

AWARD NUMBER: W81XWH-15-1-0574

TITLE: Long-Term Followup of the Delayed Effects of Acute Radiation Exposure in Primates

PRINCIPAL INVESTIGATOR: J Mark Cline, DVM, PhD

CONTRACTING ORGANIZATION: Wake Forest University Health Sciences, Winston-Salem, NC

REPORT DATE: December 2021

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE December 2021		2. REPORT TYPE Final		3. DATES COVERED 30Sep2015-29Sep2021	
4. TITLE AND SUBTITLE Long-Term Followup of the Delayed Effects of Acute Radiation Exposure in Primates				5a. CONTRACT NUMBER PR141508	
				5b. GRANT NUMBER W81XWH-15-1-0574	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) J. Mark Cline, DVM, PhD E-Mail: jmcline@wakehealth.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Wake Forest University Health Sciences Medical Center Blvd Winston-Salem, NC 27157				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT This program studies the mechanisms and consequences of delayed effects of acute radiation exposure (DEARE) in non-human primates (NHP), with complementary mechanistic and therapeutic studies in mice. The program consists of 4 projects, focused on: 1. Type 2 diabetes mellitus (T2DM); 2. Radiation-induced heart disease (RIHD); 3. Chronic immune impairment; and 4. Genomics and transcriptomics. Major findings include peripheral microvascular and bioenergetic abnormalities in NHP with T2DM; restrictive myocardial fibrosis in RIHD; dose and age-dependent immune impairment; and ongoing sequencing work which will inform us regarding the molecular pathogenesis of T2DM, RIHD, and immune impairment in the context of DEARE.					
15. SUBJECT TERMS Radiation; DEARE; heart; fat; skeletal muscle; immune system; diabetes; insulin resistance; genomics; transcriptomics; <i>Mus musculus</i> ; <i>Macaca mulatta</i> ; T lymphocytes; immune recovery; thymopoiesis					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code) USAMRDC
			Unclassified	103	

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	5
4. Impact	90
5. Changes/Problems	91
6. Products	96
7. Participants & Other Collaborating Organizations	98
8. Special Reporting Requirements	102
9. Appendices	102

1. INTRODUCTION

The **subject** of this research is the phenomenon of Delayed Effects of Acute Radiation Exposure (DEARE). The **purpose** and overarching goal of this program is to explore our recent clinically relevant observations of DEARE in a unique cohort of non-human primates (NHP), termed the Radiation Survivor Cohort (RSC), and a smaller more controlled group of NHP exposed to radiation prospectively, termed the Prospective Radiation Cohort (PRC), with complementary studies in mouse models to explore growth factor therapeutics and genomic consequences. The **scope** of this work consists of 4 projects making use of these unique NHP resources: 1. Studies of type 2 diabetes mellitus (T2DM); 2. Radiation-induced heart disease (RIHD); 3. Chronic immune impairment with restriction of the antigenic response repertoire; and 4. Genomic and transcriptomic studies which will inform us regarding the molecular pathogenesis of T2DM, RIHD, and immune impairment in the context of DEARE. Work during the first four years of this program has proceeded as planned, with slight logistical modifications but no major changes or delays.

2. KEYWORDS

Radiation; DEARE; heart; fat; skeletal muscle; immune system; diabetes; insulin resistance; genomics; transcriptomics; *Mus musculus*; *Macaca mulatta*; T lymphocytes; immune recovery; thymopoiesis

3. ACCOMPLISHMENTS

What were the major goals of the project?

Core Primate Studies (Cline)

SA1: Provide NHP Investigative Platform

Major Task 1: Regulatory Approval (months 1-6)

Milestone: Local and ACURO approvals in place (100%)

Major Task 2: Access to Long Term Radiation Survivor Cohort (months 1-6)

Milestone: Scheduling and meetings in progress (100%)

Sample collections continue (planned for months 6-60; 100% complete)

Major Task 3: Acquisition of Prospective Cohort (months 1-6)

Milestone: Animals arrived at Wake Forest 2/9/16 (100% completed).

