil% and lymphocyte% from control animals did not show significant trends over time, while those from LD10-50 dose groups were significantly increased neutrophil% and decreased lymphocyte%. Monocyte% from control animals significantly increased with age at the blood sampling, and exposed animals did not show significant difference or decreased significantly over time.

Compared to the male animals, females had lower neutrophil counts and percentages, more lymphocyte counts and percentages, and lower neutrophil-to-lymphocyte ratio. The association of time at blood sampling and dose level was also tested with a Chi-square test. Only lymphocyte count and neutrophil-to-lymphocyte ratio showed significant association of sex with age at blood

sampling and dose level ((X^2, df) = p-value :(26.116, 7) = 0.00048, (16.91, 7) = 0.018, respectively). The status of mitigator assignment had no significant effect on the longitudinal trend of any blood parameters. Age at the time of exposure was a significant covariate only for monocyte counts and monocyte % (p<0.001). The estimates calculated suggested the animals exposed at older age have a smaller number of monocytes and smaller percent monocytes compared to the animals exposed at the younger age. Only monocyte percentages had statistically significant association with age at the blood sampling and dose level (55.347, 7) <0.0001).

Table 10: Summary of results.
Estimates with p-values for each variable.

		estimate	p-value	interaction with age at sampling		
				estimate	p-value	(X^2, df) = p-value
Leukocyte count	age at sampling (m)	**-0.121**	**0.0001**			
	dose level 1 (vs unirradiated)	0.028	0.665	**0.119**	**0.015**	
	dose level 2 (vs unirradiated)	**0.134**	**0.031**	**0.097**	**0.010**	**(12.126, 3)=0.006965**
	dose level 3 (vs unirradiated)	**0.177**	**0.007**	**0.135**	**0.001**	
	sexF (vs male)	-0.030	0.405			
	mitigator (vs No)	-0.033	0.391			
	age at exposure (m)	-0.0001	0.893			
Neutrophil count	age at sampling (m)	**-0.088**	**0.004**			
	dose level 1 (vs unirradiated)	0.010	0.881	0.073	0.148	
	dose level 2 (vs unirradiated)	0.086	0.166	**0.144**	**0.0002**	**(16.247, 3)=0.001009**
	dose level 3 (vs unirradiated)	**0.147**	**0.026**	**0.123**	**0.003**	
	sexF (vs male)	**-0.078**	**0.043**			
	mitigator (vs No)	-0.035	0.378			
	age at exposure (m)	0.0002	0.797			
Lymphocyte count	age at sampling (m)	**-0.133**	**0.003**			
	dose level 1 (vs unirradiated)	0.062	0.455	**0.153**	**0.026**	
	dose level 2 (vs unirradiated)	**0.159**	**0.045**	-0.070	0.195	**(16.024, 3)=0.001121**
	dose level 3 (vs unirradiated)	**0.186**	**0.026**	0.082	0.171	
	sexF (vs male)	**0.098**	**0.042**			
	mitigator (vs No)	-0.010	0.833			
	age at exposure (m)	-0.0003	0.763			
Monocyte counts	age at sampling (m)	**0.023**	**0.039**			
	dose level 1 (vs unirradiated)	-0.041	0.067	-0.0003	0.986	
	dose level 2 (vs unirradiated)	-0.027	0.210	**0.030**	**0.027**	**(9.1772, 3)=0.02703**
	dose level 3 (vs unirradiated)	-0.025	0.263	**0.035**	**0.020**	
	sexF (vs male)	-0.009	0.481			
	mitigator (vs No)	-0.007	0.609			
	age at exposure (m)	**-0.001**	**0.001**			

Table 10 (continued)

		estimate	p-value	interaction with age at sampling		
				estimate	p-value	(X^2, df) = p-value
Neutrophil to Lymphocyte ratio	age at sampling (m)	0.012	0.713			
	dose level 1 (vs unirradiated)	-0.036	0.604	-0.034	0.543	
	dose level 2 (vs unirradiated)	-0.051	0.441	**0.132**	**0.002**	**(15.612, 3)=0.001362**
	dose level 3 (vs unirradiated)	-0.002	0.982	0.045	0.322	
	sexF (vs male)	**-0.120**	**0.003**			
	mitigator (vs No)	-0.025	0.547			
	age at exposure (m)	0.000	0.861			
Neutrophil%	age at sampling (m)	-0.035	0.976			
	dose level 1 (vs unirradiated)	-0.733	0.749	-0.728	0.704	
	dose level 2 (vs unirradiated)	-0.904	0.675	**4.732**	**0.001**	**(15.93, 3)=0.001172**
	dose level 3 (vs unirradiated)	0.671	0.767	1.629	0.302	
	sexF (vs male)	**-4.143**	**0.002**			
	mitigator (vs No)	-0.666	0.632			
	age at exposure (m)	-0.001	0.982			
Lymphocyte%	age at sampling (m)	-1.195	0.296			
	dose level 1 (vs unirradiated)	1.717	0.429	1.383	0.454	
	dose level 2 (vs unirradiated)	2.102	0.304	**-4.758**	**0.001**	**(10.096, 3)=0.0002612**
	dose level 3 (vs unirradiated)	0.989	0.644	-1.517	0.329	
	sexF (vs male)	**4.491**	**0.001**			
	mitigatorY (vs No)	0.463	0.729			
	age at exposure (m)	0.022	0.413			
Monocyte%	age at sampling (m)	**1.396**	**0.000**			
	dose level 1 (vs unirradiated)	**-1.258**	**0.002**	**-0.919**	**0.004**	
	dose level 2 (vs unirradiated)	**-1.614**	**0.000**	-0.206	0.391	**(8.8394, 3)=0.0315**
	dose level 3 (vs unirradiated)	**-1.816**	**0.000**	-0.333	0.208	
	sexF (vs male)	-0.053	0.824			
	mitigatorY (vs No)	0.002	0.995			
	age at exposure (m)	**-0.024**	**0.000**			

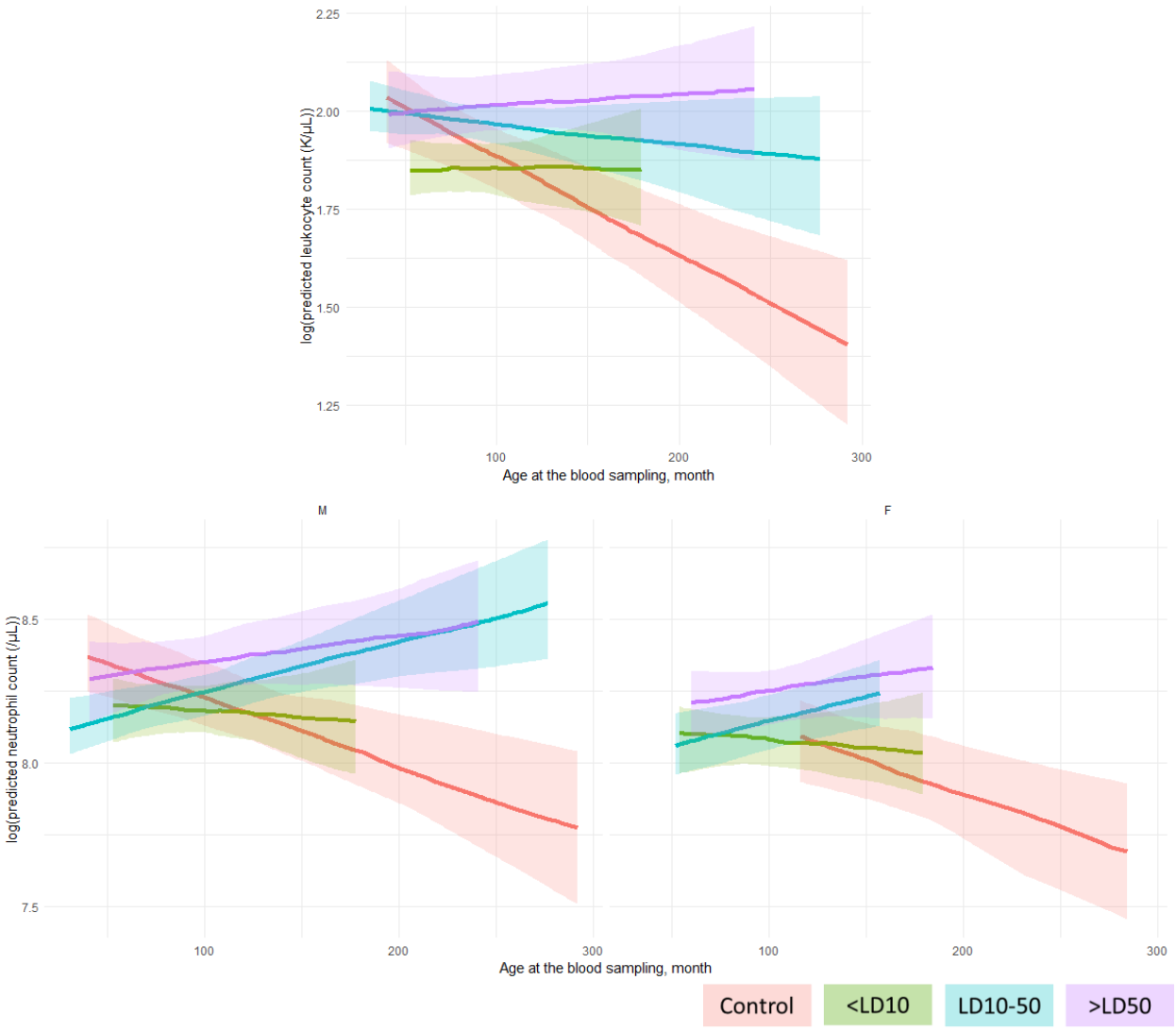

Figure 8a model trajectories for leukocyte and neutrophil counts.
Shaded area indicates 95% CI. red= control, green=<LD10, blue=LD10-50, purple=>LD50.
Note neutrophil counts were plotted separately based on sex (left: male, right: female)

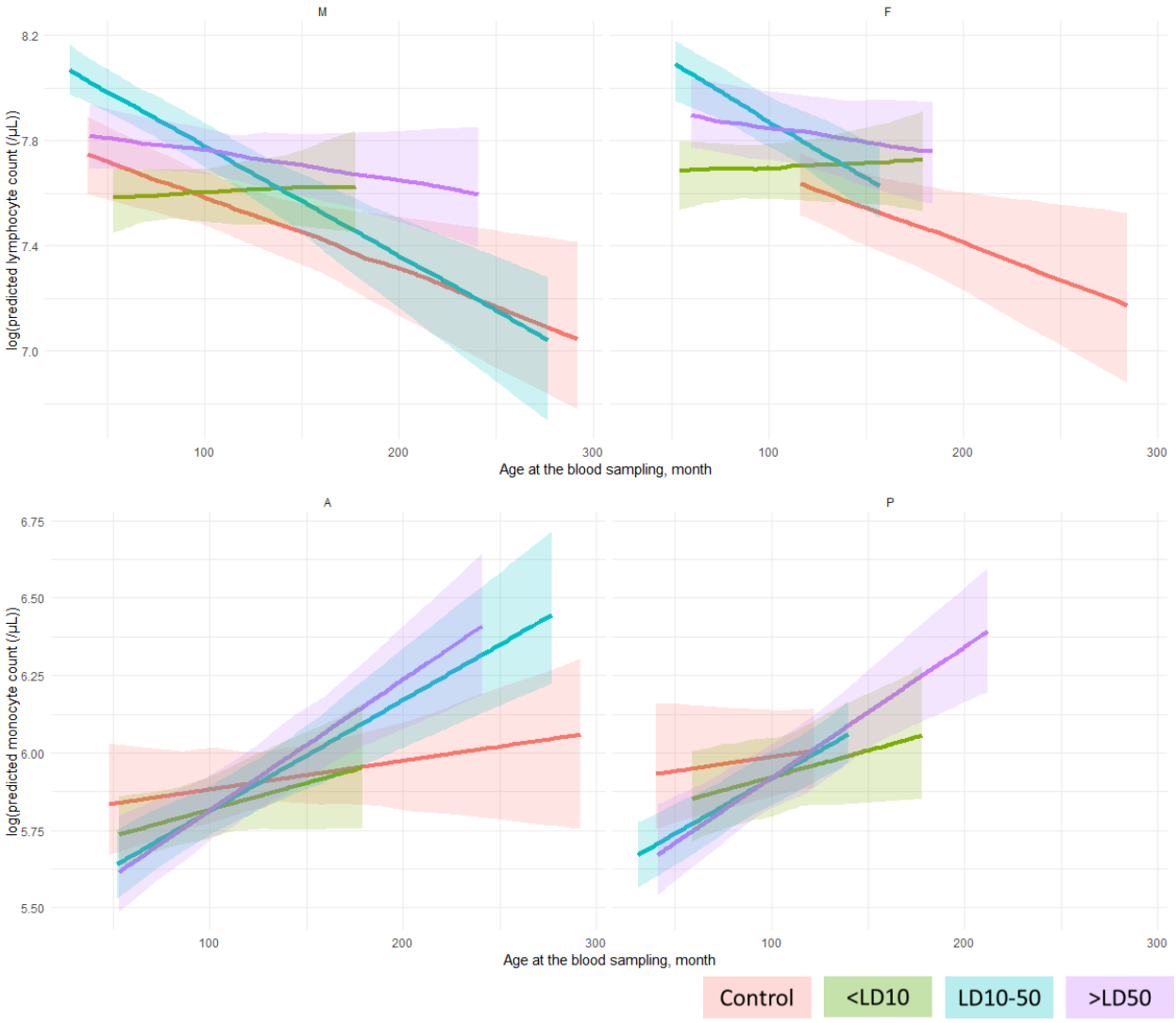

Figure 8b model trajectories for lymphocyte and monocyte counts.
Shaded area indicates 95% CI. red= control, green=<LD10, blue=LD10-50, purple=>LD50.
Note lymphocyte counts were plotted separately based on sex (left: male, right: female) and monocyte counts were plotted separately based on age at exposure (left: A=adult (>48 month old at the time of exposure), right: P=pediatric (≤48 month old at the time of exposure))

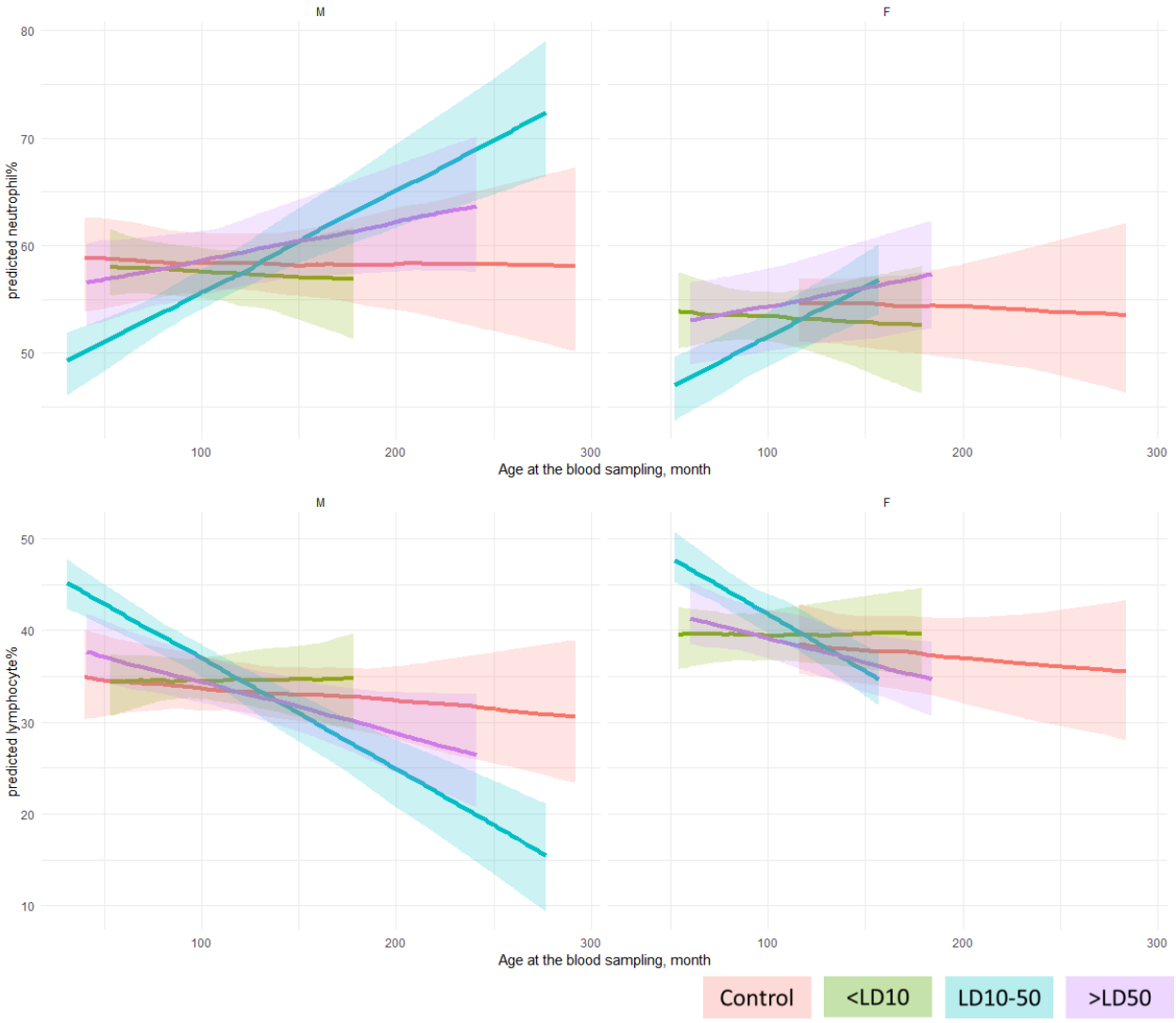

Figure 9a model trajectories for neutrophil and lymphocyte percentages.
Shaded area indicates 95% CI. red= control, green=<LD10, blue=LD10-50, purple=>LD50.
Note neutrophil and lymphocyte percentages were plotted separately based on sex (left: male, right: female).

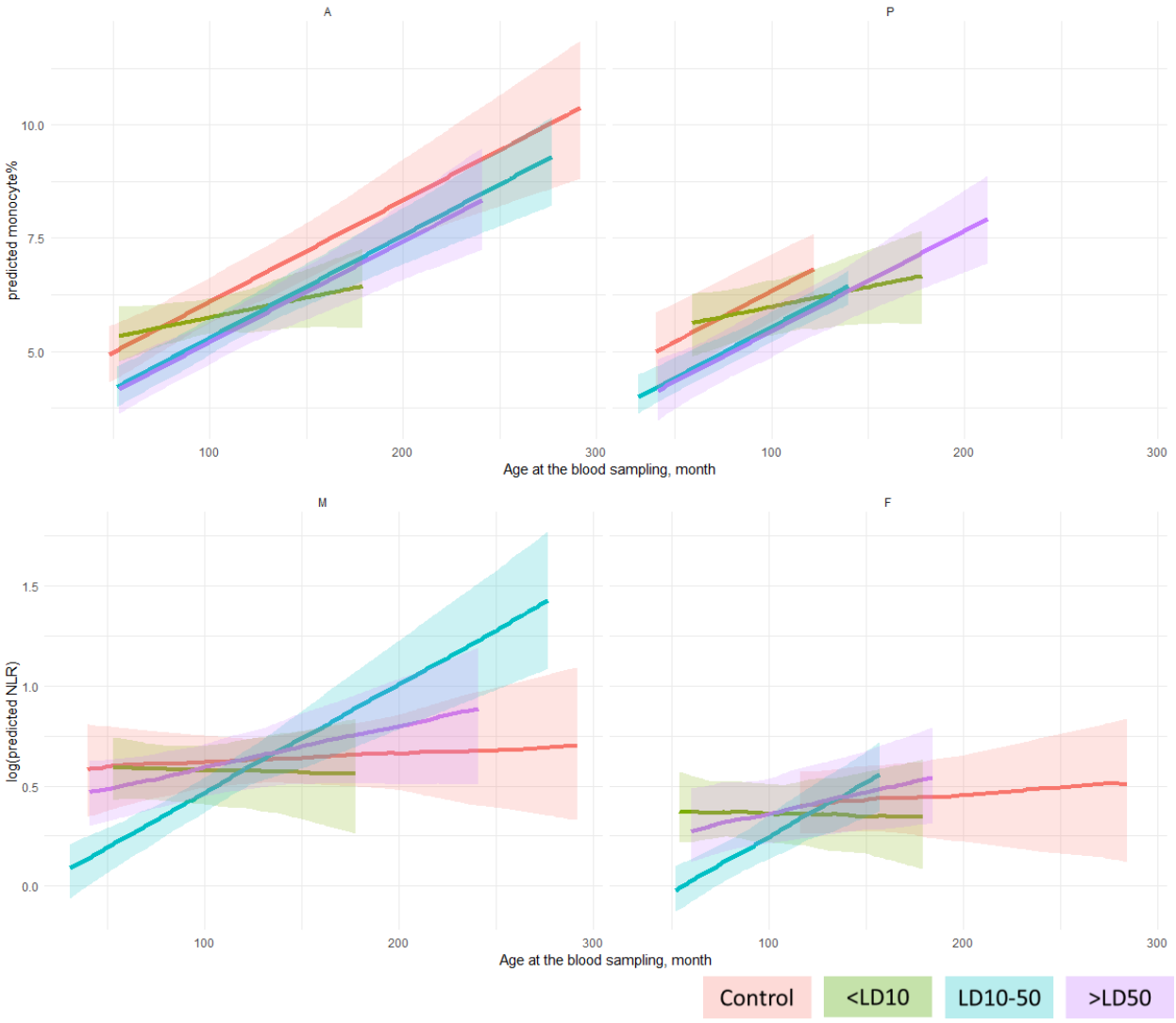

Figure 9b model trajectories for neutrophil-to lymphocyte ratio (NLR) and monocyte percentage. Shaded area indicates 95% CI. red= control, green=<LD10, blue=LD10-50, purple=>LD50. Note NLR was plotted separately based on sex (left: male, right: female) and monocyte percentage was plotted separately based on age at exposure (left: A=adult (>48 month old at the time of exposure), right: P=pediatric (≤48 month old at the time of exposure))

6 Discussion

6.1 Summary of results

The preliminary analysis presented here reports longitudinal trends in total and differential leukocyte counts using mathematical models in irradiated rhesus macaques. Estimated marginal means (EMMs) calculated by the models indicated elevated leukocyte counts in irradiated animals ($p<0.05$), associated with elevated neutrophil counts ($p<0.005$). EMMs for Neutrophil% and NLR were also statistically higher in irradiated animals than in controls ($p<0.005$). In contrast, EMM for lymphocyte% was significantly lower in Irradiated animals than in controls ($p<0.005$). EMMs for the longitudinal trends were significantly negative in lymphocyte count and lymphocyte% ($p<0.05$), and significantly positive in monocyte count, neutrophil%, and monocyte % ($p<0.05$) for both irradiated and control animals. Different from the EMMs for the leukocytes and the differentials, EMMs for longitudinal trends had no statistically significant difference between irradiated and controls.

The result from the next analysis provided more details on longitudinal effects of ionizing radiation to the hematopoietic system. The significance of dose-level factors in the longitudinal trends were statistically supported in all the leukocyte parameters. Model estimates of the leukocyte and neutrophil counts were negative over time in the control animals (figure 8). Model estimates of the leukocyte and neutrophil counts for the dose group were also negative, but did not decrease as rapidly as the control group (Figure 8), consistent with the results from the part 1 analysis. Neutrophil%, Lymphocyte%, and NLR had no significant longitudinal trend for all the dose groups except for LD10-50 group, which suggests a significant decrease in lymphocyte% and significant increase in neutrophil% and NLR. Monocyte counts and the percentages were increased

significantly as shown in the preliminary analysis. The preliminary analysis did not show any statistically significant difference among control and irradiated animals, but monocyte counts seemed increased significantly more in LD 10-50 and >LD50 groups.

All the trends calculated here are very subtle. Even with the largest estimate, which was for the interaction term of age at the blood sampling and dose level 2 group (LD10-50) for neutrophil%, the estimated change for 1 year from 15.5 years old (186 month) to 16.5 years old (198 month) will be from 65.5 % to 66.7% for the male animal with LD10-50 dose irradiation. The calculation process is shown below:

For the neutrophil% model, the only statistically significant factors are.

Intercept: 58.56,

Sex(F): -4.143,

Dose_level(2):m_sample_s: 4.732

Function estimating neutrophil% of a 186-month-old male animal with dose level2

$$\text{Neutrophil\%} = 58.56 + scale(m\_sample) \times 4.732 \qquad (2)$$

The m_sample needs to be scaled with the mean (115.7) and standard deviation (48.1) of the m_sample in this dataset:

$$scale(m\_sample) = \frac{m\_sample - 115.7}{48.1} = 1.46$$

Insert this to the function (2) and the neutrophil % will be.

$$58.56 + 1.46 \times 4.732 = 65.46$$

In the same way, neutrophil% for 198-month-old is estimated as 66.65%

Note the values provided here are merely an estimate based on the model output. LMM assigns random slopes and intercepts for each animal, which cannot be done manually. Therefore, the

calculation and values would be different when we use the model for estimating specific animal parameters. Considering all the estimates are much less than 4.73, the changes would be less than 1% per year. Since the leukocyte and the differential count and percentages has diurnal fluctuation with relative amplitude of 9.75-21.4% (Henriette et al., 2011), the long-term change is quite small even though the statistical significance was large.

One of the reasons for differences in longitudinal trends in different dose levels in the latter analysis was observed is use of a different dataset; the preliminary analysis only included 9-18 years old animals while the second part included all age ranges available (3-18 years old). The number of observations as well as the number of the animals were much larger, providing a statistically stronger analysis. The other possible reason is because the preliminary analysis included irradiated animals with 7.20-8 Gy exposure, which is a very high dose considering the rhesus macaques' lethal dose threshold, and so the animals in the study may be intrinsically resilient against ionizing radiation exposure and may have had less residual damage to the hematopoietic system. The latter analysis is consistent because the statistical significance in the increasing neutrophil%, NLR and the decreasing lymphocyte% were only supported for the LD10-50 group and not the highest dose (>LD50) group.

Confounding factors were also analyzed in the latter study. Sex was the most significant factor next to dose level for lymphocyte count, lymphocyte%, and NLR. Chi-square tests suggested females tend to have higher lymphocyte counts and lower NLR than males with age and dose level. Status of mitigator assignment at the acute phase after exposure was not significant for any leukocyte parameters. Age at the time of exposure was only significant for monocyte counts and the monocyte%, indicating the animals likely to have less monocyte counts and percentages when they exposed at the older age (See chapter 6.4).

To summarize, the main findings of this study are:

1. Subtle but long-term changes in leukocyte counts and differentials were induced by ionizing radiation exposure, and included:
    a. Leukocyte and neutrophil counts were decreasing in controls, while less so and even increasing in irradiated animals.
    b. Neutrophil% and NLR only increased in LD10-50 animals and Lymphocyte% werw decreased in LD10-50 animals.
    c. Monocyte counts increased more in irradiated animals.
2. Sex was a significant factor influencing Neutrophil count and %, Lymphocyte count and % and NLR. Age at exposure was only significant for monocyte counts and %

The trend 1.b. was only observed in LD10-50 groups possibly because the largest dataset of the LD10-50 dose group enabled to detect the slight difference in the dose group. The statistical significance was not provided, the estimates for >LD50 dose group was like those of 1b. The other possibility lies in the selection bias because >LD50 dose is very high, and those animals survived may have resilient hematopoietic system against radiation exposure. However, the >LD50 dose group had the highest disease morbidity (see 6.5), indicating the long-term effect probably exist even if the leukocyte counts and the differentials did not drastically change.

The RLEC cohort, different laboratory animals as well as human studies were reviewed in this chapter to investigate the mechanism and understand the meaning of the result.

6.2 Comparison with other study cohorts

6.2.1 RLEC study

Results in this study partially agreed with previous hematopoietic system studies using the RLEC rhesus macaque cohort. Hale et al. (2019), investigated long-term response of hematopoietic system in 29 male Rhesus Macaques with one-time WBI of 6.5-8.4 Gy matched with 14 control animals. Dose dependent elevation of leukocytes, consistent with finding 1.a, and dose dependent decrease of platelet count and T cell proportion were reported, but there was no dose-dependent difference in absolute lymphocyte count or neutrophil counts.

For rhesus macaques with 4 Gy WBI, Macintyre et al (2021) reported leukocyte, neutrophil, lymphocyte, and monocyte counts had transient depletion for about 100 days of exposure, followed by recovery within one year of exposure. They also found the effect of aging instead of TBI significantly affects the frequencies of dendric cells, CD4 and CD8 T cells and B cells. Naïve/memory T cell balance was also affected by WBI; exposed group had less naïve T cells and more effector T cells. The difference in CD8 T cell distribution between naïve and memory cells continued up to the end of the analysis, which is 664 days after exposure, while that of CD4 T cells recovered between 282 and 664 days.

French et al. (2023), reported a decrease of leukocyte counts regardless of radiation exposure associated with marginally higher counts in irradiated animals based on a statistical analysis of annual blood cell examination from 185 irradiated animals (1.1-8.5 Gy WBI) and 36 unirradiated animals. They statistically analyzed lymphocytes and monocyte subsets for the long-term effect in immune system. Classical monocytes, which is the common monocyte subset and associated with inflammation, and intermediate monocytes, which is important for antigen presenting to T cells, increased in a dose-dependent manner. Memory B cell proportion was smaller for any ages and

irradiation dose except for young animals exposed to the highest dose. While they did not observe long-term changes in absolute number of monocytes and lymphocytes, changes for some monocyte and lymphocyte subsets were sustained over 5 years after exposure.

The skewing trend towards neutrophil than lymphocytes (finding 1.b) and increasing monocyte count (finding 1.c), which will be referred as myeloid skewing in the following, were not observed in any previous studies. However, fewer T-cells (Hale et al., 2019), fewer naïve cells (Macintyre et al., 2021), fewer memory B cells, and more classical monocytes (French et al., 2023) indicate weaker adaptive immune system and an inflammatory state, which can induce more production of neutrophils and less lymphocytes. This study is the first to analyze longitudinal trends in leukocyte counts. Since all previous studies are cross-sectional, and longitudinal analysis is the best for detecting long-term changes, this study successfully observed the subtle long-term change by the ionizing radiation exposure.

6.2.2 A-bomb survivor cohort

The study result was partly consistent with longitudinal analysis studies of A-bomb survivor cohorts (Hsu et al., 2010, Yoshida et al., 2019). Hsu et al. (2010) reported the effect of radiation exposure over 2 Gy was a significant long term trend ($p<0.05$) of leukocyte, neutrophil, lymphocyte, and monocyte counts, over 40 years after exposure, which is also reported in this study. In contrast, the longitudinal analysis over 50 years of follow up demonstrated radiation-related significant change in percentages only for monocytes (Yoshida et al., 2019), while the result of this study demonstrated significant effects of radiation exposure in neutrophil, lymphocyte, monocytes percentages.

One large difference is the data size and sampling. Both A-bomb survivor studies have more than 5000 participants, which intrinsically can give stronger statistical significance than our NHP

cohort. However, the blood sampling in A-bomb survivors was every 2 years and some participants had only two repeat samples. Considering blood cell counts change in an everyday manner, every 2-year sampling is less preferable to understand the longitudinal trend compared to our NHP cohort, which had at least 6 months of sampling. The number of animals in our study cohort is one limitation, but the more frequent data possibly allowed us to understand the subtle change in the long-term trend in the percentages.

There are other possible reasons that our results did not exactly match the A-bomb survivor cohorts. The follow-up time was relatively shorter for NHPs than that for A-bomb survivors. The rate of aging in rhesus monkeys is approximately three times of that of humans (Simmons et al., 2016) and this study contains follow up time up to 16 years after radiation, corresponding to a similar proportion of the lifespan as that examined in A-bomb survivors; however, most animals were followed for a shorter period. Additionally, the effect of radiation exposure on monocyte counts and percentages reported in Yoshida et al., (2019) was stronger in the survivor cohort populations over 60 years old. Animals over 20 years old, which corresponds to 60 years old of human age were zero in the 1st analysis and only 11 in the 2nd analysis. Therefore, the NHP study cohort might be too young to express the expected effect from the A-bomb survivor cohort studies. As more observations and animals provided more statistically significant longitudinal trends in the 2nd analysis than the first, the NHP cohort could possibly show similar trends with more data available.

There are other factors that possibly impact the long-term effects of ionizing radiation exposure. The radiation dose received by the A-bomb survivor cohort is much smaller than our rhesus macaque cohort. The exposure scenario is also very different among the Macaque cohort and A-bomb survivors. While the rhesus macaques were kept in a laboratory for their entire

lifetime, the human cohort had multiple outside variables associated with their lifestyles, which indicates more confounding factors in human cohort such as exposure to pathogens which can cause long-term effect in hematopoietic system. Therefore, direct comparison of the results from A-bomb survivor study trends is impossible, while comparing the results from this study will help understand the long-term effects of ionizing radiation in the hematopoietic system.

6.3 Mechanisms of the long-term effect

6.3.1 Accelerated aging process in the irradiated animals

The longitudinal trends of leukocyte counts and differentials in both control and irradiated animals were consistent with the effect of aging in the hematopoietic system. Hematopoiesis is skewed to produce more myeloid cells than lymphoid cells during aging, resulting in myeloid lineage-skewed ratio of the leukocyte differentials (Muller et al., 2019). This pattern of aging effects on hematopoiesis and hematologic parameters has been previously reported for rhesus macaques (He et al., 2018; Yu et al., 2018; Yu et al., 2019), which is generally consistent with humans. While the difference was not statistically demonstrated in the preliminary analysis, irradiated animals had indications of accelerated hematopoietic aging. Longitudinal trends of neutrophil% were 2.45 and 2.73 for controls and irradiated animals respectively, and that of lymphocyte% were -0.098 and -0.117 for controls and irradiated respectively. In the latter analysis, statistically significantly increasing trends in neutrophil-to-lymphocyte ratio and neutrophil% in irradiated animals as well as decreasing trend of lymphocyte% in irradiated animals were observed. While the accelerated aging in hematopoietic and immune system has been suggested by many other aspects including lymphocyte subpopulations, stem cell senescence, as well as monocyte skewing, the skewing in myeloid lineage cells, such as neutrophils and monocytes, in

peripheral blood (findings a and b (see 6.1)) has never been reported. Considering this is the first study conducting longitudinal analysis of the leukocyte counts and the differentials, this study likely succeeded in capturing the subtle but significant myeloid skewing because of radiation exposure. In the following, possible mechanisms causing the myeloid skewing hematopoiesis are reviewed.

6.3.2 Clonal hematopoiesis

Clonal hematopoiesis (CH) is described as expansion of hematopoietic stem and progenitor cells carrying the same somatic mutations with no symptoms in hematologic disease (Genovese et al., 2014). The strong association of CH with aging has been demonstrated in both humans and Rhesus macaques (Jaiswal et al., 2014; Shlush 2018; Shin et al., 2022). The main mutations in CH are DNMT3A, TET2, PPM1D, and ASXL1. Epidemiologic studies have provided evidence that ionizing radiation exposure is associated with higher occurrence of CH from follow-up studies of radiation therapy patients with non-hematologic cancer as well as environmental radon exposure (Coombs et al., 2017; Bolton et al., 2020; Anthony et al., 2020). Coombs et al. (2017) first reported CH and CH-PD (CH with presumptive leukemia driver mutations) mutation increases with previous RT. They also observed a significant increase in neutrophil and monocyte counts as well as NLR>4% in association with CH and CH-PD. Among mutations common in CH, TP53 and PPM1D mutations were statistically significantly associated with RT. TP53 is tumor-suppressor gene encoding protein p53, which promotes cell cycle arrest and DNA repair upon activation through regulating several downstream signaling pathways (Ozaki and Nakagawara, 2011). A murine study reported TP53 deletion and mutation increased resistance of HSC to 2Gy IR and 5Gy TBI increased TP53 mutated bone marrow cells and HSPCs in vivo (Chen et al., 2019). TP53 mutation is also associated with myelodysplastic syndrome (MDS) and acute myeloid leukemia

(AML) with over 50% frequency in therapy related cases (Rahmé et al., 2023). Considering both MDS and AML occurs at myeloid stem cells and allow clonal expansion of immature cells (Estey, 2007), the residual HSC damage of TP53 mutation may be one mechanism reading to elevation of neutrophils.

PPM1D is one of the proteins activated by p53, dephosphorylating p53 and downregulating apoptosis (Dudgeon et al., 2013). Mutated PPM1D seen in CH produces truncated PPM1D protein at the C end (-COOH), which has enhanced p53 downregulating ability (Jan et al., 2016). The association of PPM1D mutation with radiation exposure is not fully understood; Coombs et al. (2017) reported positive association with RT and PPM1D mutation in CH patients, while Hsu et al (2018) only discovered association with radionuclide therapy and not external beam radiation therapy. The association with MDS and AML has not been reported. Overall, the mechanisms of neither clonal hematopoiesis induced by ionizing radiation nor the induction of neutrophil-skewed hematopoiesis have been fully investigated, yet the residual damage to the HSCs or HSPCs genes likely induces chronal hematopoiesis leading to deficient hematopoiesis in the exposed animals.

6.3.3 Chronic inflammation

Aged human and Rhesus macaques often develop low-grade chronic inflammation associated with natural aging processes in the immune system, often referred as “Inflammaging” (Didier et al., 2012; Kovtonyuk et al., 2016; Walker et al., 2019). Inflammaging is typically characterized as elevated levels of inflammatory markers such as IL-6, tumor necrosis factor α (TNF-α), IL-8, and IL-15, acute phase proteins such as C-reactive protein (CRP), lipoprotein a (Lp(a)), fibrinogen, and other coagulation factors (Franceschi et al.; 2000). As normal inflammatory responses induce elevation in myeloid lineage cells, inflammaging has been suggested as one of the factors triggering age-related myeloid skewing in hematopoiesis

(Kovtonyuk et al., 2016).

Subclinical chronic (systemic) inflammation has been observed in the A-bomb survivor cohort study long after exposure. Elevation of inflammation markers, such as IL-6, TNF- α, sialic acid, CRP, Erythrocyte sedimentation rate (ESR), IL-4, was observed over 50 years after the exposure with and without presenting clinically significant inflammatory disease (Neriishi et al.; 2001, Hayashi et al., 2003; Hayashi et al., 2005). For the NHP RLEC cohort, Debo et al (2016) analyzed inflammatory markers (CRP, IL-6, monocyte chemoattractant protein (MCP-1) and soluble intercellular adhesion molecule (s-ICAM)) from 20 male macaques with 6.5-8.4 Gy WBI which was assigned 5.6-9.7 years prior to the investigation. Compared with 11 control subjects, they only found statistically significant elevation in MCP-1 ($p<0.05$), but all the other inflammatory markers were higher in the irradiated animals.

Hayashi et al (2012) conducted multivariate statistical analysis of inflammatory cytokines and serum reactive oxygen species (ROS) levels with radiation exposure and aging, suggesting ROS dependent pathway related to IL-6 and CRP. ROS is produced by normal cell metabolism mainly at mitochondria but also produced and released by activated phagocytic cells such as neutrophils to the plasma to effectively destroy pathogens as well as one of the signaling molecules regulating inflammatory and immune response (Nathan et al., 2013; Herb and Schramm, 2021). However, overproduction and accumulation of ROS can cause oxidative stress to cells and tissues, damaging the DNA, lipids, and proteins (Schieber and Chandel, 2014). Persistent elevation of intracellular ROS, depending on radiation dose, was reported by the A-bomb survivor study, especially for monocytes, neutrophils, and CD8 T lymphocytes (Hayashi et al., 2021). Among several types of ROS, superoxide anion ($O_2^{\bullet-}$) was the most abundant in the immune cells. $O_2^{\bullet-}$ is mainly produced through ATP production at mitochondria. Aging is known to affect

mitochondria and increase the production of ROS. (Mittal et al., 2014; Pangrazzi et al., 2017), previous radiation exposure may affect mitochondria function and induced high production of ROS, leading to chronic inflammation.

For inflammaging, aging in monocytes has been investigated as mechanistically key for development (Baylis et al., 2013; Maeyer and Chambers 2021). Intermediate monocytes, the subtype of monocytes associated with inflammation by producing more pro-inflammatory cytokines such as IL-6 and IL-1β, increases primarily in elderly populations (Sadeghi et al., 1999). Additionally, impaired monocyte and macrophage function in the elderly, such as decreased phagocytosis and immune resolution, results in increased production of pro-inflammatory cytokine for macrophages and decreased type I IFN production, which is vital to regulate innate and adaptive response including macrophage activation (McNab et al., 2015), and decreased clearance capacity of apoptotic cells (efferocytosis). For the Rhesus Macaque cohort, increased intermediate and non-classical monocyte subsets and depleted classical monocyte subsest were observed 6 months post-irradiation with 4 Gy WBI (Michalson et al., 2019). French et al (2023) also reported persisted dose-dependent elevation of intermediate monocytes of irradiated Rhesus macaques. There seems no other investigation has been conducted on monocyte subpopulations or their functions in any other irradiated cohort including A-bomb survivors, although persistent damage to monocytes by ionizing radiation exposure can be one contributing factor to initiating chronic inflammation.

The exact mechanism of chronic inflammation by the long-term effects of ionizing radiation has not been identified yet, but damages from ionizing radiation are likely to initiate early-onset of inflammaging in the irradiated subjects than in the controls.

6.3.4 Immunosenescence

Immunosenescence is defined as age related dysfunction of immune system due to decreased function of lymphoid tissue, pro-inflammatory environment, as well as intrinsic factors in immune cells such as teromere shortening, functional alterations, and alterations in proliferation and differentiation of HSPCs (Lian et al., 2020). Immunocenescence is characterized as decrased naïve T cells and decreased function and altered subpopulation of CD4 T cells, leading to dysfunctions in adaptive immunity. Accumulating evidence from the A-bomb survivor study indicate that immunosenescence is accelerated by radiation exposure. T lymphocytes immunosenescence is observed in the alterations in both the subset population as well as the functions, resembling aging in T cell immunosenescence (Kusunoki et al., 2008). CD4 T cell decreased, Naïve CD4 and CD8 T cell decreased, and Memory CD8 T cell increased in a dose-dependent manner decades after exposure (Kusunoki et al., 2002; Yamaoka et al., 2008). Since one of the main functions of CD4 T cell is to stimulate macrophages in cellular immune response, depleted stimulation might require more macrophage production, leading to myeloid-skewed hematopoiesis in our study. The depletion of Naïve T cell pool is indicative of the decreased supply of lymphoid HSPCs.

Persistent depletion in naïve T cell population is also reported in Hodgkin's lymphoma patients treated with RT over 30 years of posttreatment (Watanabe et al., 1997). The Rhesus Macaque cohort study also reported naïve T cell depletion (Macintyre et al 2021). Decreased percentages of CD4 T cells were reported in highly exposed population after Chornobyl nuclear power plant accident as well (Chernyshov et al., 1997).

Deficiency in lymphocyte function has also been reported from the A-bomb survivor study. Weaker response of lymphocytes to several mitogens including PHA (phytohemagglutinin), MLR (mixed lymphocyte reaction), and superantigen staphylococcal enterotoxin was observed

(Akiyama et al., 1983; Akiyama et al., 1989; Kusunoki et al., 2002). These functional alterations are possibly due to the decrease in the population of CD4 T cells as well as the naïve T cell pool. However, Kusunoki et al (2001) utilized limiting dilution assay on lymphocytes from 251 A-bomb survivors with less than 0.005 Gy and 159 survivors with over 1.5 Gy, discovering a significant decrease in the CD4 T cell population capable of producing IL-2 against a T cell mitogen, concanavalin A, associated with high radiation exposure.