Major Task 4: Irradiation and Study of Prospective Cohort

Milestone: Irradiations 100% completed in Y2Q1; follow-up complete 100%.

SA2: Promote Synergy and Productivity

Major Task 5: Data management/tracking, group interactions (months 1-6)

Milestones: Data management (100% complete)

Establish monthly web meetings (100% complete)

Annual strategic retreat (100% complete)

Publications (months 6-60; 100% complete)

SA3: Facilitate Translation and Therapeutic/Preventive approaches

Major Task 6: Strategize towards clinical implementation and future funding

Milestones: Promote new grant submissions (months 36-60; 100% complete)

Progress update at end of year 2 (100% complete)

Project 1: Diabetes (Kavanagh)

SA1: Document the time course of IR-mediated reduced insulin sensitivity

Major Task 1: Prospective IR cohort

Milestones: Create a longitudinal clinical profile of DM risk with IR (0% achieved)

Local IACUC approval (100% achieved)

HRPO/ACURO Approval (100% achieved)

CT/DEXA imaging (100% achieved)

Characterization of changes in skeletal muscle structure and function (100% achieved)

Characterization of changes in adipose structure and function (100% achieved)

Major Task 2: Long-term IR Cohort

Milestones: Generate comparable clinical data in monkeys with DM as DEARE to compare with the earlier prospectively IR monkeys (100% achieved)

Insulin sensitivity data collection (100% achieved)

SA2: Examine IR-related increases in oxidation of tissue proteins over time

Major Task 3: Redox Proteomics of prospective and long-term IR tissue

Milestones: Compare and contrast early and late changes in redox proteomics following IR in mice and monkeys. Verify the role of Akt protein in DM. Identify individual or protein clusters with long-term modifications following IR. (100% achieved)

SA3: Track ECM remodelling, including fibrosis, in muscle and adipose depots after IR

Major Task 4: ECM characterization and MRI imaging

Milestones: Characterize tissue changes in both prospective and long-term IR monkeys and relate these to in vivo insulin sensitivity assessments. Validate the utility of MR imaging to detect qualitative tissue changes (100% achieved)

SA4: Evaluate therapeutic agents that reduce fibrosis and restore fat expansion to mitigate metabolic changes with IR.

Major Task 5: Mouse intervention study

Milestones: Prevent metabolic disease development in IR mice with interventions. Demonstrate reductions in protein modifications and/or tissue remodelling with treatment (100% achieved).

Project 2: Radiation-Induced Heart Disease (Register)

We proposed 3 inter-related Specific Aims across the 5 Year Grant Period.

SA 1: Determine the Effects of Total Body Irradiation on NHP Cardiac Structure and Function.

Major Task 1: Novel cardiac MRI approaches will be used to evaluate myocardial tissue fibrosis, a key component of RIHD, in order to follow changes in myocardial phenotypes in animals across time. Additional structural and functional cardiac phenotypes to be assessed by echocardiography/MRI include left ventricular (LV) mass, posterior and interventricular wall thickness, right ventricular thickness, and aortic wall thickness. Functional parameters include systolic and diastolic function, ejection fraction, aortic stiffness/pulse wave velocity, wall motion, and electrocardiography. Grid tag mapping is being used to assess strain and LV volume.