Subtle but dose-dependent increase in IgM, and IgA are observed in A-bomb survivor cohort over 40 years after exposure (Fujiwara et al., 1994). Although the mechanism of stimulated B-cell response is not fully understood, B cell population was elevated with radiation exposure (Kusunoki et al., 1998) as well as immunoglobulins are associated with inflammatory state (Hayashi et al., 2012). The impact on humoral immunity of the survivors seems less significant because there was no difference in the response to Influenza vaccine between control (<0.005 Gy), low-mid (0.005-1Gy), and high (>1 Gy) dose exposed group (Hayashi et al., 2018), except for the possibility of less clearance ability in Hepatitis B reported in Fujiwara et al. (2003). Conversely, for the Rhesus macaque cohort, Hale et al. (2019), reported selective deficiency in producing antibodies against one type of polysaccharide antigen vaccine, alongside with relatively decreased IgM production for tetanus toxoid vaccine as well as slower peak in antibody production against rabies vaccine compared to the controls. Macintyle et al (2021) also reported weaker IgG response to 10 of the 23 polysaccharide antigens in exposed animals. Much higher dose in rhesus macaque cohort (6.5-8.4 Gy in Hale et al (2019) and 4 Gy in Macintyle et al (2021)) may have induced more significant damage to the humoral immunity, but considering one of the aspects of immune senescence is weakened response to vaccines from due to T cell senescence, immunosenescence by ionizing radiation resembles immunosenescence by aging.

The mechanism underlying immunosenescence is under investigation, while there are several evidence of circulating lymphocytes and T cells from exposed cohorts carrying persistent damage from ionizing radiation including chromosome aberration (Awa, 1991; Sevan'kaev et al., 1995) and gene mutation (Hirai et al., 1995; Skandaliset al., 1998; Grant et al., 1999), which may lead to functional alteration of T lymphocytes. Yoshida et al. (2016), also reported dose-dependent telomere shortening in pheripheral blood T cells in A-bomb survivors. Telomere is a cellular senescent marker, and the shortening was observed in both naïve and memore CD4 and CD8 Tcells and not in granulocytes. Collectively, damages from previous radiation exposure to lymphocytes likely persisted and induced senescence leading to the alterations in the population and function.

So, how does immunosenescence induce myeloid skewing? The inverse association between plasma inflammatory markers and peripheral CD4 cells have been reported; IL-6 and CRP significantly increased with low percentages of CD4 T cell (Hayashi et al., 2003) and TNF-α significantly increased with low CD4 naïve T cells (Kusunoki et al., 2010). Yet, the immunosenescence mechanism inducing inflammation or any other system that may lead to myeloid skewing is not unknown. One possibility is regulatory T cells (Treg), which is 5-10% of the subpopulation of CD4 T lymphocyte and responsible for immune tolerance against autoantigens as well as suppressing immune response including inflammation through secreting IL-10 and TGF-β and promote successful tissue repairing (Lei et al., 2015; Shevyrev and Tereshchenko, 2020). Additionally, Treg possibly communicates directly and downregulates innate immune cells including neutrophils and macrophages (Okeke and Uzonna., 2019). Treg has greater radiation resistance possibly because of the low apoptotic capacity, and thus, increases the population in the acute phase after exposure, providing radioprotective feature to cancer cells after radiation therapy (Persa et al., 2015). However, several studies implicated deficiency of the Treg

function after ionizing radiation exposure. Billiard et al. (2011) found accumulation of malfunctioning Treg with significantly low Foxp3, which is the critical protein for Treg function, in the small intestine of mice with single abdominal 10 Gy irradiation over 90 days post irradiation. Cao et al. (2009), also discovered ionizing radiation exposure over 1.875 Gy decreases FOXP3 expression with other membrane proteins as well as high dose as 30 Gy strongly reduced the immunosuppressive function of Treg in vivo. There is not enough evidence to support the damage to Treg influencing the immune and hematopoietic system. Unfortunately, Treg did not correlate with TNF-α in A-bomb survivor study (Kusunoki et al., 2010), yet the malfunction of Treg could be one mechanism inducing elevation in myeloid lineage cells.

6.3.5 Residual damage to the hematopoietic system

Residual damage to the hematopoietic system; damage to the HSC and the bone marrow environment, is the primary cause of these processes above. Murine in vivo and vitro studies have been provided the evidence as well as the possible mechanisms of the residual damage to the hematopoietic system (Shao et al., 2014).

HSCs have apoptotic capacity elevated by IR exposure to prevent them from accumulating DNA damage (Wickremasinghe RG and Hoffbrand 1999; Meng et al., 2003). P53 and the downstream protein PUMA (p53 upregulated mediator of apoptosis) are activated by IR and lead HSCs to apoptosis through caspase cascade. Inhibiting or removing those genes and pathways successfully increase the IR resistance of HSCs by downregulating HSCs (Hirabayashi et al., 1997; Meng et al., 2003; Shao et al., 2010). To overcome the shortage of HSCs, HSCs need to upregulate their self-renewal, while IR is known to impair the self-renewal capacity of HSCs (Testa et al., 1985; Mauch et al., 1995; Himburg et al., 2012), which is one outcome of HSC senescence by IR. Hence, IR exposure induce HSC loss and deficient self-renewal, leading to the reduction of HSC

pool.

IR also can change the differentiation of HSCs. Wang et al., (2012) conducted in vivo study of mice with 4 Gy γ irradiation discovered IR promotes lymphoid differentiation more than myeloid differentiation through activating BATF (basic leucine zipper transcription factor) in G-CSF/STAT3 dependent manner, possibly exhausting the lymphoid-HSC pool and turning the HSC pool into myeloid biased. The BATF activation was also associated with shortened telomere and MDS associated DNA damage signal (p21), indicating residual damage to HSC can keep affecting the differentiation as well.

Senescence of HSCs is also promoted by IR exposure. Wang et al. (2006) reported irradiated mice with 6.5 Gy TBI resulted in persistent reduction of HSCs (LKS^+ and LKS^- cells) over 56 days after exposure with increased expression of cellular senescence markers, $p16^{Ink4a}$ and SA-β-gal only for LKS^+ cells. They also discovered sustained deficiency in the clonogenic function of LKS^+ cells under the cobblestone area-forming assay. Chua et al (2012) also provided evidence on persistent HSC malfunction regarding the repopulating potential of the HSCs after 7.6-7.9 TBI in vivo. There are many possible mechanisms of IR inducing HSC senescence including increased ROS production at HSCs by altered the regulation of NADPH oxidases (NOX) (Wang et al., 2010), activation of p38 pathway (Wang et al., 2011; Li et al., 2011), and elevated lnk4a and Arf gene expression (Wang et al., 2006). HSC senescence by IR does not have evidence on myeloid skewing so far, yet senescence by aging itself induce HSC more myeloid lineage (Sudo et al., 2000; Rossi et al., 2005; Beerman et al., 2010)

IR also cause BM stromal cell injury leading to the senescence. Carbonneau et al (2012) discovered the stromal cell senescence in p16/Arf, which is a cellular senescent marker, dependent manner, and nonautonomously altered B cell lymphopoiesis in association with p16/Arf

expression. Additionally, BM sinusoidal endothelial cells (SEC) was found to be so sensitive to IR that 9.5 Gy WBI can damage SECs significantly as they require transplantation to regenerate (Hooper et al., 2009). Considering SECs promotes HSC recovery after IR (Chute et al., 2007; Li et al., 2010) as well as improving repopulating capacity of aged HSCs (Poulos et al., 2017), damages to SECs can impair the recovery of HSCs and hematopoietic system. The exact mechanism of residual damage of BM cells affecting HSC needs more investigation, while HSCs are possibly affected by the bone marrow environment considering the entangled regulation and cross-talking between cells in hematopoietic system.

HSCs have a long-life span of 10-60 months, and the hematopoietic system is where all the leukocytes are produced. Residual damage to HSCs and the bone marrow niche in may be the primary cause of the long-term consequences of IR including chronic inflammation, chronal hematopoiesis, as well as immunosenescence.

6.3.6 Summary of plausible mechanisms

All the mechanisms reviewed above are not mutually exclusive and correlates with each other (Figure 10). Chronic inflammatory status induces random mutations in HSPC, and the acquired mutation in genes like *Tet2* and *Ppm1d* induces clonal hematopoiesis of cells producing pro-inflammatory cytokines (Yura et al., 2021; Avagyan and Zon 2023). Frequently mutated genes in CH such as *Tet2* and *DNMT3A* causes immunosenescence by inducing functional alterations of immune cells (Belizaire et al., 2023). *Tet2*$^{-/-}$ neutrophils have limited mobility and phagocytic function against bacterial infection. *Tet2*$^{-/-}$ B lymphocytes result in impaired plasma cell differentiation and reduced antibody production. *DNMT3A*$^{-/-}$ macrophages and dendric cells have reduced type-1 IFN, leading to limited response against viral infection. Finally, immunosenescence and chronic inflammations are also related to each other (Jurk et al., 2014;

Bektas et al., 2017). ROS and associated DNA damages, which are induced by chronic inflammation, upregulates NF-κB activation. NF-κB is a transcription factor regulating pro-inflammatory cytokine production. Activation of NF-κB by inflammatory condition promotes inflammation by producing more pro-inflammatory cytokine, which process is further stimulated in senescent T lymphocytes because such senescent cells have limited potential of responding to the NF-κB.

The mechanisms mentioned above are only examples. Large amount of studies on human and experimental animals in vivo and vitro are conducted to understand the mechanism of clonal hematopoiesis, immunosenesence, and chronic inflammation in regarding to aging. Considering the long-term effect on hematopoietic and immune system from IR resembles aging in hematopoietic and immune system, Residual damage to the HSC from IR exposure is highly likely induces and accelerates the aging through the interrelated mechanism.

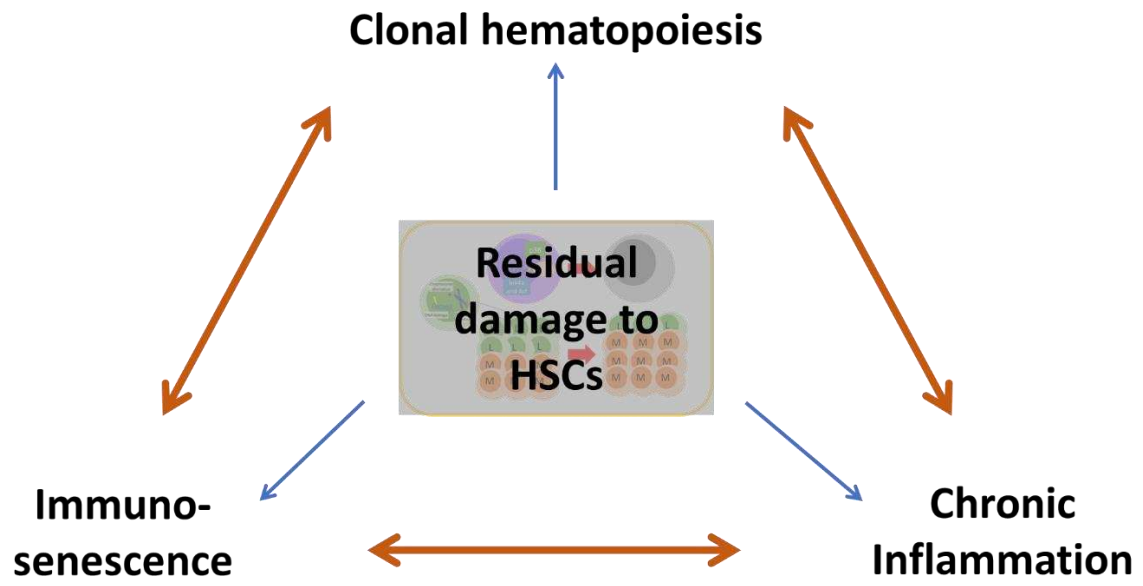

Figure 10 Interrelated mechanisms of long-term effect of IR in hematopoietic and immune system

6.4 Confounding factors

6.4.1 Sex

Radiosensitivity and the late effect risk is different depending on sex. Females are generally more radiosensitive than males based on several epidemiological evidence (Narendran et al., 2019). A-bomb survivor studies proposed higher solid cancer risk in females. Chornobyl studies suggested higher female mortality, thyroid condition including cancer, as well as higher overall cancer rate including hematopoietic cancer. These higher radiosensitivity and higher rate of disease likely indicate but also lead to more significant long-term damages in the hematopoietic/immune system in females. The mechanistic understanding is still limited, while possible reasons include females having more reproductive tissues than males (Olson, 2010), different immune response because of sex hormones and X-chromosomes, leading to better recovery in males after acute exposure as proposed in mice study (Wu et al., 2020). Additionally, murine studies provided some evidence of the difference on long-term effect of ionizing radiation such as different IR-induced gene and protein expression, IR-induced DNA methylation, apoptotic potential, as well as cell proliferative potential (Kovalchuk et al., 2004; Koturbash et al., 2008; Koturbash et al., 2011).

However, our result showed the opposite. Female animals had less neutrophil skewing than male animals. One cause may be the baseline difference, while the sexual dimorphism on neutrophils and lymphocytes are contrasting; some studies show females tend to have relatively low neutrophil% and NLR than males and some are the opposite (Howard et al., 2019; Pellegrino et al., 2023), and those studies for rhesus macaques were limited and there were no statistically significant difference was proposed so far (Koo et al., 2019). Regarding the lower morbidity of almost all the disease in female animals than males when compared within the same dose groups, our result suggests that the radiation sensitivity among different sex may be different from what it

have been suggested.

There are several plausible reasons why females could be more tolerant than males against the late effect of IR in hematopoietic and immune systems. One possible mechanism is that the difference in sex hormones. Sex hormones, especially estrogens and androgens, are known to induce thymic involution. Mice and human studies also suggested the ablation of sex hormones improves recovery after bone marrow transplantation and possibly reverse thymic aging (Sutherland et al., 2015; Hince et al., 2008). Females have more drastic depletion of estrogen level than that of androgen in males (Decaroli and Rochira, 2017). Thus, the lower levels of sex hormones may have helped female animals maintain immune homeostasis more effectively than males. The other plausible mechanism is difference in X chromosomes. X chromosomes have many genes related to immune system (Noguchi et al., 2013). Females having two pair of X chromosomes possibly give older females more resistance to infections but sensitivity to autoimmune disease compared to males (Libert et al., 2010; Klein and Flanagan, 2016) and this same mechanism may give females more tolerance to the long-term effect of IR in hematopoietic and immune system.

The recent NCRP report suggested the lung cancer risk from chronic radiation exposure is not significantly different among males and females (NCRP 2022). Also, there are no animal studies suggesting higher morbidity in any late effect diseases in females. While the cohort only includes 4 control females, which limited number may have provided less reliable results, this study suggests higher tolerance in the female animals for the long-term effect of IR exposure.

6.4.2 Age at the time of exposure

Younger population is at higher risk of DEARE because of the long-life expectancy after the exposure and the immune system under development (UNSCEAR, 2013). There are

epidemiological evidence including significantly higher all solid cancer risk in A-bomb survivor cohort (Ozasa et al., 2012), and increased thyroid, breast, brain and skin cancer, as well as leukemia in radiation therapy and diagnostic cohort (Kleinerman, 2006; Little et al., 2022).

The results of the analysis show strong effect of age at exposure in monocyte counts and percentages, suggesting exposed at younger age with high dose are likely to have higher monocyte count and percentages. This result is partially consistent with longitudinal analysis of leukocyte counts from A-bomb survivor cohort (Hsu et al., 2010), indicating strong effect of age at bombing under 20 years old, while the effect was opposite to our result. Their analysis also suggested statistically significant effect of age at bombing under 20 years old in the leukocyte count of females, neutrophil count of both sex, while these are also opposite trend of our results. The difference is possible because the A-bomb survivor cohort has larger age range the rhesus macaque cohort. The data from population exposed under 20 years old in the A-bomb survivor study are also younger at the sampling.

Previously, lower age at irradiation in the rhesus cohort was reported to only associated with low body weight, testicular atrophy, brain lesions, osteopenia, and dermatitis/alopecia (Little et al., 2022), which may lead to the high monocyte count and percentages. These condition more likely related to the residual damage from the IR exposure than chronic inflammatory state or immune disturbance, while this condition can possibly induce chronic inflammatory status. Since the animals exposed at younger are still relatively young and alive in the cohort, they may the present inflammatory related disease later in their lifetime.

Mechanisms of difference in long-term response of IR in immune and hematopoietic system among age at exposure is not fully understood. One possible mechanism is defective immune homeostasis in animals exposed at younger age. Kusunoki et al (2003) reported significantly large

CD4 memory TCR repertoire in population ≥20 years old (ATB 20+) at the bombing compared to the control or the cohort under 20 years old (ATB <20). Considering the deviation is possibly due to the clonally expanded population of memory T cells, the ATB 20+ population may have expanded the memory cell population to achieve immune homeostasis, which was disturbed by significant depletion of naïve T cell and CD4 T cell population. Since younger population generally have less experience of encountering pathogen, attaining less variety of memory T cell and the TCR, ATB <20 population may not be able to pursue the same process as the ATB 20+ cohort, resulting in less maintained immune homeostasis and less functioning immune system, leading to high production of monocytes.

This study is one of the few studies of longitudinal analysis of leukocyte counts and the differentials of IR exposed cohorts, reporting the association in elevated monocyte counts and percentages in the circulating blood. Monocytes are responsible for chronic inflammation (see chapter 3.3.2). Chronic inflammation is associated with many diseases including cancer and CVD. A-bomb survivors exposed at younger age have significantly higher risk of cancer throughout of their lifetime (Ozasa et al., 2012). The association of noncancer disease risk and age at exposure and has not yet reported (Preston et al., 2003; Ozasa et al., 2012), but this study suggests population exposed to high dose at younger age are at high risk of developing inflammatory disease because of the disturbed immune and hematopoietic system related to the high production of monocytes.

6.4.3 Mitigators

The status of mitigator assignment had no significant effect on the longitudinal trend in this study. One reason is that this analysis assigned one parameter (Y) to assignment of any mitigators. The rhesus cohort received different mitigators with different function, which may have different effect on the long-term effect, and the study design may have failed to detect the different function

of different mitigators. Another reason is that rhesus macaque cohort in this study are originated from different study projects. The later cohorts, which were younger at the age of the sampling, tend to be from studies of mitigators. Therefore, the animals with mitigators were possibly too young to develop any late effects in hematopoietic and immune system. Some of the ARS mitigators have proven to have long-term effect. For example, PEGylated IL-11 is effective for H-ARS as well as increase blood cell counts and HSPC population up to 12 months in mice study (Sharma et al., 2020). Therefore, more follow-up examination as well as detailed study design would be significant to observe the effect of mitigators on the long-term effect of IR on hematopoietic system.

6.5 Disease morbidity and inflammatory status

6.5.1 Disease morbidity in the study cohort

Incidence of morbidity, disease, and conditions observed during clinical examination are in Figure 11. The criteria used for diagnoses are in appendix D. Periodontitis had high incidence in all dose groups. Periodontitis is an age related disease common in rhesus macaques (Gonzalez et al., 2016). Reproductive organs such as testis and ovary are known for their sensitivity to ionizing radiation (ICRP 2012). The dose dependent injury and associated recovery of testicular atrophy in the RLEC animals is well investigated and reported at Schaaf et al., 2023. Cataracts are an age associated disease in rhesus macaques, but lenses of the eyes are highly radiosensitive organs and the WBI is associated with cataracts (Sonneveld et al., 1994; Zierhut et al., 2000; ICRP 2012). The kidney and brain tissues are known to display late effects of ionizing radiation because of slow cell proliferation (Barnett et al., 2009). Renal fibrosis and brain injury in the RLEC have been investigated and reported elsewhere (Cohen et al., 2020; Andrews et al., 2020). For these tissues

displaying late effects, chronic inflammation likely not the cause, but chronic inflammation could be induced as one of the consequences of these diseases.

Figure 11 Incidence (%) of disease and conditions in the study cohort

The high morbidity of testicular atrophy in >LD50 groups may be one reason that no statistically significant difference in lymphocyte%, neutrophil% and neutrophil to lymphocyte

ratio was observed in the >LD50 dose group. As mentioned in 6.4.1, sex hormones, such as androgens and estrogens, induce thymic involution, the ablation of which can improve recovery of immune deficient condition. The >LD50 dose group had the highest testicular atrophy morbidity. Malfunctioning reproductive organs possibly downregulated androgen releases, and thus helped the animals overcome long-term effects in the hematopoietic and immune system after radiation exposure. Additionally, LD10-50 dose groups had the lowest morbidity of ovary dysfunction. Relatively normally functioning ovaries provided higher estrogen level to female animals in the LD10-50 group, leading to less tolerance of the immune system and more prevalent long-term effects in the LD10-50 dose group.

Chronic inflammation suggested from the analysis in this study is itself an underlying cause of many diseases including cardiovascular disease, cancer, diabetes mellitus, chronic hepatic and renal disease, as well as autoimmune diseases (Furman et al., 2019; Pahwa et al., 2022). Among those, hypertension, heart disease, tumor, as well as diabetes are often reported in RELC cohort and A-bomb survivor cohort and possibly closely related to chronic inflammation. In the following, the association between chronic inflammation and hypertension, heart disease, tumor, as well as diabetes mellitus are reviewed.

6.5.2 Neoplasia

The incidence of neoplasia was small, but there was a clear increase in the incidence of cancer reported for the RLEC (Sills et al., 2021). Sarcoma was about 40% of the neoplasm diagnosis, and the most common outcomes were malignant nerve sheath tumors and malignant glomus tumors. Both tumors are uncommon in aged, non-irradiated rhesus macaques, and not commonly observed with radiation exposure (Broerse et al., 2000; Simmons et al., 2011). Carcinoma was only approximately 20% of the diagnosis, while carcinoma is most prevalent in

neoplasms of aging, non-irradiated animals. The most common carcinoma diagnosis included renal cell and hepatocellular carcinomas. While the cancer diagnosis occurred at most of the body sites, skin and subcutis was the most common composing 40% of the diagnosis.

The A-bomb survivor study also demonstrated a high incidence and excess risk of neoplasms from radiation exposure (Preston et al., 2007). However, the type of cancer in the A-bomb survivor study was very different from the RLEC. Squamous cell carcinoma and adenocarcinoma composed over 70% of the cancer diagnosis, while sarcomas are less than 1% of the diagnosis for A-bomb survivors. Approximately 27% of A-bomb survivor cancers were stomach cancer, followed by lung, colon, and liver. The difference in the size of cohort, dose and age range were possibly factors of the difference in the type and site of neoplasia, while the A-bomb survivor cohort is consists of a Japanese population, which is known to have a high incidence in stomach cancer, highly likely one reason for differences.

Chronic inflammation is an underlying cause of cancer development but also triggered by cancer development by various mechanisms (Coussens and Werb, 2000; Singh et al., 2019; Greten and Grivennikov, 2019). For example, Reactive oxygen and nitrogen species produced by macrophages in chronic inflammatory condition can generate mutagenic agents such as peroxynitrite, inducing DNA mutations in proliferating epithelial and stroma cells (Maeda and Akaike, 1998). Inflammatory cytokines such as TNF-α not only induce DNA damage but also helps initiation, growth, and metastasis of cancer (Smyth et al., 2004; Yoshimura 2006). Additionally, tumor cells and cells in tumor microenvironment produce more pro-inflammatory cytokines (Jackson et al., 2002; Lázár-Molnár et al., 2004; Pollard 2004). Sills et al. (2021) did not find any statistical significance in the first cancer incidence among dose groups but discovered the dose-dependent incidence in developing more than one tumor with the highest in LD50-90 group

of 47.1%, and 25.0% in over LD90 group. The elevated trend in myeloid cells reported in this dissertation may be one of the causes and consequences of cancer development in the RELC cohort.

6.5.3 Cardiovascular disease

Epidemiological studies have proven increased risk of cardiovascular disease (CVD) in A-bomb survivors (Yamada et al., 2004; Shimizu et al., 2010), Mayak nuclear workers (Azizova et al., 2010), Chornobyl emergency workers (Kashcheev et al., 2017), British nuclear worker cohorts (McGeoghegan et al., 2008). Cardiovascular diseases include different diagnosis, while the most prevalent and studied was ischemic heart diseases. Ischemic heart disease is commonly caused by atherosclerosis, which is plaque development in the coronary artery and associated depressed oxygen supply to the myocardium of heart (Gofman et al., 1966). The risk factor includes Age, BMI, alcohol and tobacco use, cholesterol, hypertension, as well as diabetes mellitus (Hajar, 2017). Hypertensive heart disease, rheumatic heart disease, as well as heart failure were also a significant risk in A-bomb survivors, which may be related to the limited medical resources for diagnosis and the lifestyle of the cohort including high salt consumption and poor hygienic conditions (Ozasa et al., 2017).

In this study cohort, the incidence of heart disease seems to increase in a dose-dependent manner, except for the high incidence in the control animals (Fig.9). Control animals tend to be older animals compared to the other dose groups, which may be one reason for the high incidence since CVD is also an age-related disease of rhesus macaques. Mean age at first CVD diagnosis was much younger in exposed animals than control animals (Control: 11.3, <LD10: 10.5, LD10-50: 9.7, and >LD50: 9.4 years old), implecating an impact of ionizing radiation exposure. The incidence of hypertension was higher in irradiated animals (Figure 11). However, hypertension

does not seem correlated with heart disease since only 24% of animals with heart condition had hypertension diagnosis.

Debo et al. (2016) investigated cardiac morphology, function, as well as circulating biomarkers from RLEC male animals. The Debo et al (2016) cohortstudied animals 6-10 years post irradiation which had recieved 6.5-8.4 Gy whole-body gamma irradiation and were compared with age matched controls. Histological assessment revealed the prevalence of myocardial fibrosis in exposed animals. Echocardiography showed that the left ventricular diameter was significantly decreased in exposed animals. These results agreed with other investigations on mice and rhesus macaques with WBI exposure (Wondergem et al., 1999; Unthank et al., 2015). They also reported higher heart rates in irradiated animals, while no other indicator of functional deterioration of hearts were observed including irregular blood pressure. Investigations on coronary arteries were not included in the study, but significantly high MCP-1 was reported. MCP-1 is significantly correlated with coronary atherosclerosis (Register et al., 2005).

Mechanisms of CVD induced by PBI such as thorax radiation therapy for breast cancer and Hodgkin's lymphoma is well understood through animal experiments (Stewart et al., 2013). High radiosensitivity of endothelial cells are the main cause of initiating atherosclerosis development in coronary arteries and also results in damage to the microvasculature of myocardium, leading to cell death and myocardial fibrosis. In contrast, the mechanisms for CVD by WBI is not well understood (ICRP 2012). Different mechanisms other than direct damage to the myocardium and endothelial cells of coronary artery may be related to the development of CVD. Chronic inflammation as well as impaired immune system by IR exposure is suspected as one causes of CVD. The association of chronic inflammation and CVD, especially for atherosclerosis, is well documented in patients with autoimmune condition (Arida et al., 2018; Kobiyama and Ley, 2018).

Viral and bacterial infections also induce CVDs (Lowe 2001; Triant., 2013; Dhakal et al., 2020). Additionally, type 2 diabetes mellitus (T2DM), which is one of the late effect diseases of acute radiation exposure (see the next chapter), is also one leading cause of CVD and ischemic heart disease. Debo et al. (2016) did not observe any association of T2DM and cardiac structural changes in RLEC. Etiology of WBI induced CVDs is highly likely to be multifactorial and requires more investigation. Results from Debo et al. (2016) and the chronic inflammatory condition revealed in this analysis suggests the RLEC rhesus cohort is useful for exploring the mechanistic understanding of CVD as DEARE.

6.5.4 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) has been suspected as a late-effect disease after TBI. T2DM was reported in childhood cancer survivors with RT, and had higher incidence with WBI than PBI (Meacham et al., 2009). Studies on A-bomb survivors demonstarted a high prevalence of diabetes mellitus, while the effect of IR is not consistent and distribution of type 1 and 2 is unclear especially since studies were missing information and incomplete (Ito et al., 1994; Tatsukawa et al., 2022). The T2DM incidence in our RLEC study cohort (Figure 11) is relatively small, while there are several reports of subsets of the RLEC cohort developing type 2 diabetes mellitus as DEARE.

T2DM of irradiated Rhesus macaques had insulin resistance and hypertriglyceridemia but not obesity (Kavanagh et al., 2015; Bacarella et al., 2019). The irradiated animals with and without T2DM were both leaner and had less fat mass than controls. Adipocytes from irradiated animals were significantly larger with increased lipolysis, indicating irradiated animals cannot produce enough adipocytes and are storing more lipids in limited number of cells. Thus, irradiated animals may not be able to respond to metabolic needs properly with changes in blood sugar level, leading

to T2DM. The other possible mechanism is in skeletal muscle tissue. Muscle is responsible for metabolizing 90% of blood glucose. Kavanagh reported the irradiated and non T2DM animals were leaner and had less fat mass than irradiated T2DM animals. Additionally, muscle fibrosis has been observed as a late effect of radiation exposure in RT cancer therapy patients as well as the RLEC cohort (Yarnold et al., 2010; Stubblefield., 2011; Fanning et al., 2017). Therefore, loss of muscle tissue or function may have induced T2DM in irradiated animals.

Considering the inflammatory cytokine level was not necessarily associated with T2DM in the two RLEC studies above, chronic inflammatory status may not be a cause of T2DM here but still could be one of the consequences. T2DM can be caused by chronic inflammation but also can induce chronic inflammation (Akash et al., 2013). For example, β cells of pancreas, responsible for producing, storage, and secretion of insulin, produces the pro-inflammatory cytokine, IL-1β, and damages β cells under T2DM condition (Maedler et al., 2002). Additionally, inflammation is known to be linked with obesity and T2DM (Van Greevenbroek et al., 2013; Esser et al., 2014). Chronic inflammation induced by IR exposure may be one cause of radiation induced T2DM without obesity.

7 Future perspectives

This study design and analysis has limitations. Limitations and possible future study directions are addressed in this chapter. Firstly, the assumption of linear trends of leukocyte counts and the differentials may not be true. Non-linear mixed effect models or generalized additive models could possibly be used for analysis, although nonlinear models generally make interpretation more challenging. Finding the threshold or time of initiation of late effects was not the object of this study, but utilizing nonlinear models may help investigate the possible timeline of late effects.

Secondly, the analysis did not include disease and health status, so the influence of these factors on the longitudinal trend is unknown. Joint regression models may be a useful addition to future analysis with more data. Joint regression models are composed of linear mixed models for longitudinal outcomes and a survival function for certain event (Tsiatis and Davidian, 2004; Cekic et al., 2019), so that we can analyze the effect of a certain event, which will be death or mortality of a certain disease for the RLEC animals, to the longitudinal trend of leukocyte counts and the differentials. Unfortunately, joint regression analysis did not improve the analysis in this study possibly because limited information was available regarding death. Death in the cohort included natural death as well as euthanasia for experimental purpose and pathological reasons. Finally, now we know that long-term effects on hematopoietic system exist and appears in leukocyte biomarkers, the next question would be how this long-term effect is associated with specific disease incidence, the severity and comorbidity. Linear mixed models cannot discern this informaion, and more sophisticated methods including machine learning (ML) and AI would be required. ML/AI would also make it possible to provide risk estimation of diseases based on the exposed dose, leukocyte counts and the differentials, and other cofounding factors.

Limitation also lies in the rhesus macaque cohort and the dataset. The study cohort is consisted of animals that survived acute radiation syndrome, which intrinsically induces selection bias to the analysis. The selection bias could be stronger for animals exposed to high dose because the acute syndrome would be more severe with higher dose. Secondly, the RLEC animals die by natural death or euthanasia much earlier than expected, considering the average (approximately 25 years) and maximum lifespans (40 years) of rhesus macaques (Roth et al., 2004). The reason for early death is unknown, while the complete follow-up of acute radiation exposure may not be achievable because of the limited life span. Difference in irradiation protocol including different radiation source and energy (1.25 MeV Co-60 gamma vs 6 MV Linac X-ray), as well as animal positioning (anterior-posterior/posterior-anterior vs bilateral) may induce differences in the dose delivery and homogeneity to the body (Singh et al., 2019; Gibbons, 2020), possibly leading to differences in late effects. No random distribution of age, sex, use of mitigator is another limitation of this cohort. The <LD10 dose group had relatively younger animals, which may be one cause of the smaller and less significant long-term effect in the dose group. Despite these limitations, the RLEC cohort is still very advantageous for studying the long-term effects of IR especially because of the richness in repetition and variety of measurements based on intensive health examinations. As more data become available in the future, the limitations will become less significant.

8 Conclusion

This study successfully investigated the hypothesis using longitudinal analysis of leukocyte counts and differentials from RLEC rhesus macaques.

Hypothesis 1: Long-term hematopoietic effects from acute radiation exposure occurs in NHP cohorts. The first analysis in chapter 4 provided evidence on long-term effects of ionizing radiation to hematopoietic system, which agrees overall with hypothesis 1. The long-term effects were further investigated and confirmed with more data in the second analysis in chapter 5.

Hypothesis 2: High-exposed cohorts have stronger effects than low dose cohorts (<LD50). The second analysis addressed the effect of different dose levels. Higher dose (>LD10 dose) animals had a more significant dose effect compared to the lower dose exposed animals, while the longitudinal effect on the highest dose group (>LD50) was less significant and prevalent compared to the middle dose group (LD10-50), which did not agree with the hypothesis. The possible reason includes selection bias and the limited dataset for >LD50, which was not enough to provide statistical significance.

Hypothesis 3: Sex, age at the time of exposure, and mitigator may also affect the long-term response. The second analysis also investigated confounding factors including sex, age at the time of exposure, and the use of mitigator. The significance of sex was observed in neutrophil counts and percentages, lymphocyte counts and percentages, as well as neutrophil-to lymphocyte ratio. The sex difference of higher neutrophils and lower lymphocytes in male animals indicates more significant late effects in male animals, which was the opposite of the hypothesis. Possible causes may be the baseline sex difference in the balance and count of neutrophil and lymphocytes. Female radiosensitivity may not be as large as suggested in other studies. The factor of age at the time of exposure was significant only for monocyte counts and percentages, indicating the late

effects are more significant in animals exposed at younger age, which was consistent with the hypothesis. There was no statistical significance in the factor of mitigator use, which disagreed with the hypothesis and the possible reason is the study design included different types of mitigators as one factor as well as the unbalanced dataset where mitigators were assigned to animals in the cohort that were relatively younger.

Many possible mechanisms were hyothesised to cause the monocyte elevation and neutrophil skewing in >LD10 dose exposed animals. Clonal hematopoiesis, chronic inflammation, immunosenescence, as well as residual damage to the hematopoietic system are suggested in the literature as having an association with radiation exposure, and it is highly likely they contribute to thelate effects suggested in this study in a interrelated way. The monocyte elevation and neutrophil skewing can also be caused by aging in the hematopoietic system and immune system, indicating this study is agrees with previous studies that ionizing radiation exposure generally induces acceleration of aging in the hematopoietic and immune system.

Monocytes are the main mediator of chronic inflammation, and the elevation is a biomarker of chronic inflammation. The increase of disease morbidity in a dose related manner in the study cohort suggested radiation induced chronic inflammation as one of the causes since chronic inflammation is related to many diseases including neoplasia, cardiovascular disease, and diabetes mellitus. Other conditions in radiosensitive organs such as kidney, testis, and ovary were not likely induced by chronic inflammation, while late effects could induce systemic inflammation in animals.

The study results partially agreed with analysis of the A-bomb survivor cohort study. The effect of radiation exposure in longitudinal trends of monocyte counts and percentages, as well as neutrophil and lymphocyte counts were also observed in A-bomb survivors, while the neutrophil

skewing was not observed, and the monocyte percentages were only significant in older populations. The comparison indicates more significant late effects in the rhesus macaque cohort. The reason for greater and more effects could be the much higher dose in the rhesus macaque cohort than A-bomb survivors. There are differences in disease morbidity including different tumor type prevalence. These difference indicate the advantages of utilizing the RLEC cohort for evaluation of the long-term risk of radiation exposure. A-bomb survivor cohort studies have been the base of many radiation protections and regulations. However, A-bomb survivors are composed of a Japanese population and their lifestyle and nutrition are very different even from modern Japanese population. For the DEARE prognosis, these lifestyle factors are indispensable. Additionally, accumulating evidence suggests the significance of genetics in radiosensitivity and disease morbidity, which may further question relying on the data from A-bomb survivor cohort as representative of the general world population. Rhesus macaques share 95-97.5 % of genes with humans, and the immune and hematopoietic system are very similar. Advantageous features of the RLEC include controlled lifestyles and extensive health records, and findings from RLEC animals should be incorporated more in regulatory suggestions.

The study result also indicates that the lower dose cohort (<LD10) had almost no significant adverse long-term effects on the hematopoietic system, while animals in the dose group tend to be younger, and more data would be required to confirm the long-term effects. The analysis and dataset have limitations, but most limitations can be solved with more data, which will be available with time. Developing ML/AI methods with this dataset would be more informative and could contribute more to the understanding the long-term effect of ionizing radiation.

Literature Cited

Abel GA, Hays JT, Decker PA, Croghan GA, Kuter DJ, Rigotti NA. Effects of biochemically confirmed smoking cessation on white blood cell count. In Mayo Clinic Proceedings 2005 Aug 1 (Vol. 80, No. 8, pp. 1022-1028). Elsevier.

Akash MS, Rehman K, Chen S. Role of inflammatory mechanisms in pathogenesis of type 2 diabetes mellitus. Journal of cellular biochemistry. 2013 Mar;114(3):525-31.

Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000 Mar 9;404(6774):193-7.

Al-Jumayli M, Brown SL, Chetty IJ, Extermann M, Movsas B. The biological process of aging and the impact of ionizing radiation. InSeminars in radiation oncology 2022 Apr 1 (Vol. 32, No. 2, pp. 172-178). WB Saunders.

Andrews RN, Bloomer EG, Olson JD, Hanbury DB, Dugan GO, Whitlow CT, Cline JM. Non-human primates receiving high-dose total-body irradiation are at risk of developing cerebrovascular injury years postirradiation. Radiation research. 2020 Sep 16;194(3):277-87.

Anthony KM, Manson JE, Jaiswal S, Natarajan P, Desai P, Hayden KM, Bick AG, Carty CL, Collins JM, Love SA, Stewart J. Abstract P450: Radon is Associated With Clonal Hematopoiesis of Indeterminate Potential in the Women's Health Initiative. Circulation. 2020 Mar 3;141(Suppl_1): AP450-.

Arida A, Protogerou AD, Kitas GD, Sfikakis PP. Systemic inflammatory response and atherosclerosis: the paradigm of chronic inflammatory rheumatic diseases. International journal of molecular sciences. 2018 Jun 27;19(7):1890.

Armstrong GT, Stovall M, Robison LL. Long-term effects of radiation exposure among adult survivors of childhood cancer: results from the childhood cancer survivor study. Radiation research. 2010 Dec 1;174(6b):840-50.

Avagyan S, Zon LI. Clonal hematopoiesis and inflammation–the perpetual cycle. Trends in Cell Biology. 2022 Dec 31.

Awa AA. Persistent chromosome aberrations in the somatic cells of A-bomb survivors, Hiroshima and Nagasaki. Journal of Radiation Research. 1991 Mar 1;32(Suppl_1):265-74.

Azizova TV, Muirhead CR, Druzhinina MB, Grigoryeva ES, Vlasenko EV, Sumina MV, O'Hagan JA, Zhang W, Haylock RG, Hunter N. Cardiovascular diseases in the cohort of workers first employed at Mayak PA in 1948–1958. Radiation research. 2010 Aug 1;174(2):155-68.

Bacarella N, Ruggiero A, Davis AT, Uberseder B, Davis MA, Bracy DP, Wasserman DH, Cline JM, Sherrill C, Kavanagh K. Whole body irradiation induces diabetes and adipose insulin resistance in nonhuman primates. International Journal of Radiation Oncology* Biology* Physics. 2020 Mar 15;106(4):878-86.

Bailey SM, Luxton JJ, McKenna MJ, Taylor LE, George KA, Jhavar SG, Swanson GP. Ad Astra–telomeres in space!. International Journal of Radiation Biology. 2022 Mar 4;98(3):395-403.

Baral E, Blomgren H, Petrini B, Wasserman J. Blood lymphocytes in breast cancer patients following radiotherapy and surgery. International Journal of Radiation Oncology* Biology* Physics. 1977 Mar 1;2(3-4):289-95.

Barnett GC, West CM, Dunning AM, Elliott RM, Coles CE, Pharoah PD, Burnet NG. Normal tissue reactions to radiotherapy: towards tailoring treatment dose by genotype. Nature Reviews Cancer. 2009 Feb;9(2):134-42.

Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software. 2015; 67(1):1–48.

Bates, I., Bain, B. J., Bates, I., Laffan, M. A. (2017). 2 - Reference Ranges and Normal Values. In Dacie and Lewis Practical Haematology (12th edition) (pp. 8–17)., Elsevier health sciences.

Baylis D, Bartlett DB, Patel HP, Roberts HC. Understanding how we age: insights into inflammaging. Longevity & healthspan. 2013 Dec;2(1):1-8.

Beerman I, Bhattacharya D, Zandi S, Sigvardsson M, Weissman IL, Bryder D, Rossi DJ. Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. Proceedings of the National Academy of Sciences. 2010 Mar 23;107(12):5465-70.

Bektas A, Schurman SH, Sen R, Ferrucci L. Human T cell immunosenescence and inflammation in aging. Journal of leukocyte biology. 2017 Oct;102(4):977-88.

Belizaire R, Wong WJ, Robinette ML, Ebert BL. Clonal haematopoiesis and dysregulation of the immune system. Nature Reviews Immunology. 2023 Mar 20:1-6.

Billiard F, Buard V, Benderitter M, Linard C. Abdominal γ-radiation induces an accumulation of function-impaired regulatory T cells in the small intestine. International Journal of Radiation Oncology* Biology* Physics. 2011 Jul 1;80(3):869-76.

Boisgontier MP, Cheval B. The anova to mixed model transition. Neuroscience & Biobehavioral Reviews. 2016 Sep 1;68:1004-5.

Bolton KL, Ptashkin RN, Gao T, Braunstein L, Devlin SM, Kelly D, Patel M, Berthon A, Syed A, Yabe M, Coombs CC. Cancer therapy shapes the fitness landscape of clonal hematopoiesis. Nature genetics. 2020 Nov;52(11):1219-26.

Broerse JJ, Bartstra RW, van Bekkum DW, van der Hage MH, Zurcher C, van Zwieten MJ, Hollander CF. The carcinogenic risk of high dose total body irradiation in non-human primates. Radiotherapy and Oncology. 2000 Mar 1;54(3):247-53.

Bovelli D, Plataniotis G, Roila FE. Cardiotoxicity of chemotherapeutic agents and radiotherapy-related heart disease: ESMO Clinical Practice Guidelines. Annals of oncology. 2010 May 1;21:v277-82.

Brown G, Ceredig R. Modeling the hematopoietic landscape. Frontiers in cell and developmental biology. 2019 Jun 18;7:104.

Bupp MR. Sex, the aging immune system, and chronic disease. Cellular immunology. 2015 Apr 1;294(2):102-10.

Cao M, Cabrera R, Xu Y, Liu C, Nelson D. Gamma irradiation alters the phenotype and function of CD4+ CD25+ regulatory T cells. Cell biology international. 2009 May 1;33(5):565-71.

Capitanio JP, Mendoza SP, Lerche NW. Individual differences in peripheral blood immunological and hormonal measures in adult male rhesus macaques (Macaca mulatta): evidence for temporal and situational consistency. American Journal of Primatology. 1998;44(1):29-41.

Carbonneau CL, Despars G, Rojas-Sutterlin S, Fortin A, Le O, Hoang T, Beauséjour CM. Ionizing radiation–induced expression of INK4a/ARF in murine bone marrow–derived stromal cell populations interferes with bone marrow homeostasis. Blood, The Journal of the American Society of Hematology. 2012 Jan 19;119(3):717-26.

Cardis E, Gilbert ES, Carpenter L, Howe G, Kato I, Armstrong BK, Beral V, Cowper G, Douglas A, Fix J, Fry SA. Effects of low doses and low dose rates of external ionizing radiation: cancer mortality among nuclear industry workers in three countries. Radiation research. 1995 May 1;142(2):117-32.