Scheduled Dates: Months 6-54

Milestones: Percent completion: 95% in the context of 5-year plan

Detailed cardiac ultrasound (US) evaluations were conducted and images acquired on the RSC (year 01, year 02, year 03, year 04, year 05) and on the PRC (2016 baseline, 20 weeks (March 2017), 1 year (October 2017), 2 years (October 2019) with the final scans at the 3.25 year timepoint (January 2020). Echocardiographic image analysis was conducted using the scanner for images collected in the RSC and PRC using US image analysis software (TOMTEC) which we purchased for the project. Drs. Register and staff were trained in its use. Data from baseline which was collected and measured on the GE instrument were validated for reproducibility by repeat reads using the TOMTEC software. M Mode measurements were assessed on the GE US instrument with ongoing plans to move those analyses to the TOMTEC. Cardiac MRI (cMRI) image analysis has been completed by skilled technicians with extensive epidemiologic experience. New software was purchased and staff trained in cMRI measures. Tissue collections from RSC subjects going to necropsy and from PRC subjects were completed. We carefully evaluated characteristics of hearts from RSC monkeys that have come to necropsy, including assessments of 1) coronary artery atherosclerosis, 2) myocardial fibrosis (Trichrome), myocardial cell populations (endothelial cells, fibroblasts, and macrophage subpopulations, 3) myocardial gene expression using RNAseq (global) and Nanostring technology (immune targeted); 4) myocardial small RNA levels using miRNAseq (global) and Nanostring miRNA (targeted) profiling (underway). Ongoing studies are further characterizing cellular phenotypes in myocardium from the PRC subjects. We continue to conducting the same studies in the hearts obtained from the PRC which went to necropsy in June 2020 after several months' delay due to the COVID-19 pandemic. Remaining analyses will be supported by other funding.

SA 2: Identify Novel Molecular Biomarkers of Cardiovascular Injury and RIHD

Major Task 2: Identify and evaluate emerging serum markers of cardiac damage implicated in cardiomyocyte death (microRNAs such as miR133a) and extracellular matrix remodeling (type I and III collagen propeptides and degradation products). Circulating miRNA levels and serum extracellular matrix turnover biomarkers are being evaluated in relation to structural and functional phenotypes and histologic and biomolecular features of myocardial tissues and differentially expressed myocardial mRNA and miRNA which will be identified through expression profiling of RSC and PRC heart tissues obtained at necropsy. Two platforms for miRNA analysis were tested and data have been analyzed for the RSC and analysis is underway for the PRC.

Scheduled Dates: Months 6-60

Milestones: Percent completion: 85%

RSC: blood samples obtained and processed; PRC: blood samples collected, processed, and archived for future biochemical analyses. Molecular and cellular profiling of PRC biomarkers is in its final stages. Remaining analyses will be supported by other funding.

SA 3: Determine the Efficacy of Candidate Mitigating Agents on RIHD.

Major Task 3: Assessment of Mitigating Agents. We propose to retrospectively and prospectively assess cardiac and coronary artery injury in NHP in studies of mitigating agents, using animals in the de novo cohort, the long-term cohort, and from prospective studies of growth factors (e.g. HGH), superoxide dismutase mimetics, and novel therapeutics (e.g. platelet analogs). We will use the pathologic and biomarker approaches noted above, including in vivo assessments. These results will be critical to our understanding of RIHD.

Scheduled Dates: Months 6-60

Milestones: Percent completion: 95% complete in the context of a 5-year plan (assessed effects of a mitigating agent (superoxide dismutase analogue, hexyl MnSOD) on myocardial gene expression, (manuscript in development), performed histologic staining of coronary arteries in available hearts and completed assessments of atherosclerotic lesion size, collected cardiac tissues from a Project 1 mouse study and cardiac tissue from rhesus monkeys. Myocardium from the selected studies from the RSC have been phenotyped for atherosclerosis. Remaining analyses will be supported by other funding.

Project 3: Immunological Injury and Recovery (Chen/Sempowski)

SA 1: Define the roles of thymopoiesis and peripheral expansion in overall T cell reconstitution after radiation-induced injury based on radiation dose.

Major Task 1: NHP Studies

Goals: 1.1. Phenotypic analyses in long-term cohort (100% complete)
1.2. sjTREC in long-term cohort (100% complete)
1.3. Phenotypic analyses in prospective cohort (100% complete)
1.4. sjTREC in prospective cohort (100% complete)
1.5. Determine primary immune response to influenza and pneumovax vaccines (100% complete)
1.6. Determine recall immune response to vaccines (100% complete)

Major Task 2: Murine Studies

Goals: 2.1. Local IACUC Approval (100% complete)
2.2. Determine the roles of thymopoiesis and peripheral expansion in overall T cell recovery in the classic radiation injury model (100% complete)

2.3. Determine the roles of thymopoiesis and peripheral expansion in overall T cell recovery in the second radiation injury model (100% complete)

SA 2: Determine if therapeutic agents identified in our previous studies are able to promote or accelerate overall T cell immunity after radiation injury.