Cardis E, Hatch M. The Chernobyl accident—an epidemiological perspective. Clinical Oncology. 2011 May 1;23(4):251-60.

Caruana EJ, Roman M, Hernández-Sánchez J, Solli P. Longitudinal studies. Journal of thoracic disease. 2015 Nov;7(11):E537.

Cekic S, Aichele S, Brandmaier AM, Köhncke Y, Ghisletta P. A tutorial for joint modeling of longitudinal and time-to-event data in R. arXiv preprint arXiv:1909.05661. 2019 Sep 12.

Chen S, Wang Q, Yu H, Capitano ML, Vemula S, Nabinger SC, Gao R, Yao C, Kobayashi M, Geng Z, Fahey A. Mutant p53 drives clonal hematopoiesis through modulating epigenetic pathway. Nature communications. 2019 Dec 11;10(1):5649.

Cheng CK, Chan J, Cembrowski GS, Van Assendelft OW. Complete blood count reference interval diagrams derived from NHANES III: stratification by age, sex, and race. Laboratory Hematology. 2004 Jan 1;10(1):42-53.

Chernyshov VP, Vykhovanets EV, Slukvin II, Antipkin YG, Vasyuk AN, Strauss KW. Analysis of blood lymphocyte subsets in children living on territory that received high amounts of fallout from Chernobyl accident. Clinical immunology and immunopathology. 1997 Aug 1;84(2):122-8.

Chua HL, Plett PA, Sampson CH, Joshi M, Tabbey R, Katz B, MacVittie TJ, Orschell CM. Long-term hematopoietic stem cell damage in a murine model of the hematopoietic syndrome of the acute radiation syndrome. Health physics. 2012 Oct;103(4):356.

Chua HL, Plett PA, Fisher A, Sampson CH, Vemula S, Feng H, Sellamuthu R, Wu T, MacVittie TJ, Orschell CM. Lifelong residual bone marrow damage in murine survivors of the hematopoietic acute radiation syndrome (H-ARS): a compilation of studies comprising the Indiana University experience. Health physics. 2019 Apr;116(4):546.

Chute JP, Muramoto GG, Salter AB, Meadows SK, Rickman DW, Chen B, Himburg HA, Chao NJ. Transplantation of vascular endothelial cells mediates the hematopoietic recovery and survival of lethally irradiated mice. Blood. 2007 Mar 15;109(6):2365-72.

Cohen EP, Olson JD, Tooze JA, Bourland JD, Dugan GO, Cline JM. Detection and quantification of renal fibrosis by computerized tomography. PloS one. 2020 Feb 13;15(2):e0228626.

Coombs CC, Zehir A, Devlin SM, Kishtagari A, Syed A, Jonsson P, Hyman DM, Solit DB, Robson ME, Baselga J, Arcila ME. Therapy-related clonal hematopoiesis in patients with non-hematologic cancers is common and associated with adverse clinical outcomes. Cell stem cell. 2017 Sep 7;21(3):374-82.

Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002 Dec 19;420(6917):860-7.

Ceredig R, Rolink AG, Brown G. Models of haematopoiesis: seeing the wood for the trees. Nature Reviews Immunology. 2009 Apr;9(4):293-300.

Crucian B, Stowe RP, Mehta S, Quiriarte H, Pierson D, Sams C. Alterations in adaptive immunity persist during long-duration spaceflight. npj Microgravity. 2015 Sep 3;1(1):1-0.

Dainiak N. Hematologic consequences of exposure to ionizing radiation. Experimental hematology. 2002 Jun 1;30(6):513-28.

DeBo RJ, Lees CJ, Dugan GO, Caudell DL, Michalson KT, Hanbury DB, Kavanagh K, Cline JM, Register TC. Late effects of total-body gamma irradiation on cardiac structure and function in male rhesus macaques. Radiation research. 2016 Jul 1;186(1):55-64.

Decaroli MC, Rochira V. Aging and sex hormones in males. Virulence. 2017 Jul 4;8(5):545-70.

de Haan G, Lazare SS. Aging of hematopoietic stem cells. Blood, The Journal of the American Society of Hematology. 2018 Feb 1;131(5):479-87.

Dhakal BP, Sweitzer NK, Indik JH, Acharya D, William P. SARS-CoV-2 infection and cardiovascular disease: COVID-19 heart. Heart, lung and Circulation. 2020 Jul 1;29(7):973-87.

De Maeyer RP, Chambers ES. The impact of ageing on monocytes and macrophages. Immunology letters. 2021 Feb 1;230:1-0.

Didier ES, Sugimoto C, Bowers LC, Khan IA, Kuroda MJ. Immune correlates of aging in outdoor-housed captive rhesus macaques (Macaca mulatta). Immunity & Ageing. 2012 Dec;9:1-5.

Douple EB, Mabuchi K, Cullings HM, Preston DL, Kodama K, Shimizu Y, Fujiwara S, Shore RE. Long-term radiation-related health effects in a unique human population: lessons learned from the atomic bomb survivors of Hiroshima and Nagasaki. Disaster medicine and public health preparedness. 2011 Mar;5(S1):S122-33.

Dudgeon C, Shreeram S, Tanoue K, Mazur SJ, Sayadi A, Robinson RC, Appella E, Bulavin DV. Genetic variants and mutations of PPM1D control the response to DNA damage. Cell Cycle. 2013 Aug 15;12(16):2656-64.

Edwards LJ. Modern statistical techniques for the analysis of longitudinal data in biomedical research. Pediatric pulmonology. 2000 Oct;30(4):330-44.

Esser N, Legrand-Poels S, Piette J, Scheen AJ, Paquot N. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. Diabetes research and clinical practice. 2014 Aug 1;105(2):141-50.

Estey E. Acute myeloid leukemia and myelodysplastic syndromes in older patients. Journal of clinical oncology. 2007 May 10;25(14):1908-15.

Even Y, Yi L, Chang CK, Rossi FM. After migration into blood circulation, hematopoietic stem cells can stably self-renew and maintain bone marrow while being skewed toward myeloid lineage when submitted to serial transplantation. bioRxiv. 2021 Mar 4:2021-03.

Fanning KM, Pfisterer B, Davis AT, Presley TD, Williams IM, Wasserman DH, Cline JM, Kavanagh K. Changes in microvascular density differentiate metabolic health outcomes in monkeys with prior radiation exposure and subsequent skeletal muscle ECM remodeling. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2017 Sep 1;313(3):R290-7.

Farese AM, Cohen MV, Katz BP, Smith CP, Jackson III W, Cohen DM, MacVittie TJ. A nonhuman primate model of the hematopoietic acute radiation syndrome plus medical management. Health physics. 2012 Oct;103(4).

Farese AM, Drouet M, Herodin F, Bertho JM, Thrall KD, Authier S, Doyle-Eisele M, MacVittie TJ. Acute radiation effects, the H-ARS in the non-human primate: a review and new data for the cynomolgus macaque with reference to the rhesus macaque. Health Physics. 2021 Oct 1;121(4):304-30.

Fiedler K, Brunner C. Mechanisms controlling hematopoiesis. Hematology-Science and Practice. 2012 Mar 2:3-28.

Fliedner TM, Graessle D, Paulsen C, Reimers K. Structure and function of bone marrow hemopoiesis: mechanisms of response to ionizing radiation exposure. Cancer Biotherapy and Radiopharmaceuticals. 2002 Aug 1;17(4):405-26.

Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, De Benedictis G. Inflamm-aging: an evolutionary perspective on immunosenescence. Annals of the New York Academy of Sciences. 2000 Jun;908(1):244-54.

Furman D, Campisi J, Verdin E, Carrera-Bastos P, Targ S, Franceschi C, Ferrucci L, Gilroy DW, Fasano A, Miller GW, Miller AH. Chronic inflammation in the etiology of disease across the life span. Nature medicine. 2019 Dec;25(12):1822-32.

Fujiwara S, Carter RL, Akiyama M, Akahoshi M, Kodama K, Shimaoka K, Yamakido M. Autoantibodies and immunoglobulins among atomic bomb survivors. Radiation research. 1994 Jan 1;137(1):89-95.

Fujiwara S, Sharp GB, Cologne JB, Kusumi S, Akahoshi M, Kodama K, Suzuki G, Yoshizawa H. Prevalence of hepatitis B virus infection among atomic bomb survivors. Radiation research. 2003 Jun 1;159(6):780-6.

Garcia TP, Marder K. Statistical approaches to longitudinal data analysis in neurodegenerative diseases: Huntington's disease as a model. Current neurology and neuroscience reports. 2017 Feb;17(2):14.

Genovese G, Kähler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF, Chambert K, Mick E, Neale BM, Fromer M, Purcell SM. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. New England Journal of Medicine. 2014 Dec 25;371(26):2477-87.

Ghiaur G and Jones RJ. Hematopoiesis. In: Schmaier AH, Lazarus HM, editors. Concise guide to hematology. John Wiley & Sons; 2019. p. 5-13

Gibbons JP. Khan's the physics of radiation therapy. Sixth edition. Lippincott Williams & Wilkins; 2020.

Gilbert ES. Invited commentary: studies of workers exposed to low doses of radiation. American Journal of Epidemiology. 2001 Feb 15;153(4):319-22.

Gofman JW, Young W, Tandy R. Ischemic heart disease, atherosclerosis, and longevity. Circulation. 1966 Oct;34(4):679-97.

Gollamudi J, Huang AY, and Stavrou EX. Physiological Roles of Leukocytes and Disorders. In: Schmaier AH, Lazarus HM, editors. Concise guide to hematology. John Wiley & Sons; 2019. p. 209-224

Gonzalez OA, Orraca L, Kensler TB, Gonzalez-Martinez J, Maldonado E, Ebersole JL. Familial periodontal disease in the cayo santiago rhesus macaques. American journal of primatology. 2016 Jan;78(1):143-51.

Grant BW, Trombley LM, Hunter TC, Nicklas JA, O'Neill JP, Albertini RJ. HPRT mutations in vivo in human CD 34+ hematopoietic stem cells. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 1999 Dec 17;431(2):183-98.

Greten FR, Grivennikov SI. Inflammation and cancer: triggers, mechanisms, and consequences. Immunity. 2019 Jul 16;51(1):27-41.

Grootens J, Ungerstedt JS, Nilsson G, Dahlin JS. Deciphering the differentiation trajectory from hematopoietic stem cells to mast cells. Blood advances. 2018 Sep 9;2(17):2273.

Haas S, Trumpp A, Milsom MD. Causes and consequences of hematopoietic stem cell heterogeneity. Cell stem cell. 2018 May 3;22(5):627-38.

Hajar R. Risk factors for coronary artery disease: historical perspectives. Heart views: the official journal of the Gulf Heart Association. 2017 Jul;18(3):109.

Hall EJ, Giaccia AJ. Radiobiology for the radiologist. Eight edition. Wolters Kluwer. 2019.

Hale, L. Hale LP, Rajam G, Carlone GM, Jiang C, Owzar K, Dugan G, Caudell D, Chao N, Cline JM, Register TC, Sempowski GD. Late effects of total body irradiation on hematopoietic recovery and immune function in rhesus macaques. PLoS One. 2019 Feb 13;14(2):e0210663.

Hancock SL, Tucker MA, Hoppe RT. Factors affecting late mortality from heart disease after treatment of Hodgkin's disease. Jama. 1993 Oct 27;270(16):1949-55.

Hayashi T, Kusunoki Y, Hakoda M, Morishita Y, Kubo Y, Maki M, Kasagi F, Kodama K, Macphee DG, Kyoizumi S. Radiation dose-dependent increases in inflammatory response markers in A-bomb survivors. International journal of radiation biology. 2003 Jan 1;79(2):129-36.

Hayashi T, Morishita Y, Kubo Y, Kusunoki Y, Hayashi I, Kasagi F. Long-term effects of radiation dose on inflammatory markers in atomic bomb survivors. Am J Med 2005; 118(1):83-6.

Hayashi T, Morishita Y, Khattree R, Misumi M, Sasaki K, Hayashi I, Yoshida K, Kajimura J, Kyoizumi S, Imai K, Kusunoki Y. Evaluation of systemic markers of inflammation in atomic-bomb survivors with special reference to radiation and age effects. The FASEB Journal. 2012 Nov;26(11):4765.

Hayashi T, Lynch HE, Geyer S, Yoshida K, Furudoi K, Sasaki K, Morishita Y, Nagamura H, Maki M, Hu Y, Hayashi I. Impact of early life exposure to ionizing radiation on influenza vaccine response in an elderly Japanese cohort. Vaccine. 2018 Oct 29;36(45):6650-9.

Hayashi T, Furukawa K, Morishita Y, Hayashi I, Kato N, Yoshida K, Kusunoki Y, Kyoizumi S, Ohishi W. Intracellular reactive oxygen species level in blood cells of atomic bomb survivors is increased due to aging and radiation exposure. Free Radical Biology and Medicine. 2021 Aug 1;171:126-34.

Haybittle JL, Brinkley D, Houghton J, A'Hern RP, Baum M. Postoperative radiotherapy and late mortality: evidence from the Cancer Research Campaign trial for early breast cancer. British Medical Journal. 1989 Jun 17;298(6688):1611-4.

He Z, Allers C, Sugimoto C, Ahmed N, Fujioka H, Kim WK, Didier ES, Kuroda MJ. Rapid turnover and high production rate of myeloid cells in adult rhesus macaques with compensations during aging. The Journal of Immunology. 2018 Jun 15;200(12):4059-67.

Herb M, Schramm M. Functions of ROS in macrophages and antimicrobial immunity. Antioxidants. 2021 Feb 19;10(2):313.

Himburg HA, Muramoto GG, Daher P, Meadows SK, Russell JL, Doan P, Chi JT, Salter AB, Lento WE, Reya T, Chao NJ. Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. Nature medicine. 2010 Apr;16(4):475-82.

Hince M, Sakkal S, Vlahos K, Dudakov J, Boyd R, Chidgey A. The role of sex steroids and gonadectomy in the control of thymic involution. Cellular immunology. 2008 Jan 1;252(1-2):122-38.

HIRABAYASHI Y, MATSUDA M, MATUMURA T, MITSUI H, SASAKI H, TUKADA T, AIZAWA SI, YOSHIDA K, INOUE T. The p53-deficient hemopoietic stem cells: their resistance to radiation-apoptosis, but lasted transiently. Leukemia (08876924). 1997 Apr 3;11.

Hirai Y, Kusunoki Y, Kyoizumi S, Awa AA, Pawel DJ, Nakamura N, Akiyama M. Mutant frequency at the HPRT locus in peripheral blood T-lymphocytes of atomic bomb survivors. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 1995 Jul 1;329(2):183-96.

Hsu WL, Tatsukawa Y, Neriishi K, Yamada M, Cologne J, Fujiwara S. Longitudinal trends of total white blood cell and differential white blood cell counts of atomic bomb survivors. Journal of radiation research. 2010 Jul 1;51(4):431-9.

Hsu JI, Dayaram T, Tovy A, De Braekeleer E, Jeong M, Wang F, Zhang J, Heffernan TP, Gera S, Kovacs JJ, Marszalek JR. PPM1D mutations drive clonal hematopoiesis in response to cytotoxic chemotherapy. Cell stem cell. 2018 Nov 1;23(5):700-13.

Hoffman R, Benz Jr EJ, Silberstein LE, Heslop H, Anastasi J, Weitz J. Hematology: basic principles and practice. Elsevier Health Sciences; 2013.

Hooper AT, Butler JM, Nolan DJ, Kranz A, Iida K, Kobayashi M, Kopp HG, Shido K, Petit I, Yanger K, James D. Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. Cell stem cell. 2009 Mar 6;4(3):263-74.

Howard R, Kanetsky PA, Egan KM. Exploring the prognostic value of the neutrophil-to-lymphocyte ratio in cancer. Scientific reports. 2019 Dec 23;9(1):19673.

International Commission on Radiological Protection, 2012. ICRP Statement on Tissue Reactions / Early and Late Effects of Radiation in Normal Tissues and Organs – Threshold Doses for Tissue Reactions in a Radiation Protection Context. ICRP Publication 118. Ann. ICRP 41(1/2)

Imelda Bates, S. Mitchell Lewis, Chapter 2 - Reference ranges and normal values, Editor(s): Barbara J. Bain, Imelda Bates, Michael A. Laffan, S. Mitchell Lewis, Dacie and Lewis Practical Haematology (Eleventh Edition), Churchill Livingstone.

Ito C. Trends in the prevalence of diabetes mellitus among Hiroshima atomic bomb survivors. Diabetes research and clinical practice. 1994 Oct 1;24:S29-35.

Jackson MW, Roberts JS, Heckford SE, Ricciardelli C, Stahl J, Horsfall DJ, Tilley WD. A potential autocrine role for vascular endothelial growth factor in prostate cancer. Cancer research. 2002 Feb 1;62(3):854-9.

Jagannathan-Bogdan M, Zon LI. Hematopoiesis. Development. 2013 Jun 15;140(12):2463-7.

Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG, Lindsley RC, Mermel CH, Burtt N, Chavez A, Higgins JM. Age-related clonal hematopoiesis associated with adverse outcomes. New England Journal of Medicine. 2014 Dec 25;371(26):2488-98.

Jaiswal S, Ebert BL. Clonal hematopoiesis in human aging and disease. Science. 2019 Nov 1;366(6465):eaan4673.

Jurk D, Wilson C, Passos JF, Oakley F, Correia-Melo C, Greaves L, Saretzki G, Fox C, Lawless C, Anderson R, Hewitt G. Chronic inflammation induces telomere dysfunction and accelerates ageing in mice. Nature communications. 2014 Jun 24;5(1):4172.

Kajimura J, Lynch HE, Geyer S, French B, Yamaoka M, Shterev ID, Sempowski GD, Kyoizumi S, Yoshida K, Misumi M, Ohishi W. Radiation-and age-associated changes in peripheral blood dendritic cell populations among aging atomic bomb survivors in Japan. Radiation research. 2018 Jan 1;189(1):84-94.

Kamiya K, Ozasa K, Akiba S, Niwa O, Kodama K, Takamura N, Zaharieva EK, Kimura Y, Wakeford R. Long-term effects of radiation exposure on health. The lancet. 2015 Aug 1;386(9992):469-78.

Kashcheev VV, Chekin SY, Karpenko SV, Maksioutov MA, Menyaylo AN, Tumanov KA, Kochergina EV, Kashcheeva PV, Gorsky AI, Shchukina NV, Lovachev SS. Radiation risk of cardiovascular diseases in the cohort of Russian emergency workers of the Chernobyl accident. Health physics. 2017 Jul 1;113(1):23-9.

Kavanagh K, Dendinger MD, Davis AT, Register TC, DeBo R, Dugan G, Cline JM. Type 2 diabetes is a delayed late effect of whole-body irradiation in nonhuman primates. Radiation research. 2015 Apr 1;183(4):398-406.

Kaur A, Vemalapally N, Severson G, Gulani J, Bolduc D, Moroni M. Development of a pediatric model of hematopoietic acute radiation syndrome (H-ARS) and countermeasure testing using the Gottingen minipigs. injury. 2017;4:5.

Kleinerman RA. Cancer risks following diagnostic and therapeutic radiation exposure in children. Pediatric radiology. 2006 Sep;36(Suppl 2):121-5.

Kobiyama K, Ley K. Atherosclerosis: a chronic inflammatory disease with an autoimmune component. Circulation research. 2018 Oct 26;123(10):1118-20.

Kovtonyuk LV, Fritsch K, Feng X, Manz MG, Takizawa H. Inflamm-aging of hematopoiesis, hematopoietic stem cells, and the bone marrow microenvironment. Frontiers in immunology. 2016 Nov 14;7:502.

Koo BS, Lee DH, Kang P, Jeong KJ, Lee S, Kim K, Lee Y, Huh JW, Kim YH, Park SJ, Jin YB. Reference values of hematological and biochemical parameters in young-adult cynomolgus monkey (Macaca fascicularis) and rhesus monkey (Macaca mulatta) anesthetized with ketamine hydrochloride. Laboratory animal research. 2019 Dec;35(1):1-6.

Kossenko MM, Nikolayenko LA, Yepifanova SB, Ostroumova YV. Chronic radiation sickness among Techa riverside residents. Armed Forces Radiobiology Research Institute Contract Report, Bethesda, Maryland. 1998 Feb 1:11-7.

Koturbash I, Kutanzi K, Hendrickson K, Rodriguez-Juarez R, Kogosov D, Kovalchuk O. Radiation-induced bystander effects in vivo are sex specific. Mutation

Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2008 Jul 3;642(1-2):28-36.

Koturbash I, Zemp F, Kolb B, Kovalchuk O. Sex-specific radiation-induced microRNAome responses in the hippocampus, cerebellum and frontal cortex in a mouse model. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2011 Jun 17;722(2):114-8.

Kovalchuk O, Ponton A, Filkowski J, Kovalchuk I. Dissimilar genome response to acute and chronic low-dose radiation in male and female mice. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2004 Jun 4;550(1-2):59-72.

Kumar V, Abbas AK, Fausto N, Aster JC. Robbins and Cotran pathologic basis of disease, professional edition e-book. Elsevier health sciences; 2014 Aug 27.

Kusunoki Y, Kyoizumi S, Hirai Y, Suzuki T, Nakashima E, Kodama K, Seyama T. Flow cytometry measurements of subsets of T, B and NK cells in peripheral blood lymphocytes of atomic bomb survivors. Radiation research. 1998 Aug 1;150(2):227-36.

Kusunoki Y, Hayashi T, Morishita Y, Yamaoka M, Maki M, Hakoda M, Kodama K, Bean MA, Kyoizumi S. T-cell responses to mitogens in atomic bomb survivors: a decreased capacity to produce interleukin 2 characterizes the T cells of heavily irradiated individuals. Radiation research. 2001 Jan 1;155(1):81-8.

Kusunoki Y, Yamaoka M, Kasagi F, Hayashi T, Koyama K, Kodama K, MacPhee DG, Kyoizumi S. T cells of atomic bomb survivors respond poorly to stimulation by Staphylococcus aureus toxins in vitro: Does this stem from their peripheral lymphocyte populations having a diminished naive CD4 T-cell content?. Radiation research. 2002 Dec 1;158(6):715-24.

Kusunoki Y, Yamaoka M, Kasagi F, Hayashi T, MacPhee DG, Kyoizumi S. Long-lasting changes in the T-cell receptor V beta repertoires of CD4 memory T-cell populations in the peripheral blood of radiation-exposed people. British journal of haematology. 2003 Sep;122(6):975-84.

Kusunoki Y, Hayashi T. Long-lasting alterations of the immune system by ionizing radiation exposure: implications for disease development among atomic bomb survivors. International journal of radiation biology. 2008 Jan 1;84(1):1-4.

Kusunoki Y, Yamaoka M, Kubo Y, Hayashi T, Kasagi F, Douple EB, Nakachi K. T-cell immunosenescence and inflammatory response in atomic bomb survivors. Radiation research. 2010 Dec 1;174(6b):870-6.

Kurjane N, Bruvere R, Shitova O, Romanova T, Jaunalksne I, Kirschfink M, Sochnevs A. Analysis of the Immune Status in Latvian Chernobyl Clean-up Workers with Nononcological Thyroid Diseases. Scandinavian Journal of Immunology. 2001 Nov;54(5):528-33.

Laird NM, Ware JH. Random-effects models for longitudinal data. Biometrics. 1982 Dec 1:963-74.

Laurier D, Richardson DB, Cardis E, Daniels RD, Gillies M, O'Hagan J, Hamra GB, Haylock R, Leuraud K, Moissonnier M, Schubauer-Berigan MK. The international nuclear workers study (INWORKS): a collaborative epidemiological study to improve knowledge about health effects of protracted low-dose exposure. Radiation protection dosimetry. 2017 Apr 1;173(1-3):21-5.

Lázár-Molnár E, Hegyesi H, Tóth S, Falus A. Autocrine and paracrine regulation by cytokines and growth factors in melanoma. Cytokine. 2000 Jun 1;12(6):547-54.

Lei H, Schmidt-Bleek K, Dienelt A, Reinke P, Volk HD. Regulatory T cell-mediated anti-inflammatory effects promote successful tissue repair in both indirect and direct manners. Frontiers in pharmacology. 2015 Sep 2;6:184.

Lenth R. emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.8.5. 2023. Available from: https://CRAN.R-project.org/package=emmeans.

Leuraud K, Richardson DB, Cardis E, Daniels RD, Gillies M, O'hagan JA, Hamra GB, Haylock R, Laurier D, Moissonnier M, Schubauer-Berigan MK. Ionising radiation and risk of death from leukaemia and lymphoma in radiation-monitored workers (INWORKS): an international cohort study. The Lancet Haematology. 2015 Jul 1;2(7):e276-81.

Li B, Bailey AS, Jiang S, Liu B, Goldman DC, Fleming WH. Endothelial cells mediate the regeneration of hematopoietic stem cells. Stem cell research. 2010 Jan 1;4(1):17-24.

Li D, Wang Y, Wu H, Lu L, Zhang H, Chang J, Zhai Z, Zhang J, Wang Y, Zhou D, Meng A. Mitigation of ionizing radiation-induced bone marrow suppression by p38 inhibition and G-CSF administration. Journal of radiation research. 2011 Nov 1;52(6):712-6.

Li K, Peng YG, Yan RH, Song WQ, Peng XX, Ni X. Age-dependent changes of total and differential white blood cell counts in children. Chinese medical journal. 2020 Aug 20;133(16):1900-7.

Lian J, Yue Y, Yu W, Zhang Y. Immunosenescence: a key player in cancer development. Journal of hematology & oncology. 2020 Dec;13:1-8.

Libert C, Dejager L, Pinheiro I. The X chromosome in immune functions: when a chromosome makes the difference. Nat Rev Immunol 10:594–604; 2010. DOI:10.1038/nri2815.

Little MP. Risks of radiation-induced cancer at high doses and dose rates. Journal of Radiological Protection. 1993 Mar 1;13(1):3.

Little MP, De Vathaire F, Charles MW, Hawkins MM, Muirhead CR. Variations with time and age in the risks of solid cancer incidence after radiation exposure in childhood. Statistics in medicine. 1998 Jun 30;17(12):1341-55.

Little MP. Risks associated with ionizing radiation: Environmental pollution and health. British medical bulletin. 2003 Dec 1;68(1):259-75.

Little MP. Threshold and other departures from linear-quadratic curvature in the non-cancer mortality dose-response curve in the Japanese atomic bomb survivors. Radiation and environmental biophysics. 2004 Jul;43:67-75.

Little MP, Brenner AV, Grant EJ, Sugiyama H, Preston DL, Sakata R, Cologne J, Velazquez-Kronen R, Utada M, Mabuchi K, Ozasa K. Age effects on radiation response: Summary of a recent symposium and future perspectives. International Journal of Radiation Biology. 2022 Nov 2;98(11):1673-83.

Little MP, Wakeford R, Bouffler SD, Abalo K, Hauptmann M, Hamada N, Kendall GM. Review of the risk of cancer following low and moderate doses of sparsely ionising radiation received in early life in groups with individually estimated doses. Environment international. 2022 Jan 15;159:106983.

Little MP, Brenner AV, Grant EJ, Sugiyama H, Preston DL, Sakata R, Cologne J, Velazquez-Kronen R, Utada M, Mabuchi K, Ozasa K. Age effects on radiation response: Summary of a recent symposium and future perspectives. International Journal of Radiation Biology. 2022 Nov 2;98(11):1673-83.

Liu C, Cripe TP, Kim MO. Statistical issues in longitudinal data analysis for treatment efficacy studies in the biomedical sciences. Molecular Therapy. 2010 Sep 1;18(9):1724-30.

Liu XD, Ma SM, Liu SZ. Effects of 0.075 Gy x-ray irradiation on the expression of IL-10 and IL-12 in mice. Physics in Medicine & Biology. 2003 Jun 17;48(13):2041.

Lowe GD. The relationship between infection, inflammation, and cardiovascular disease: an overview. Annals of periodontology. 2001 Dec;6(1):1-8.

Luxin W, Yongru Z, Zufan T, Weihui H, Deqing C, Yongling Y. Epidemiological investigation of radiological effects in high background radiation areas of Yangjiang, China. Journal of radiation research. 1990 Mar 1;31(1):119-36.

Luxton JJ, McKenna MJ, Lewis A, Taylor LE, George KA, Dixit SM, Moniz M, Benegas W, Mackay MJ, Mozsary C, Butler D. Telomere length dynamics and DNA damage responses associated with long-duration spaceflight. Cell Reports. 2020 Dec 8;33(10).

Lumniczky K, Impens N, Armengol G, Candéias S, Georgakilas AG, Hornhardt S, Martin OA, Rödel F, Schaue D. Low dose ionizing radiation effects on the immune system. Environment international. 2021 Apr 1;149:106212.

Macintyre AN, French MJ, Sanders BR, Riebe KJ, Shterev ID, Wiehe K, Hora B, Evangelous T, Dugan G, Bourland JD, Cline JM. Long-term recovery of the adaptive immune system in rhesus macaques after total body irradiation. Advances in Radiation Oncology. 2021 Sep 1;6(5):100677.

MacKinney Jr AA. Effect of aging on the peripheral blood lymphocyte count. Journal of Gerontology. 1978 Mar 1;33(2):213-6.

MacVittie TJ, Bennett AW, Cohen MV, Farese AM, Higgins A, Hankey KG. Immune cell reconstitution after exposure to potentially lethal doses of radiation in the nonhuman primate. Health physics. 2014 Jan 1;106(1):84-96.

MacVittie TJ, Farese AM, Jackson III W. The hematopoietic syndrome of the acute radiation syndrome in rhesus macaques: A systematic review of the lethal dose response relationship. Health physics. 2015 Nov 1;109(5):342-66.

MacVittie TJ. Where are the medical countermeasures against the ARS and DEARE? A current topic relative to an animal model research platform, radiation exposure context, the acute and delayed effects of acute exposure, and the FDA animal rule. International Journal of Radiation Biology. 2023 Mar 10:1-5.

Maeda H, Akaike T. Nitric oxide and oxygen radicals in infection, inflammation, and cancer. BIOCHEMISTRY C/C OF BIOKHIMIIA. 1998 Jul 1;63:854-65.

Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA, Donath MY. Glucose-induced β cell production of IL-1β contributes to glucotoxicity in human pancreatic islets. The Journal of clinical investigation. 2002 Sep 15;110(6):851-60.

Mauch P, Constine L, Greenberger J, Knospe W, Sullivan J, Liesveld JL, Deeg HJ. Hematopoietic stem cell compartment: acute and late effects of radiation therapy and chemotherapy. International Journal of Radiation Oncology* Biology* Physics. 1995 Mar 30;31(5):1319-39.

McGeoghegan D, Binks K, Gillies M, Jones S, Whaley S. The non-cancer mortality experience of male workers at British Nuclear Fuels plc, 1946–2005. International journal of epidemiology. 2008 Jun 1;37(3):506-18.

McNab F, Mayer-Barber K, Sher A, Wack A, O'garra A. Type I interferons in infectious disease. Nature Reviews Immunology. 2015 Feb;15(2):87-103.

Meacham LR, Sklar CA, Li S, Liu Q, Gimpel N, Yasui Y, Whitton JA, Stovall M, Robison LL, Oeffinger KC. Diabetes mellitus in long-term survivors of childhood cancer: increased risk

associated with radiation therapy: a report for the childhood cancer survivor study. Archives of internal medicine. 2009 Aug 10;169(15):1381-8.

Michalson KT, Macintyre AN, Sempowski GD, Bourland JD, Howard TD, Hawkins GA, Dugan GO, Cline JM, Register TC. Monocyte polarization is altered by total-body irradiation in male rhesus macaques: implications for delayed effects of acute radiation exposure. Radiation research. 2019 Aug 1;192(2):121-34.

Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. Antioxidants & redox signaling. 2014 Mar 1;20(7):1126-67.

Monroy RL, MacVittie TJ, Darden JH, Schwartz GN, Patchen ML. The rhesus monkey: a primate model for hemopoietic stem cell studies. Exp Hematol. 1986 Nov 1;14(10):904-11.

Müller L, Di Benedetto S, Pawelec G. The immune system and its dysregulation with aging. Biochemistry and cell biology of ageing: Part II clinical science. 2019:21-43.

Murphy K, Weaver C. Janeway's immunobiology. Garland science; 2016 Mar 1.

Nagasawa N, Tamakoshi K, Yatsuya H, Hori Y, Ishikawa M, Murata C, Zhang H, Wada K, Otsuka R, Mabuchi T, Kondo T. Association of white blood cell count and clustered components of metabolic syndrome in Japanese men. Circulation journal. 2004;68(10):892-7.

Narendran N, Luzhna L, Kovalchuk O. Sex difference of radiation response in occupational and accidental exposure. Frontiers in genetics. 2019 May 3;10:260.

Nathan C, Cunningham-Bussel A. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. Nature Reviews Immunology. 2013 May;13(5):349-61.

National Council on Radiation Protection and Measurements. Evaluation of a Sex-Specific Difference in Lung Cancer Radiation Risk and Approaches for Improving Lung Cancer

Radiation Risk Projection (With a Focus on Application to Space Activities). Bethesda, MD: NCRP; NCRP Commentary No. 32; 2022.

Neriishi K, Nakashima E, Delongchamp RR. Persistent subclinical inflammation among A-bomb survivors. International journal of radiation biology. 2001 Jan 1;77(4):475-82.

Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, McBride OW, Leonard WJ. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. Cell 73:147–157; 1993. DOI:10.1016/0092-8674(93)90167-o.

Ochiai K, Hayama SI, Nakiri S, Nakanishi S, Ishii N, Uno T, Kato T, Konno F, Kawamoto Y, Tsuchida S, Omi T. Low blood cell counts in wild Japanese monkeys after the Fukushima Daiichi nuclear disaster. Scientific reports. 2014 Jul 24;4(1):5793.

Okeke EB, Uzonna JE. The pivotal role of regulatory T cells in the regulation of innate immune cells. Frontiers in immunology. 2019 Apr 9;10:680.

Olson, M. Atomic Radiation is More Harmful to Women. Takoma Park, MD: Nuclear Information And Resource Service. 2011.

Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell. 2008 Feb 22;132(4):631-44.

Ozaki T, Nakagawara A. Role of p53 in cell death and human cancers. Cancers. 2011 Mar 3;3(1):994-1013.

Ozasa K, Shimizu Y, Suyama A, Kasagi F, Soda M, Grant EJ, Sakata R, Sugiyama H, Kodama K. Studies of the mortality of atomic bomb survivors, Report 14, 1950–2003: an overview of cancer and noncancer diseases. Radiation research. 2012 Mar 1;177(3):229-43.

Ozasa K, Takahashi I, Grant EJ, Kodama K. Cardiovascular disease among atomic bomb survivors. International journal of radiation biology. 2017 Oct 3;93(10):1145-50.

Paganetti H. A review on lymphocyte radiosensitivity and its impact on radiotherapy. Frontiers in Oncology. 2023 Aug 3;13:1201500.

Pahwa R, Goyal A, Jialal I. Chronic Inflammation. In: StatPearls. StatPearls Publishing, Treasure Island (FL); 2022. PMID: 29630225.

Pangrazzi L, Meryk A, Naismith E, Koziel R, Lair J, Krismer M, Trieb K, Grubeck Loebenstein B. "Inflamm-aging" influences immune cell survival factors in human bone marrow. European journal of immunology. 2017 Mar;47(3):481-92.

Park T. A comparison of the generalized estimating equation approach with the maximum likelihood approach for repeated measurements. Statistics in Medicine. 1993 Sep 30;12(18):1723-32.

Parkin DM, Cardis E, Masuyer E, Friedl HP, Hansluwka H, Bobev D, Ivanov E, Sinnaeve J, Augustin J, Plesko I, Storm HH. Childhood leukaemia following the Chernobyl accident: the European childhood leukaemia-lymphoma incidence study (ECLIS). European Journal of Cancer. 1993 Jan 1;29(1):87-95.

Patterson AM, Vemula S, Plett PA, Sampson CH, Chua HL, Fisher A, Wu T, Sellamuthu R, Feng H, Katz BP, DesRosiers CM. Age and sex divergence in hematopoietic radiosensitivity in aged mouse models of the hematopoietic acute radiation syndrome. Radiation Research. 2022 Sep 1;198(3):221-42.

P. Chang, JS Hwang, MC Hung, TH Hu, SD Lee, BF Hwang W. Chronic low-dose gamma-radiation exposure and the alteration of the distribution of lymphocyte subpopulations in

residents of radioactive buildings. International journal of radiation biology. 1999 Jan 1;75(10):1231-9.

Persa E, Balogh A, Sáfrány G, Lumniczky K. The effect of ionizing radiation on regulatory T cells in health and disease. Cancer letters. 2015 Nov 28;368(2):252-61.

Pellegrino R, Paganelli R, Di Iorio A, Bandinelli S, Moretti A, Iolascon G, Sparvieri E, Tarantino D, Ferrucci L. Temporal trends, sex differences, and age-related disease influence in Neutrophil, Lymphocyte count and Neutrophil to Lymphocyte-ratio: results from InCHIANTI follow-up study. Immunity & Ageing. 2023 Sep 4;20(1):46.

Plett PA, Sampson CH, Chua HL, Jackson W, Vemula S, Sellamuthu R, Fisher A, Feng H, Wu T, MacVittie TJ, Orschell CM. The H-ARS dose response relationship (DRR): validation and variables. Health physics. 2015 Nov;109(5):391.

Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nature Reviews Cancer. 2004 Jan 1;4(1):71-8.

Posner MR, Reinherz E, Lane H, Mauch P, Hellman S, Schlossman SF. Circulating lymphocyte populations in Hodgkin's disease after mantle and paraaortic irradiation.

Poulos MG, Ramalingam P, Gutkin MC, Llanos P, Gilleran K, Rabbany SY, Butler JM. Endothelial transplantation rejuvenates aged hematopoietic stem cell function. The Journal of clinical investigation. 2017 Nov 1;127(11):4163-78.

Preston DL, Kusumi S, Tomonaga M, Izumi S, Ron E, Kuramoto A, Kamada N, Dohy H, Matsui T, Nonaka H, Thompson DE. Cancer incidence in atomic bomb survivors. Part III: Leukemia, lymphoma and multiple myeloma, 1950-1987. Radiation research. 1994 Feb 1;137(2s):S68-97.

Preston DL, Shimizu Y, Pierce DA, Suyama A, Mabuchi K. Studies of mortality of atomic bomb survivors. Report 13: Solid cancer and noncancer disease mortality: 1950–1997. Radiation research. 2003 Oct 1;160(4):381-407.

Preston DL, Pierce DA, Shimizu Y, Cullings HM, Fujita S, Funamoto S, Kodama K. Effect of recent changes in atomic bomb survivor dosimetry on cancer mortality risk estimates. Radiation research. 2004 Oct 1;162(4):377-89.

Preston DL, Ron E, Tokuoka S, Funamoto S, Nishi N, Soda M, Mabuchi K, Kodama K. Solid cancer incidence in atomic bomb survivors: 1958–1998. Radiation research. 2007 Jul 1;168(1):1-64.

Rahmé R, Braun T, Manfredi JJ, Fenaux P. TP53 Alterations in Myelodysplastic Syndromes and Acute Myeloid Leukemia. Biomedicines. 2023 Apr 11;11(4):1152.

Rees, G.S., Daniel, C.P., Morris, S.D., Whitehouse, C.A., Binks, K., MacGregor, D.H. and Tawn, E.J., 2004. Occupational exposure to ionizing radiation has no effect on T- and B-cell total counts or percentages of helper, cytotoxic and activated T-cell subsets in the peripheral circulation of male radiation workers. International journal of radiation biology, 80(7), pp.493-498.

Reece, J. B., Urry, L. A., Cain, M. L., Wasserman, S. A., Minorsky, P. V., & Jackson, R. B. (2014). Campbell biology (Vol. 9). Boston: Pearson.

Richardson RB. Ionizing radiation and aging: rejuvenating an old idea. Aging (Albany NY). 2009 Nov;1(11):887.

Rossi DJ, Bryder D, Zahn JM, Ahlenius H, Sonu R, Wagers AJ, Weissman IL. Cell intrinsic alterations underlie hematopoietic stem cell aging. Proceedings of the National Academy of Sciences. 2005 Jun 28;102(26):9194-9.

Roth GS, Mattison JA, Ottinger MA, Chachich ME, Lane MA, Ingram DK. Aging in rhesus monkeys: relevance to human health interventions. Science. 2004 Sep 3;305(5689):1423-6.

Sadeghi HM, Schnelle JF, Thomas JK, Nishanian P, Fahey JL. Phenotypic and functional characteristics of circulating monocytes of elderly persons. Experimental gerontology. 1999 Dec 1;34(8):959-70.

Sakai A, Ohira T, Hosoya M, Ohtsuru A, Satoh H, Kawasaki Y, Suzuki H, Takahashi A, Kobashi G, Ozasa K, Yasumura S. White blood cell, neutrophil, and lymphocyte counts in individuals in the evacuation zone designated by the government after the Fukushima Daiichi Nuclear Power Plant accident: the Fukushima Health Management Survey. Journal of Epidemiology. 2015 Jan 5;25(1):80-7.

Schaaf GW, Justice JN, Quillen EE, Cline JM. Resilience, aging, and response to radiation exposure (RARRE) in nonhuman primates: a resource review. GeroScience. 2023 May 16:1-9.

Schaaf GW, Olson JD, Dugan GO, Pray BA, Cleary JA, Cline JM. Dose-Dependent Testicular Injury and Recovery after Total-Body Irradiation in Rhesus Monkeys. Radiation Research. 2023 Sep 13.

Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Current biology. 2014 May 19;24(10):R453-62.

Schmaier AH. Introduction to Hematology. In: Schmaier AH, Lazarus HM, editors. Concise guide to hematology. John Wiley & Sons; 2019. p. 1-3

Seed TM, Inal C, Dobson ME, Ghose S, Hilyard E, Tolle D, Fritz TE. Accommodative responses to chronic irradiation: effects of dose, dose rate, and pharmacological response modifiers. Military medicine. 2002 Feb 1;167(suppl_1):82-6.

Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. Wiley Interdisciplinary Reviews: Systems Biology and Medicine. 2010 Nov;2(6):640-53.

Sennels HP, Jørgensen HL, Hansen AL, Goetze JP, Fahrenkrug J. Diurnal variation of hematology parameters in healthy young males: the Bispebjerg study of diurnal variations. Scandinavian journal of clinical and laboratory investigation. 2011 Nov 1;71(7):532-41.

Senyuk OF, Kavsan VM, Müller WE, Schröder HC. Long-term effects of low-dose irradiation on human health. Cellular and molecular biology (Noisy-le-Grand, France). 2002 Jun 1;48(4):393-409.

Sevan'kaev AV, Lloyd DC, Braselmann H, Edwards AA, Moiseenko VV, Zhloba AA. A survey of chromosomal aberrations in lymphocytes of Chernobyl liquidators. Radiation Protection Dosimetry. 1995 Feb 1;58(2):85-91.

Shao L, Sun Y, Zhang Z, Feng W, Gao Y, Cai Z, Wang ZZ, Look AT, Wu WS. Deletion of proapoptotic Puma selectively protects hematopoietic stem and progenitor cells against high-dose radiation. Blood, The Journal of the American Society of Hematology. 2010 Jun 10;115(23):4707-14.

Shao L, Feng W, Li H, Gardner D, Luo Y, Wang Y, Liu L, Meng A, Sharpless NE, Zhou D. Total body irradiation causes long-term mouse BM injury via induction of HSC premature senescence in an Ink4a-and Arf-independent manner. Blood, The Journal of the American Society of Hematology. 2014 May 15;123(20):3105-15.

Sharma NK, Holmes-Hampton GP, Kumar VP, Biswas S, Wuddie K, Stone S, Aschenake Z, Wilkins WL, Fam CM, Cox GN, Ghosh SP. Delayed effects of acute whole body lethal radiation exposure in mice pre-treated with BBT-059. Scientific Reports. 2020 Apr 22;10(1):6825.