Major Tasks 3 & 4: Mitigator Studies

Goals: 3.1 & 4.1. Determine the effect of HGH/IGF-1/IL-7 on phenotypic T cell reconstitution (100% complete)
3.2 & 4.2. Determine the effect of HGH/IGF-1/IL-7 on functional T cell recovery (50% complete)
3.3 & 4.3. Determine the mechanism by which HGH/IGF-1/IL-7 promotes T cell reconstitution (49-60 months, 100% complete)

Project 4: Genomic Sequencing and Stem Cell Lines (Dave)

Aim 1: Define the genetic mutations and gene expression signatures induced by DEARE

Major Task 1: Exome Sequencing

Milestones:

DNA Sample extraction (months 1-36) - 100% complete

Exome analysis (months 13-60) - 100% complete

Major Task 2: Transcriptome Sequencing

RNAseq (months 6-48) - 100% complete

RNAseq analysis (months 12-60) - 100% complete

Aim 2: Determine the molecular basis of lymphoid immunodeficiency induced by DEARE.

Major Task 1: Generate iPSCs

Milestones: iPSC from controls complete 100% complete

Milestones: iPSC from genetic variants 100% complete

Major Task 2: Differentiate iPSCs into lymphoid lineages

Milestone(s) Achieved: B cell characterization 100% complete

Milestone(s) Achieved: T cell characterization 100% complete

What was accomplished under these goals?

Core Primate Studies (Cline)

1) Major activities:

Monthly review of goals and plans with all investigators; allocation of effort for personnel; physical examinations, imaging, and blood sampling for all projects.

Characteristics of our animal populations are briefly described below.

2) Specific objectives:

As per SOW. Regulatory approval maintenance, monthly meetings, planning, writing.

3) Results/key outcomes:

- Goals and plans reviewed with all investigators
- Monthly web conferences continuing
- Secure server folders established for data exchange
- Sampling and data acquisition on schedule

4) Other achievements:

A brief overview of the resource and synopsis of major findings is presented below.

The Radiation Survivor Cohort (RSC)

The population of the RSC is shown graphically in Figure 1. Briefly, this cohort consists of rhesus macaques (*Macaca mulatta*) irradiated as juveniles or adults, in the range of 1.14-8.5 Gy via single total body exposure, at a dose delivery of 60-80 cGy/min. The cohort is supported by the National Institute of Allergy and Infectious Disease (NIAID), which is leveraged by this DOD Focused Program award. Animals were acquired between 2007 and 2020 through an “adoption” strategy from multiple sites. The RSC currently consists of 144 irradiated animals and 37 controls (corresponding numbers for all animals ever in the cohort are 184 irradiated and 51 controls). The population has a 2:1 male:female ratio. Animals are housed socially whenever possible, primarily in pens, and fed a custom-formulated “Typical American Diet”. All animals receive daily environmental enrichment and are part of a behavioral management program.

Approximately one-third of the animals were given mitigating agents (e.g. antibiotics or growth factors) during the acute period of hematopoietic injury. This cohort is studied using a multidisciplinary approach to long-term morbidity/mortality. Notably, some animals are now 14 years out from irradiation (1/2 of their lifespan), providing a unique long-term view of DEARE.