Shevyrev D, Tereshchenko V. Treg heterogeneity, function, and homeostasis. Frontiers in immunology. 2020 Jan 14;10:3100.

Shimizu Y, Kodama K, Nishi N, Kasagi F, Suyama A, Soda M, Grant EJ, Sugiyama H, Sakata R, Moriwaki H, Hayashi M. Radiation exposure and circulatory disease risk: Hiroshima and Nagasaki atomic bomb survivor data, 1950-2003. Bmj. 2010 Jan 15;340.

Shin TH, Zhou Y, Chen S, Cordes S, Grice MZ, Fan X, Lee BC, Aljanahi AA, Hong SG, Vaughan KL, Mattison JA. A macaque clonal hematopoiesis model demonstrates expansion of TET2-disrupted clones and utility for testing interventions. Blood, The Journal of the American Society of Hematology. 2022 Oct 20;140(16):1774-89.

Shlush LI. Age-related clonal hematopoiesis. Blood, The Journal of the American Society of Hematology. 2018 Feb 1;131(5):496-504.

Shukla HS, Hughes LE, Whitehead RH, Newcombe RG. Long-term follow-up of general immune competence in breast cancer: II. Sequential pre-and post-treatment levels: A 10 year study. Cancer Immunology, Immunotherapy. 1986 Jan;21:6-11.

Sills WS, Tooze JA, Olson JD, Caudell DL, Dugan GO, Johnson BJ, Kock ND, Andrews RN, Schaaf GW, Lang RA, Cline JM. Total-body irradiation is associated with increased incidence of mesenchymal neoplasia in a radiation late effects cohort of rhesus macaques (Macaca mulatta). International Journal of Radiation Oncology* Biology* Physics. 2022 Jul 1;113(3):661-74.

Simmons HA, Mattison JA. The incidence of spontaneous neoplasia in two populations of captive rhesus macaques (Macaca mulatta). Antioxidants & redox signaling. 2011 Jan 15;14(2):221-7.

Simmons HA. Age-associated pathology in rhesus macaques (Macaca mulatta). Veterinary pathology. 2016 Mar;53(2):399-416.

Singh N, Baby D, Rajguru JP, Patil PB, Thakkannavar SS, Pujari VB. Inflammation and cancer. Annals of African medicine. 2019 Jul;18(3):121.

Singh R, Oinam AS, Trivedi G, Kainth HS, Shahi JS, Singh B, Kapoor R. A comparative study for surface dose evaluation in conventional treatment of carcinoma breast patients irradiated with Co-60 and 6 MV radiation beam. Journal of Cancer Research and Therapeutics. 2019 Jul 1;15(5):1035-41.

Skandalis A, Da Cruz AD, Curry J, Nohturfft A, Curado MP, Glickman BW. Molecular analysis of T-lymphocyte HPRT-mutations in individuals exposed to ionizing radiation in Goiânia, Brazil. Environmental and molecular mutagenesis. 1997;29(2):107-16.

Smucny DA, Allison DB, Ingram DK, Roth GS, Kemnitz JW, Kohama SG, Lane MA. Changes in blood chemistry and hematology variables during aging in captive rhesus macaques (Macaca mulatta). Journal of medical primatology. 2001 Jun;30(3):161-73.

Smyth MJ, Cretney E, Kershaw MH, Hayakawa Y. Cytokines in cancer immunity and immunotherapy. Immunological reviews. 2004 Dec;202(1):275-93.

Snoeck HW. Aging of the hematopoietic system. Current opinion in hematology. 2013 Jul 1;20(4):355-61.

So EY, Jeong EM, Wu KQ, Dubielecka PM, Reginato AM, Quesenberry PJ, Liang OD. Sexual dimorphism in aging hematopoiesis: an earlier decline of hematopoietic stem and progenitor cells in male than female mice. Aging (Albany NY). 2020 Dec 12;12(24):25939.

Sonneveld P, Peperkamp E, van Bekkum DW. Incidence of cataracts in rhesus monkeys treated with whole-body irradiation. Radiology. 1979 Oct;133(1):227-9.

Stepanova E, Karmaus W, Naboka M, Vdovenko V, Mousseau T, Shestopalov VM, Vena J, Svendsen E, Underhill D, Pastides H. Exposure from the Chernobyl accident had adverse effects on erythrocytes, leukocytes, and platelets in children in the Narodichesky region, Ukraine: A 6-year follow-up study. Environmental Health. 2008 Dec;7:1-3.

Stubblefield MD. Radiation fibrosis syndrome: neuromuscular and musculoskeletal complications in cancer survivors. PM&R. 2011 Nov 1;3(11):1041-54.

Stewart FA, Seemann I, Hoving S, Russell NS. Understanding radiation-induced cardiovascular damage and strategies for intervention. Clinical oncology. 2013 Oct 1;25(10):617-24.

Sudo K, Ema H, Morita Y, Nakauchi H. Age-associated characteristics of murine hematopoietic stem cells. The Journal of experimental medicine. 2000 Nov 6;192(9):1273-80.

Sutherland JS, Goldberg GL, Hammett MV, Uldrich AP, Berzins SP, Heng TS, Blazar BR, Millar JL, Malin MA, Chidgey AP, Boyd RL. Activation of thymic regeneration in mice and humans following androgen blockade. The Journal of Immunology. 2005 Aug 15;175(4):2741-53.

Tak T, Drylewicz J, Conemans L, de Boer RJ, Koenderman L, Borghans JA, Tesselaar K. Circulatory and maturation kinetics of human monocyte subsets in vivo. Blood, The Journal of the American Society of Hematology. 2017 Sep 21;130(12):1474-7.

Tatsukawa Y, Cordova K, Yamada M, Ohishi W, Imaizumi M, Hida A, Sposto R, Sakata R, Fujiwara S, Nakanishi S, Yoneda M. Incidence of diabetes in the atomic bomb survivors: 1969-2015. The Journal of Clinical Endocrinology & Metabolism. 2022 May 1;107(5):e2148-55.

Testa NG, Hendry JH, Molineux G. Long-term bone marrow damage in experimental systems and in patients after radiation or chemotherapy. Anticancer research. 1985 Jan 1;5(1):101-10.

Toivanen A, Granberg I, Nordman E. Lymphocyte subpopulations in patients with breast cancer after postoperative radiotherapy. Cancer. 1984 Dec 15;54(12):2919-23.

Tong J, Hei TK. Aging and age-related health effects of ionizing radiation. Radiation Medicine and Protection. 2020 Mar 1;1(1):15-23.

Triant VA. Cardiovascular disease and HIV infection. Current HIV/AIDS Reports. 2013 Sep;10:199-206.

Tsiatis AA, Davidian M. Joint modeling of longitudinal and time-to-event data: an overview. Statistica Sinica. 2004 Jul 1:809-34.

United Nations Scientific Committee on the Effects of Atomic Radiation. (2008). Effects of Ionizing Radiation, United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) 2006 Report, Volume I: Report to the General Assembly, Scientific Annexes A and B. United Nations.

United Nations Scientific Committee on the Effects of Atomic Radiation. (2008). Effects of Ionizing Radiation, United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) 2006 Report, Volume 2: Scientific Annexes C, D, and E. United Nations.

United Nations Scientific Committee on the Effects of Atomic Radiation. (2013). Scientific annex B: effects of ionizing radiation on children. Volume II: sources, effects, and risks of ionizing radiation. Vienna: United Nations Scientific Committee on the Effects of Atomic Radiation.

Unthank JL, Miller SJ, Quickery AK, Ferguson EL, Wang M, Sampson CH, Chua HL, DiStasi MR, Feng H, Fisher A, Katz BP. Delayed effects of acute radiation exposure in a murine model of the H-ARS: multiple-organ injury consequent to< 10 Gy total body irradiation. Health physics. 2015 Nov;109(5):511.

Urushihara Y, Suzuki T, Shimizu Y, Ohtaki M, Kuwahara Y, Suzuki M, Uno T, Fujita S, Saito A, Yamashiro H, Kino Y. Haematological analysis of Japanese macaques (Macaca fuscata) in the area affected by the Fukushima Daiichi Nuclear Power Plant accident. Scientific reports. 2018 Nov 13;8(1):16748.

Van Greevenbroek MM, Schalkwijk CG, Stehouwer CD. Obesity-associated low-grade inflammation in type 2 diabetes mellitus: causes and consequences. Neth J Med. 2013 May 1;71(4):174-87.

Van Zant G, Liang Y. Concise review: hematopoietic stem cell aging, life span, and transplantation. Stem cells translational medicine. 2012 Sep 1;1(9):651-7.

Walker EM, Slisarenko N, Gerrets GL, Kissinger PJ, Didier ES, Kuroda MJ, Veazey RS, Jazwinski SM, Rout N. Inflammaging phenotype in rhesus macaques is associated with a decline in epithelial barrier-protective functions and increased pro-inflammatory function in CD161-expressing cells. Geroscience. 2019 Dec;41:739-57.

Wang Y, Schulte BA, LaRue AC, Ogawa M, Zhou D. Total body irradiation selectively induces murine hematopoietic stem cell senescence. Blood. 2006 Jan 1;107(1):358-66.

Wang Y, Liu L, Pazhanisamy SK, Li H, Meng A, Zhou D. Total body irradiation causes residual bone marrow injury by induction of persistent oxidative stress in murine hematopoietic stem cells. Free Radical Biology and Medicine. 2010 Jan 15;48(2):348-56.

Wang Y, Liu L, Zhou D. Inhibition of p38 MAPK attenuates ionizing radiation-induced hematopoietic cell senescence and residual bone marrow injury. Radiation research. 2011 Dec 1;176(6):743-52.

Wang J, Sun Q, Morita Y, Jiang H, Groß A, Lechel A, Hildner K, Guachalla LM, Gompf A, Hartmann D, Schambach A. A differentiation checkpoint limits hematopoietic stem cell self-renewal in response to DNA damage. Cell. 2012 Mar 2;148(5):1001-14.

Wang G, Xu C, Li S, Zhang D, Chen Y, Liu J, Liu N. The Influence of Low-Dose Occupational Radiation Exposure on Peripheral Blood Cells in a Cohort of Chinese Medical Radiation Workers. Radiation Protection Dosimetry. 2022 Mar;198(4):246-56.

Watanabe N, De Rosa SC, Cmelak A, Hoppe R, Herzenberg LA, Herzenberg LA, Roederer M. Long-term depletion of naive T cells in patients treated for Hodgkin's disease. Blood, The Journal of the American Society of Hematology. 1997 Nov 1;90(9):3662-72.

Wersal C, Keller A, Weiss C, Giordano FA, Abo-Madyan Y, Tuschy B, Sütterlin M, Wenz F, Sperk E. Long-term changes in blood counts after intraoperative radiotherapy for breast cancer—single center experience and review of the literature. Translational Cancer Research. 2019 Sep;8(5):1882.

Wickremasinghe RG, Hoffbrand AV. Biochemical and genetic control of apoptosis: relevance to normal hematopoiesis and hematological malignancies. Blood, The Journal of the American Society of Hematology. 1999 Jun 1;93(11):3587-600.

Wilson A, Laurenti E, Trumpp A. Balancing dormant and self-renewing hematopoietic stem cells. Current opinion in genetics & development. 2009 Oct 1;19(5):461-8.

Wondergem J, Persons K, Zurcher C, Frölich M, Leer JW, Broerse J. Changes in circulating atrial natriuretic peptide in relation to the cardiac status of Rhesus monkeys after total-body irradiation. Radiotherapy and Oncology. 1999 Oct 1;53(1):67-75.

Wu L, Liu YJ. Development of dendritic-cell lineages. Immunity. 2007 Jun 22;26(6):741-50.

Wu T, Plett PA, Chua HL, Jacobsen M, Sandusky GE, MacVittie TJ, Orschell CM. Immune reconstitution and thymic involution in the acute and delayed hematopoietic radiation syndromes. Health physics. 2020 Nov;119(5):647.

Wu T, Orschell CM. The Delayed Effects of Acute Radiation Exposure (DEARE): Characteristics, mechanisms, animal models, and promising medical countermeasures. International Journal of Radiation Biology. 2023 Mar 15:1-4.

Xia HJ, Zhang GH, Wang RR, Zheng YT. The influence of age and sex on the cell counts of peripheral blood leukocyte subpopulations in Chinese rhesus macaques. Cellular & molecular immunology. 2009 Dec;6(6):433-40.

Xie L, Xu F, Liu S, Ji Y, Zhou Q, Wu Q, Gong W, Cheng K, Li J, Li L, Fang L. Age-and sex-based hematological and biochemical parameters for Macaca fascicularis. PloS one. 2013 Jun 10;8(6):e64892.

Yamada M, Lennie Wong F, Fujiwara S, Akahoshi M, Suzuki G. Noncancer disease incidence in atomic bomb survivors, 1958–1998. Radiation research. 2004 Jun 1;161(6):622-32.

Yamaoka M, Kusunoki Y, Kasagi F, Hayashi T, Nakachi K, Kyoizumi S. Decreases in percentages of naive CD4 and CD8 T cells and increases in percentages of memory CD8 T-cell subsets

in the peripheral blood lymphocyte populations of A-bomb survivors. Radiation research. 2004 Mar 1;161(3):290-8.

Yarnold J, Brotons MC. Pathogenetic mechanisms in radiation fibrosis. Radiotherapy and oncology. 2010 Oct 1;97(1):149-61.

Yarilin AA, Belyakov IM, Kusmenok OI, Arshinov VY, Simonova AV, Nadezhina NM, Gnezditskaya EV. Late T cell deficiency in victims of the Chernobyl radiation accident: possible mechanisms of induction. International journal of radiation biology. 1993 Jan 1;63(4):519-28.

Yoshida K, French B, Yoshida N, Hida A, Ohishi W, Kusunoki Y. Radiation exposure and longitudinal changes in peripheral monocytes over 50 years: the Adult Health Study of atomic bomb survivors. British Journal of Haematology. 2019 Apr;185(1):107-15.

Yoshimura A. Signal transduction of inflammatory cytokines and tumor development. Cancer science. 2006 Jun;97(6):439-47.

Yu KR, Espinoza DA, Wu C, Truitt L, Shin TH, Chen S, Fan X, Yabe IM, Panch S, Hong SG, Koelle S. The impact of aging on primate hematopoiesis as interrogated by clonal tracking. Blood, The Journal of the American Society of Hematology. 2018 Mar 15;131(11):1195-205.

Yu W, Hao X, Yang F, Ma J, Zhao Y, Li Y, Wang J, Xu H, Chen L, Liu Q, Duan S. Hematological and biochemical parameters for Chinese rhesus macaque. PLoS One. 2019 Sep 17;14(9):e0222338.

Yura Y, Miura-Yura E, Katanasaka Y, Min KD, Chavkin N, Polizio AH, Ogawa H, Horitani K, Doviak H, Evans MA, Sano M. The cancer therapy-related clonal hematopoiesis driver

gene Ppm1d promotes inflammation and non-ischemic heart failure in mice. Circulation research. 2021 Sep 3;129(6):684-98.

Zahorec R. Neutrophil-to-lymphocyte ratio, past, present and future perspectives. Bratisl Lek Listy. 2021 Jan 1;122(7):474-88.

Zhang W, Muirhead CR, Hunter N. Age-at-exposure effects on risk estimates for non-cancer mortality in the Japanese atomic bomb survivors. Journal of Radiological Protection. 2005 Dec 6;25(4):393.

Zierhut D, Lohr F, Schraube P, Huber P, Wenz F, Haas R, Fehrentz D, Flentje M, Hunstein W, Wannenmacher M. Cataract incidence after total-body irradiation. International Journal of Radiation Oncology* Biology* Physics. 2000 Jan 1;46(1):131-5.

Appendix

A. List of Abbreviations

ARS acute radiation syndrome

BM bone marrow

CBC complete blood counts

CD cluster of differentiation

CH clonal hematopoiesis

CI confidential interval

CLP common lymphoid progenitor cells

CMP common myeloid progenitor cells

CVD cardiovascular disease

DC dendritic cells

DEARE delayed effect of acute radiation exposure

EMM estimated marginal mean

HSC hematopoietic stem cell

- LT-HSC long-term HSC
- ST-HSC short-term HSC

IL Interleukin

IFN interferon

IR ionizing radiation

LMM liner mixed model

MPP multipotent progenitor cell

NHP non-human primates

NLR neutrophil-to-lymphocyte ratio

RLEC radiation late effect study cohort

ROS reactive oxygen species

RT radiation therapy

RM rhesus macaques

SEC sinusoidal endothelial cells

SD standard deviation

TBI total body irradiation

TNF tumor necrotic factor

T2DM type 2 diabetes mellitus

WBC white blood cell

WBI whole body irradiation

B. Colorado State University IACUC inter-institutional agreement

Colorado State University
Institutional Animal Care and Use Committee
Inter-Institutional Agreement
(CSU IACUC IIA)

This form should be completed when animal work will take place at an institution other than Colorado State University (CSU) and:

- **CSU is funding animal use activity or purchasing live vertebrate animals[1]—either directly or through a subcontract or subaward**
- **Or CSU representatives are directly participating in the work involving animals.**

Such collaboration has the potential to cause uncertainty involving animal use. Therefore, this form is designed to serve as a written agreement between CSU and the Collaborating Institution addressing the responsibility for animal care and use, animal ownership, and IACUC[2] review and oversight.

Please complete the applicable fields on this document and email the partially completed agreement and required attachments to: *RICRO_IACUC@mail.colostate.edu*

Name of CSU Investigator: Thomas E. Johnson
Name of Collaborating Institution Point of Contact: J. Mark Cline
Name of Collaborating Institution Providing IACUC Oversight: Wake Forest School of Medicine
Collaborating Institution USDA Registration # (as applicable): 825
Collaborating Institution PHS Animal Welfare Assurance# (as applicable): D16-00248(A3391-01)
Collaborating Institution AAALAC Accreditation Status (as applicable): Accredited September 13, 2017 Accredited #00008
The Officials signing below agree that Colorado State University (CSU) will rely on the designated IACUC of Wake Forest School of Medicine for the review and continuing oversight of its use of animals for the project described below:

This agreement covers the following specific IACUC protocol(s) at the *Collaborating Institution*:
Grant/Award/ Project Title: Radiation Countermeasures Long Term Response of Rhesus Macaques
Grant/Award/Project Principal Investigator: J. Mark Cline
Sponsor/Funding Agency (if any): NIH/NIAID
Sponsor Contract/Award Number (if any): U19 AI67798
IACUC Protocol[3] Principal Investigator: J. Mark Cline
IACUC Protocol Title: Radiation Countermeasures Long Term Response of Rhesus Macaques
IACUC Protocol Number: A19-028
IACUC Protocol Approval Date: Tuesday, October 22, 2019

[1] Animals are defined here as live vertebrate animals used for the purposes of research, teaching, and/or testing.
[2] IACUC or equivalent body that performs this review and oversight
[3] IACUC Protocol or equivalent document

Revised June 21, 2019

1

Please check all that apply:

☒ The animals involved in the activity will be owned by the Collaborating Institution.

☐ The animals involved in the activity will be owned by Colorado State University but housed at the Collaborating Institution.

☐ Free-ranging, wild animals will be used in their natural habitat.

☐ Other (please describe): Click here to enter text.

Please provide a brief description of the nature of the collaboration in the space below.

Peripheral blood smears that have been taken and since archived by the collaborating institution are useable for a radiation biomarker study that is being conducted at CSU. Only fixed tissue will be needed by researchers at CSU and no contact will be made directly with the Rhesus Macaques. In addition, the work that will be conducted at CSU with the samples provided by Wake Forest will be funded by NIOSH/CDC and not the NIH funding listed in the IACUC protocol.

Please attach *official documentation of approval by the Collaborating Institution IACUC* to this document.

Grant – Protocol Congruency

If funded by a subaward or subcontract on a US Public Health Service (PHS) sponsored project[4], the Collaborating Institution certifies the animal use as described in the protocol and grant are congruent:

☑ Yes ☐ No ☒ N/A

The CSU IACUC also requires that the Collaborating Institution provide, as applicable:

- Documentation of IACUC approval for modifications to the approved protocol as well as triennial reviews of the protocols;
- Prompt notification of review and reporting of[5]:
  - any unexpected/adverse events that occur during the conduct of the research activities associated with this IIA that directly impact animal welfare,
  - any incidents of noncompliance with PHS Policy, the *Guide for the Care and Use of Laboratory Animals*, or any suspension of this activity by the IACUC;
- CSU requires that the collaborating institution provide notification of change in PHS Assurance status or AAALAC, International Accreditation status;
- For institutions not located in the US, a copy of the approved animal care and use protocol.

CSU IACUC may review the attached documentation and determine if a duplicative review is warranted or this form is sufficient to ensure animal welfare.

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[4] PHS agencies: National Institutes of Health (NIH), Food and Drug Administration (FDA), Center for Disease Control and Prevention (CDC), Health and Human Services Biomedical Advanced Research and Development Authority (HHS BARDA), Veterans Affairs (VA), and National Science Foundation (NSF).

[5] Guidance on Prompt Reporting to OLAW under the PHS Policy on Humane Care and Use of Laboratory Animals: https://grants.nih.gov/grants/guide/notice-files/NOT-OD-05-034.html

CSU Signatures:

Signature of CSU Investigator — Date: 21 NOV 19

Thomas E. Johnson
Print Name of CSU Investigator

Signature of Signatory Official for CSU — Date: 12/20/19
Alan Rudolph, MBA, PhD[6]
CSU Vice President for Research and Institutional Official (IO)

Collaborating Institution Signatures:

Signature of Collaborating Institution IACUC Protocol PI — Date: 11/27/19

J. Mark Cline
Print Name of Collaborating Institution IACUC Protocol PI

Signature of Signatory Official for Collaborating Institution — Date: 12/17/19

Chris O'Byrne
Print Name of Signatory Official for Collaborating Institution

[6] Christa Johnson, PhD, Associate Vice President for Research, signature delegate for the IO.

C. *Diagnostic criteria for disease and conditions*

Organ/Disease	Diagnostic criteria
Testis	Testis volume <10ml each after 7 years of age
Ovary	Vaginal discharge, Uterine abnormality
Cataracts	Any lens opacity on annual slit-lamp exam
Kidney	Blood Urea Nitrogen (BUN) >30mg/dL or Serum creainine (Cr) >1.1 mg/dL Loss of renal volume >50%
Brain	MRI lesions visible on susceptivility weighted imaging (SWI)
Lung	Pulmonary consolidation on CT scan or emphysema >25%; percent blood oxygen saturation (SPO_2) <80%; Respiratory rate>80bpm
Hypertension	maximum arterial pressure (MAP) >120
Heart	Murmur detected on auscultation or echocardiography adult stroke volume <5ml and cardiac output (CO) <0.5L/min at >7y
Tumor	Any neoplastic disease by imaging or biopsy
Diabetes	Hemoglobin A1c >6.5% 3 fasting blood glucose measurements >100mg/dL any non-fasted blood glucose >200 mg/dL

D. *R codes*

Preliminary analysis

Setup

```
knitr::opts_chunk$set(echo = TRUE)
library(readxl)
library(ggplot2)
library(tidyverse)
library(lme4)
library(lmerTest)
library(pbkrtest)
library(emmeans)
library(dplyr)
library(scales)
Data_all <- read_excel("Data for R_manuscript_2.xlsx")

#change unit of abs neut/lymph/mono
Data_all$abs_neut=Data_all$abs_neut/1000
range(Data_all$abs_neut)
Data_all$abs_lymph=Data_all$abs_lymph/1000
range(Data_all$abs_lymph)
Data_all$abs_mono=Data_all$abs_mono/1000
range(Data_all$abs_mono)

#scale m_sample
Data_all$m_sample_s=scale(Data_all$m_sample)
```

```
#assign irradiation status as factor
Data_all$irr=as.factor(Data_all$irr)
```

Outlier elimination and dataset for each blood parameters

```
Data_wbc=cbind.data.frame(id=Data_all$id,irr=Data_all$irr, m_sample=Data_all
$m_sample,m_sample_s=Data_all$m_sample_s,wbc=Data_all$wbc)
lower_wbc <- quantile(Data_wbc$wbc, 0.01)
upper_wbc <- quantile(Data_wbc$wbc, 0.99)
Data_wbc= Data_wbc %>% filter(wbc<upper_wbc,wbc>lower_wbc)

Data_absneut=cbind.data.frame(id=Data_all$id,irr=Data_all$irr,m_sample=Data_a
ll$m_sample,m_sample_s=Data_all$m_sample_s,abs_neut=Data_all$abs_neut)
lower_absneut <- quantile(Data_absneut$abs_neut, 0.01)
upper_absneut <- quantile(Data_absneut$abs_neut, 0.99)
Data_absneut= Data_absneut %>% filter(abs_neut<upper_absneut,abs_neut>lower_a
bsneut)

Data_abslymph=cbind.data.frame(id=Data_all$id,irr=Data_all$irr,m_sample=Data_
all$m_sample,m_sample_s=Data_all$m_sample_s,abs_lymph=Data_all$abs_lymph)
lower_abslymph <- quantile(Data_abslymph$abs_lymph, 0.01)
upper_abslymph <- quantile(Data_abslymph$abs_lymph, 0.99)
Data_abslymph= Data_abslymph %>% filter(abs_lymph<upper_abslymph,abs_lymph>lo
wer_abslymph)

Data_absmono=cbind.data.frame(id=Data_all$id,irr=Data_all$irr,m_sample=Data_a
ll$m_sample,m_sample_s=Data_all$m_sample_s,abs_mono=Data_all$abs_mono)
lower_absmono <- quantile(Data_absmono$abs_mono, 0.01)
upper_absmono <- quantile(Data_absmono$abs_mono, 0.99)
Data_absmono= Data_absmono %>% filter(abs_mono<upper_absmono,abs_mono>lower_a
bsmono)

Data_neut=cbind.data.frame(id=Data_all$id,irr=Data_all$irr,m_sample_s=Data_al
l$m_sample_s,m_sample=Data_all$m_sample,neut=Data_all$neut)
lower_neut <- quantile(Data_neut$neut, 0.01)
upper_neut <- quantile(Data_neut$neut, 0.99)
Data_neut= Data_neut %>% filter(neut<upper_neut,neut>lower_neut)

Data_lymph=cbind.data.frame(id=Data_all$id,irr=Data_all$irr,m_sample=Data_all
$m_sample,m_sample_s=Data_all$m_sample_s, lymph=Data_all$lymph)
lower_lymph <- quantile(Data_lymph$lymph, 0.01)
upper_lymph <- quantile(Data_lymph$lymph, 0.99)
Data_lymph= Data_lymph %>% filter(lymph<upper_lymph,lymph>lower_lymph)

Data_mono=cbind.data.frame(id=Data_all$id,irr=Data_all$irr,m_sample=Data_all
$m_sample,m_sample_s=Data_all$m_sample_s, mono=Data_all$mono)
lower_mono <- quantile(Data_mono$mono, 0.01)
upper_mono <- quantile(Data_mono$mono, 0.99)
Data_mono= Data_mono %>% filter(mono<upper_mono,mono>lower_mono)
```

```
Data_NLR=cbind.data.frame(id=Data_all$id,irr=Data_all$irr,m_sample=Data_all$m
_sample,m_sample_s=Data_all$m_sample_s,NLR=Data_all$NLR)
lower_NLR <- quantile(Data_NLR$NLR, 0.01)
upper_NLR <- quantile(Data_NLR$NLR, 0.99)
Data_NLR= Data_NLR%>% filter(NLR<upper_NLR,NLR>lower_NLR)
```

Analysis

```
## Leukocyte count
#linear mixed model
lmm_wbc<- lmer(log(wbc) ~m_sample_s*factor(irr)+(1+m_sample_s|id)+0,data = Da
ta_wbc, control=lmerControl(optimizer="bobyqa"))
plot(lmm_wbc)
summary(lmm_wbc)
#estimated trends
emtrends(lmm_wbc, pairwise ~ irr,
         var = "m_sample_s")
test(emtrends(lmm_wbc, pairwise ~ irr,
         var = "m_sample_s") )
#estimated means
emmeans(lmm_wbc, pairwise ~ irr | m_sample_s)
pairs(emmeans(lmm_wbc, pairwise ~ irr | m_sample_s))

## Absolute neutrophil count
#linear mixed model
lmm_absneut<- lmer(log(abs_neut) ~m_sample_s*factor(irr)+(1+m_sample_s|id)+0,
data = Data_absneut, control=lmerControl(optimizer="bobyqa"))
plot(lmm_absneut)
summary(lmm_absneut)
#estimated trends
emtrends(lmm_absneut, pairwise ~ irr,
         var = "m_sample_s")
test(emtrends(lmm_absneut, pairwise ~ irr,
         var = "m_sample_s") )
#estimated means
emmeans(lmm_absneut, pairwise ~ irr | m_sample_s)
pairs(emmeans(lmm_absneut, pairwise ~ irr | m_sample_s))

##Absolute lymphocyte count
#linear mixed model
lmm_abslymph<- lmer(log(abs_lymph) ~m_sample_s*factor(irr)+(1+m_sample_s|id)+
0,data = Data_abslymph, control=lmerControl(optimizer="bobyqa"))
plot(lmm_abslymph)
summary(lmm_abslymph)
#estimated trends
emtrends(lmm_abslymph, pairwise ~ irr,
         var = "m_sample_s")
test(emtrends(lmm_abslymph, pairwise ~ irr,
         var = "m_sample_s") )
#estimated means
emmeans(lmm_abslymph, pairwise ~ irr | m_sample_s)
```

```
pairs(emmeans(lmm_abslymph, pairwise ~ irr | m_sample_s))

## Absolute monocyte count
#linear mixed model
lmm_absmono<- lmer(log(abs_mono) ~m_sample_s*factor(irr)+(1+m_sample_s|id)+0,
data = Data_absmono, control=lmerControl(optimizer="bobyqa"))
plot(lmm_absmono)
summary(lmm_absmono)
#estimated trends
emtrends(lmm_absmono, pairwise ~ irr,
         var = "m_sample_s")
test(emtrends(lmm_absmono, pairwise ~ irr,
         var = "m_sample_s") )
#estimated means
emmeans(lmm_absmono, pairwise ~ irr | m_sample_s)
pairs(emmeans(lmm_absmono, pairwise ~ irr | m_sample_s))

##Neutrophil %
#linear mixed model
lmm_neut<- lmer(neut ~m_sample_s*factor(irr)+(1+m_sample_s|id)+0,data = Data_
neut, control=lmerControl(optimizer="bobyqa"))
plot(lmm_neut)
summary(lmm_neut)
#estimated trends
emtrends(lmm_neut, pairwise ~ irr,
         var = "m_sample_s")
test(emtrends(lmm_neut, pairwise ~ irr,
         var = "m_sample_s") )
#estimated means
emmeans(lmm_neut, pairwise ~ irr | m_sample_s)
pairs(emmeans(lmm_neut, pairwise ~ irr | m_sample_s))

## Lymphocyte %
#linear mixed model
lmm_lymph<- lmer(log(lymph) ~m_sample_s*factor(irr)+(1+m_sample_s|id)+0,data
= Data_lymph, control=lmerControl(optimizer="bobyqa"))
plot(lmm_lymph)
summary(lmm_lymph)
#estimated trends
emtrends(lmm_lymph, pairwise ~ irr,
         var = "m_sample_s")
test(emtrends(lmm_lymph, pairwise ~ irr,
         var = "m_sample_s") )
#estimated means
emmeans(lmm_lymph, pairwise ~ irr | m_sample_s)
pairs(emmeans(lmm_lymph, pairwise ~ irr | m_sample_s))

## Monocyte %
#linear mixed model
lmm_mono<- lmer(mono ~m_sample_s*factor(irr)+(1+m_sample_s|id)+0,data = Data_
```

```
mono, control=lmerControl(optimizer="bobyqa"))
plot(lmm_mono)
summary(lmm_mono)
#estimated trends
emtrends(lmm_mono, pairwise ~ irr,
         var = "m_sample_s")
test(emtrends(lmm_mono, pairwise ~ irr,
         var = "m_sample_s") )
#estimated means
emmeans(lmm_mono, pairwise ~ irr | m_sample_s)
pairs(emmeans(lmm_mono, pairwise ~ irr | m_sample_s))

## NLR
#linear mixed model
lmm_NLR<- lmer(log(NLR) ~m_sample_s*factor(irr)+(1+m_sample_s|id)+0,data = Da
ta_NLR, control=lmerControl(optimizer="bobyqa"))
plot(lmm_NLR)
summary(lmm_NLR)
#estimated trends
emtrends(lmm_NLR, pairwise ~ irr,
         var = "m_sample_s")
test(emtrends(lmm_NLR, pairwise ~ irr,
         var = "m_sample_s") )
#estimated means
emmeans(lmm_NLR, pairwise ~ irr | m_sample_s)
pairs(emmeans(lmm_NLR, pairwise ~ irr | m_sample_s))
```

Analysis part 2

Setup, Data processing

```
knitr::opts_chunk$set(echo = TRUE)
library(readxl)
library(ggplot2)
library(tidyverse)
library(lme4)
library(lmerTest)
library(pbkrtest)
library(emmeans)
library(dplyr)
library(scales)
library(ggeffects)
library(gtools)
Data_all <- read_excel("Data for R_manuscript_3.xlsx")

#change unit of abs neut/lymph/mono
Data_all$abs_neut=Data_all$abs_neut/1000
Data_all$abs_lymph=Data_all$abs_lymph/1000
Data_all$abs_mono=Data_all$abs_mono/1000

#transform id, sex, miti to factor
Data_all$id=as.factor(Data_all$id)
```

```
Data_all$sex=factor(Data_all$sex,levels = c("M", "F"))
Data_all$miti=as.factor(Data_all$miti)

#scale m_sample
Data_all$m_sample_s=scale(Data_all$m_sample)

#add dose level factor
fun1<-function(x){
  if(x>=5.5){
    if(x<=6.8){ans="2"}
    else{ans="3"}
  }
  else if (x>0){ans="1"}
  else {ans="0"}}
Data_all$dlevel<-sapply(Data_all$dose,fun1)
Data_all$dlevel=as.factor(Data_all$dlevel)

#add age at the time of exposure
#control animals don't have age at radiation, so impute as first observed age
tmp = by(Data_all[,c("id", "m_sample")], Data_all[,"id"], function(x)
    {
    x[which.min(x$m_sample),]
    })
tmp = data.frame(do.call("rbind", tmp)); names(tmp) = c("id", "t1")
Data_all = merge(Data_all, tmp, by = "id", all.x = T)
Data_all$age1 = Data_all$m_airrad
Data_all[which(is.na(Data_all$age1)), "age1"]
  = Data_all[which(is.na(Data_all$age1)), "t1"]
```

Analysis

```
## leukocyte count
# linear mixed model
lmm_wbc_1<- lmer(log(wbc) ~m_sample_s*dlevel+sex+miti+age1+(1+m_sample_s|id),
                 data = Data_all,REML = T)
plot(lmm_wbc_1) #check residual distribution
summary(lmm_wbc_1) #get estimates and p-values
## check significance of interaction (m_sample*dlevel)
lmm_wbc_1a<- lmer(log(wbc) ~m_sample_s*dlevel+sex+miti+age1+(1+m_sample_s|i
d),
                  data = Data_all, REML = F)
lmm_wbc_1b<- lmer(log(wbc) ~m_sample_s+dlevel+sex+miti+age1+(1+m_sample_s|i
d),
                  data = Data_all, REML = F)
anova(lmm_wbc_1a,lmm_wbc_1b)

## absolute neutrophil count
# linear mixed model
lmm_absneut_1<- lmer(log(abs_neut+1) ~m_sample_s*dlevel+sex+miti+age1+(1+m_sa
mple_s|id),data = Data_all)
plot(lmm_absneut_1)
```

```
summary(lmm_absneut_1)
## check the significance of interaction (m_sample*dlevel)
lmm_absneut_1a<- lmer(log(abs_neut+1) ~m_sample_s*dlevel+sex+miti+age1+
                        (1+m_sample_s|id), data = Data_all,REML = F)
lmm_absneut_1b<- lmer(log(abs_neut+1) ~m_sample_s+dlevel+sex+miti+age1+
                        (1+m_sample_s|id), data = Data_all,REML = F)
anova(lmm_absneut_1a,lmm_absneut_1b)
## check the significance of interaction (m_sample*dlevel*sex)
lmm_absneut_1.5a<- lmer(log(abs_neut+1) ~m_sample_s*dlevel*sex+miti+age1+
                          (1+m_sample_s|id),data = Data_all, REML = F)
lmm_absneut_1.5b<- lmer(log(abs_neut+1) ~m_sample_s*dlevel+sex+miti+age1+
                          (1+m_sample_s|id),data = Data_all,REML = F)
anova(lmm_absneut_1.5a,lmm_absneut_1.5b)

## absolute lymphocyte count
# linear mixed model
lmm_abslymph_1<- lmer(log(abs_lymph) ~m_sample_s*dlevel+sex+miti+age1+(1+m_sa
mple_s|id),data = Data_all)
plot(lmm_abslymph_1)
summary(lmm_abslymph_1)
# check the significance of interaction (m_sample*dlevel)
lmm_abslymph_1a<- lmer(log(abs_lymph) ~m_sample_s*dlevel+sex+miti+age1+
                         (1+m_sample_s|id),data = Data_all,REML=F)
lmm_abslymph_1b<- lmer(log(abs_lymph) ~m_sample_s+dlevel+sex+miti+age1+
                         (1+m_sample_s|id),data = Data_all,REML=F)
anova(lmm_abslymph_1a,lmm_abslymph_1b)
# check the significance of interaction (m_sample*dlevel*sex)
lmm_abslymph_1.5a<- lmer(log(abs_lymph) ~m_sample_s*dlevel*sex+miti+age1+
                           (1+m_sample_s|id),data = Data_all,REML=F)
lmm_abslymph_1.5b<- lmer(log(abs_lymph) ~m_sample_s*dlevel+sex+miti+age1+
                           (1+m_sample_s|id),data = Data_all,REML=F)
anova(lmm_abslymph_1.5a, lmm_abslymph_1.5b)

## absolute monocyte count
# linear mixed model
lmm_absmono_1<- lmer(log(abs_mono+1) ~m_sample_s*dlevel+sex+miti+age1+(1+m_sa
mple_s|id),data = Data_all)
plot(lmm_absmono_1)
summary(lmm_absmono_1)
# check the significance of interaction (m_sample*dlevel)
lmm_absmono_1a<- lmer(log(abs_mono+1) ~m_sample_s*dlevel+sex+miti+age1+
                        (1+m_sample_s|id), data = Data_all,REML = F)
lmm_absmono_1b<- lmer(log(abs_mono+1) ~m_sample_s+dlevel+sex+miti+age1+
                        (1+m_sample_s|id), data = Data_all,REML = F)
anova(lmm_absmono_1a,lmm_absmono_1b)
# check the significance of interaction (m_sample*dlevel*age1)
lmm_absmono_1.5a<- lmer(log(abs_mono+1)~m_sample_s*dlevel*age1+sex+miti+
                          (1+m_sample_s|id),data = Data_all,REML = F)
lmm_absmono_1.5b<- lmer(log(abs_mono+1) ~m_sample_s*dlevel+age1+sex+miti+
                          (1+m_sample_s|id),data = Data_all,REML = F)
```

```
anova(lmm_absmono_1.5a,lmm_absmono_1.5b)

## neutrophil%
# linear mixed model
lmm_neut_1<- lmer(neut ~m_sample_s*dlevel+sex+miti+age1+
                    (1+m_sample_s|id),data = Data_all)
plot(lmm_neut_1)
summary(lmm_neut_1)
# check the significance of interaction (m_sample*dlevel)
lmm_neut_1a<- lmer(neut~m_sample_s*dlevel+sex+miti+age1
                   +(1+m_sample_s|id),data = Data_all,REML = F)
lmm_neut_1b<- lmer(neut~m_sample_s+dlevel+sex+miti+age1
                   +(1+m_sample_s|id),data = Data_all,REML = F)
anova(lmm_neut_1a,lmm_neut_1b)
# check the significance of interaction (m_sample*dlevel*sex)
lmm_neut_1.5a<- lmer(neut ~m_sample_s*dlevel*sex+miti+age1+
                       (1+m_sample_s|id),data = Data_all, REML = F)
lmm_neut_1.5b<- lmer(neut ~m_sample_s*dlevel+sex+miti+age1+
                       (1+m_sample_s|id),data = Data_all,REML = F)
anova(lmm_neut_1.5a,lmm_neut_1.5b)

## lymphocyte%
# linear mixed model
lmm_lymph_1<- lmer(lymph ~m_sample_s*dlevel+sex+miti+age1+
                     (1+m_sample_s|id),data = Data_all,REML=T)
plot(lmm_lymph_1)
summary(lmm_lymph_1)
# check the significance of interaction (m_sample*dlevel)
lmm_lymph_1a<- lmer(lymph ~m_sample_s*dlevel+sex+miti+age1+
                      (1+m_sample_s|id),data = Data_all,REML=F)
lmm_lymph_1b<- lmer(lymph ~m_sample_s+dlevel+sex+miti+age1+
                      (1+m_sample_s|id),data = Data_all,REML=F)
anova(lmm_lymph_1a,lmm_lymph_1b)
# check the significance of interaction (m_sample*dlevel*sex)
lmm_lymph_1.5a<- lmer(lymph ~m_sample_s*dlevel*sex+miti+age1+
                        (1+m_sample_s|id),data = Data_all,REML=F)
lmm_lymph_1.5b<- lmer(lymph ~m_sample_s*dlevel+sex+miti+age1+
                        (1+m_sample_s|id),data = Data_all,REML=F)
anova(lmm_lymph_1.5a, lmm_lymph_1.5b)

## monocyte%
# linear mixed model
lmm_mono_1<- lmer(mono ~m_sample_s*dlevel+sex+miti+age1+
                    (1+m_sample_s|id),data = Data_all)
plot(lmm_mono_1)
summary(lmm_mono_1)
# check the significance of interaction (m_sample*dlevel)
lmm_mono_1a<- lmer(mono ~m_sample_s*dlevel+sex+miti+age1+
                     (1+m_sample_s|id),data = Data_all,REML = F)
lmm_mono_1b<- lmer(mono ~m_sample_s+dlevel+sex+miti+age1+
```

```
                    (1+m_sample_s|id),data = Data_all,REML = F)
anova(lmm_mono_1a,lmm_mono_1b)
# check the significance of interaction (m_sample*dlevel*age1)
lmm_mono_1.5a<- lmer(mono~m_sample_s*dlevel*age1+sex+miti+
                       (1+m_sample_s|id),data = Data_all,REML = F )
lmm_mono_1.5b<- lmer(mono ~m_sample_s*dlevel+age1+sex+miti+
                       (1+m_sample_s|id),data = Data_all,REML = F)
anova(lmm_mono_1.5a,lmm_mono_1.5b)

## NLR
# linear mixed model
lmm_nlr_1<- lmer(log(NLR+1) ~m_sample_s*dlevel+sex+miti+age1+
                   (1+m_sample_s|id),data = Data_all)
plot(lmm_nlr_1)
summary(lmm_nlr_1)
# check the significance of interaction (m_sample*dlevel)
lmm_nlr_1a<- lmer(log(NLR+1) ~m_sample_s*dlevel+sex+miti+age1+
                    (1+m_sample_s|id),data = Data_all,REML = F)
lmm_nlr_1b<- lmer(log(NLR+1) ~m_sample_s+dlevel+sex+miti+age1+
                    (1+m_sample_s|id),data = Data_all,REML = F)
anova(lmm_nlr_1a,lmm_nlr_1b)
# check the significance of interaction (m_sample*dlevel*sex)
lmm_nlr_1.5a<- lmer(log(NLR+1) ~m_sample_s*dlevel*sex+miti+age1+
                      (1+m_sample_s|id),data = Data_all, REML = F)
lmm_nlr_1.5b<- lmer(log(NLR+1) ~m_sample_s*dlevel+sex+miti+age1+
                      (1+m_sample_s|id),data = Data_all,REML = F)
anova(lmm_nlr_1.5a,lmm_nlr_1.5b)
```