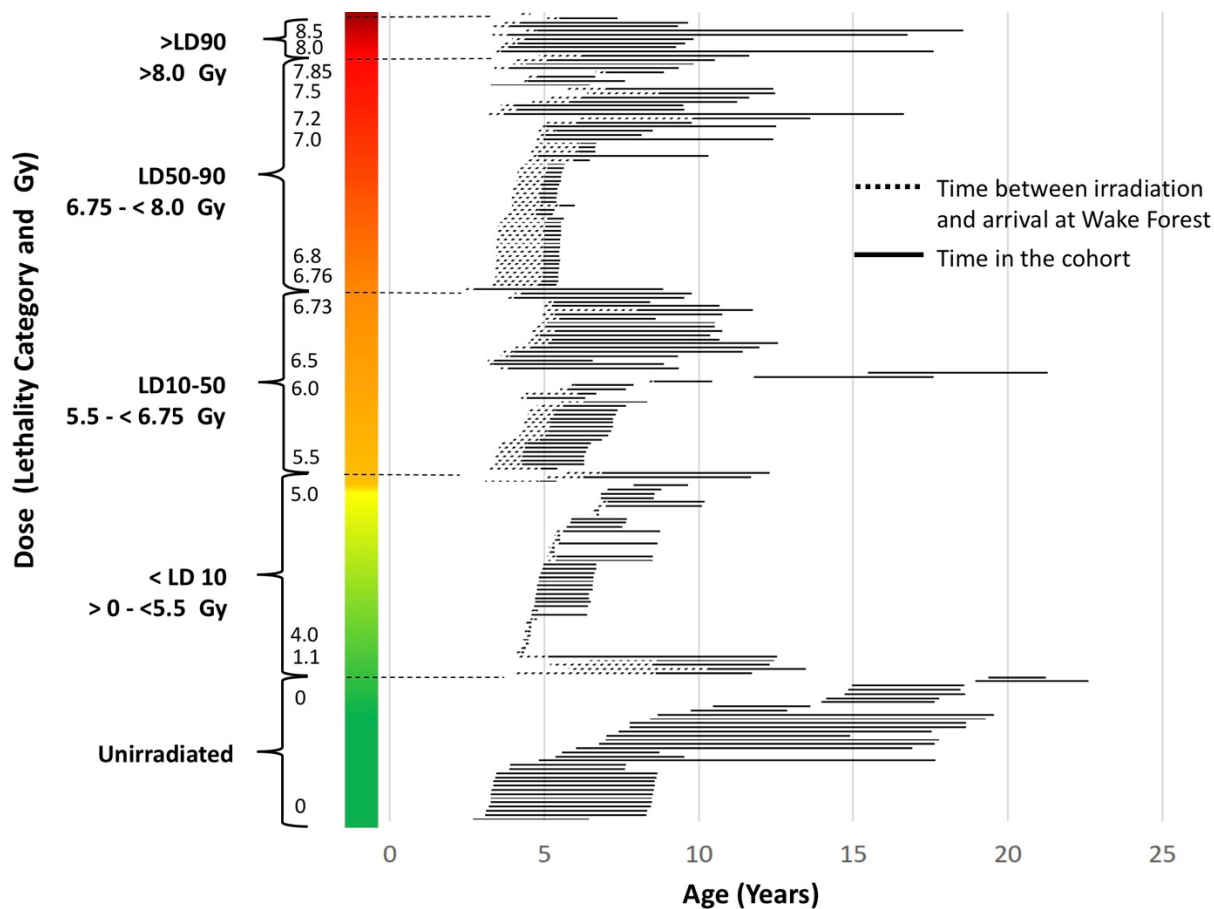


Figure 1: Age and dose distribution of the Radiation Survivor Cohort Each horizontal bar represents a single animal; the left end of the bar is the age at which the animal was irradiated, and the right end of the bar is the animal's current age or age at time of death.

Animals in the RSC undergo a regular schedule of medical care and assessment, including but not limited to:

Daily

- Twice-daily clinical observations
- Environmental enrichment

Monthly

- Physical exam
- Blood collection for experimental measures and archive
- Urine collection for experimental measures and archive
- Body weight

Semiannually

- Complete blood counts
- Clinical chemistry panels
- Testing for diabetes mellitus (hemoglobin A1c, glucose, with glucose tolerance testing and minimal model testing on a subset)
- Anthropometrics (trunk length, waist diameter)

Annually

- Flow cytometry
- Bone marrow aspirate
- Echocardiography

- Computed tomography scan (whole body)
- Renal ultrasound
- Gastrointestinal endoscopy and biopsy
- Bronchoalveolar lavage
- Cranial MRI (subset, 3 year rotation)
- Ocular exam

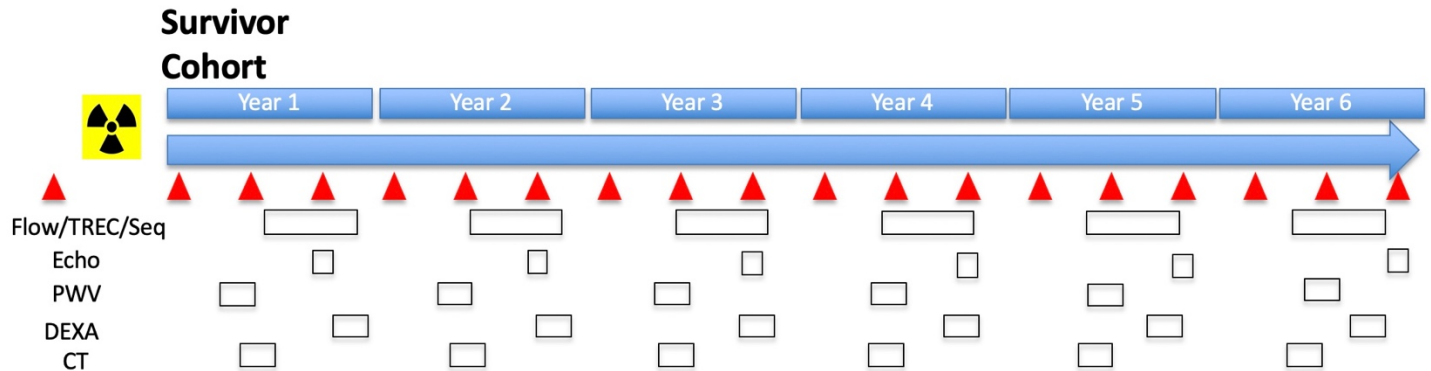


Figure 2: Diagram representing the annual schedule of clinical assessments in the RSC. Red triangles indicate blood collection dates. Abbreviations: Flow = flow cytometric measurement of lymphocyte subsets; TREC = T cell receptor excision circles; Seq = DNA and RNA sequencing collections from peripheral blood; Echo = echocardiography; PWV pulse wave velocity; DEXA = dual energy x-ray absorptiometry; CT = computed tomography.

Morbidity

We have established clinical criteria for identification and management of animals with health abnormalities. Criteria for diagnosis of different patterns of morbidity in the RSC are summarized in Table 1, below.

Table 1. RSC diagnostic thresholds, including prevalence, criteria for diagnosis and euthanasia.

Organ/ Disease	Irradiated	Control	Criteria for Diagnosis	Criteria for Euthanasia or Removal
Diabetes	9.2% (13/141)	0% (0/36)	Hemoglobin A1c (HbA1c) >6.5% Three fasting blood glucose measurements > 100 mg/dL Any non-fasted blood glucose > 200 mg/dL Long term treatment	Long term Insulin Treatment
Kidney	19.1% (27/141)	8.3% (3/36)	BUN > 30 mg/dl or Cr > 1.1 mg/dl Loss of renal volume >50% Urolithiasis	Clinical Renal Failure/Uremia
Lung	6.3% (9/141)	5.6% (2/36)	Pulmonary consolidation on CT scan Bullous emphysema >25% Hypoxia (SPO2 < 80%) Respiratory rate >80 breaths per minute	Respiratory distress
GI	14.9% (21/141)	5.6% (2/36)	Any lesion on endoscopy Diarrhea (severity code >2 for >5 days)	Chronic diarrhea with weight loss
Skin	13.5% (19/141)	2.6% (1/38)	Persistent dermatitis, alopecia or loss of pigmentation	Severe extensive dermatitis
Heart/CV	27.7% (39/141)	16.7% (6/36)	Murmur detected on auscultation or echocardiography Mean arterial pressure >120 Adult stroke volume <5 mls & Cardiac output <0.5 L/min at >7 y	Congestive heart failure or substantial dysfunction
Brain (any lesion)	12% (17/141)	0% (0/36)	MRI lesions visible on SWI Neurologic abnormality on clinical exam	Clinical neurologic abnormality, large infarct or edema on MRI
Eyes (cataracts)	39.0% (55/141)	25.0% (9/36)	Any lens opacity on annual slit-lamp exam	n/a
Behavior	17.7% (25/141)	33.3% (12/36)	Any behavioral abnormality requiring individual management plan	Unmanageable self-injurious behavior or depression
Neoplasia	16% (23/141)	0% (0/36)	Any neoplastic disease (imaging or biopsy)	Malignancy
Testicular atrophy	48.6% (36/74)	0% (0/20)	Testis volume <10 ml each after 7 years of age; Vol = (p/6)(LxW^2)	n/a
Bone	41.8% (59/141)	2.8% (1/36)	Adult bone mineral content (BMC) < 274g or density (BMD) < 0.373 g/cc	Fracture
Obese Underweight	11.3% (16/141) 33.3% (47/141)	33.3% (12/36) 16.7% (6/36)	Obesity: Waist Circumference > 45 cm or DEXA Body Fat > 30% Underweight: Waist Circumference < 25 cm or DEXA Adult Body Fat < 12.3%	Unintended weight loss >25% in non-obese animal

Abbreviations: BMC = bone mineral content; BMD = bone mineral density; BUN = blood urea nitrogen; Cr = serum creatinine; CT = computed tomography scan; CV = cardiovascular; DEXA = dual emission x-ray absorptiometry; HbA1c = hemoglobin A1c; MRI = magnetic resonance imaging; NSD = no significant difference; SPO2 = oxygen saturation.

Patterns of Multi-Systemic Morbidity

The categorical morbidity data shown in Table 1 are displayed below in order to display the relationships between radiation dose and late disease outcomes. Figure 3 shows the number of concurrent co-morbid conditions in individual animals, which increases with radiation dose in an exponential curve. Figure 4 shows a chord plot indicating which specific disorders tend to occur together. The most telling element of Figure 4 is the category of "No other", indicating those irradiated animals that have only one abnormality; few animals are in this category.

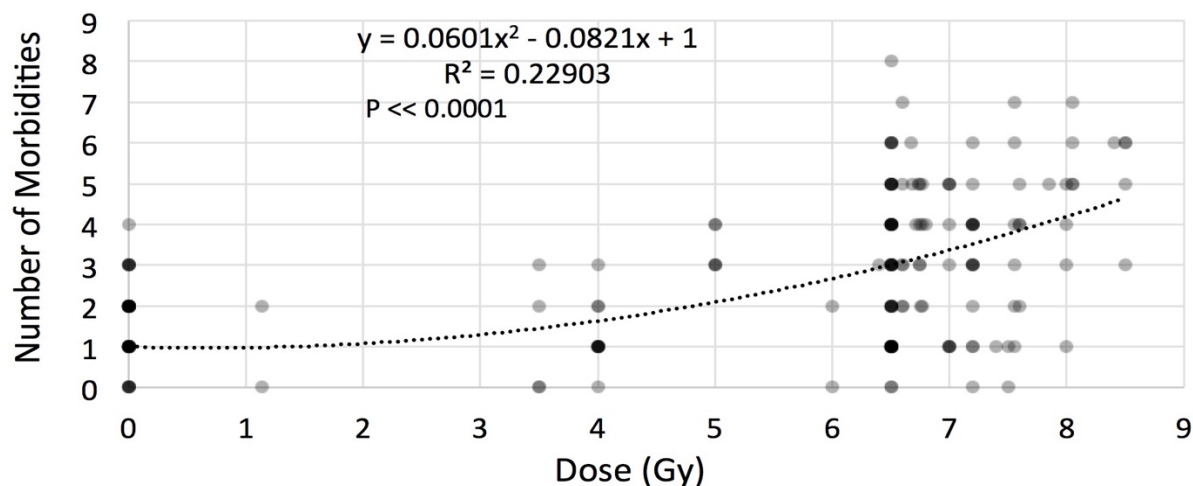


Figure 3. Numbers of disease morbidities plotted against radiation dose for individual animals in the long-term radiation survivor cohort. Darker dots indicate multiple animals at the same plot coordinates. There is a highly significant quadratic relationship between radiation dose and numbers of co-morbid conditions.

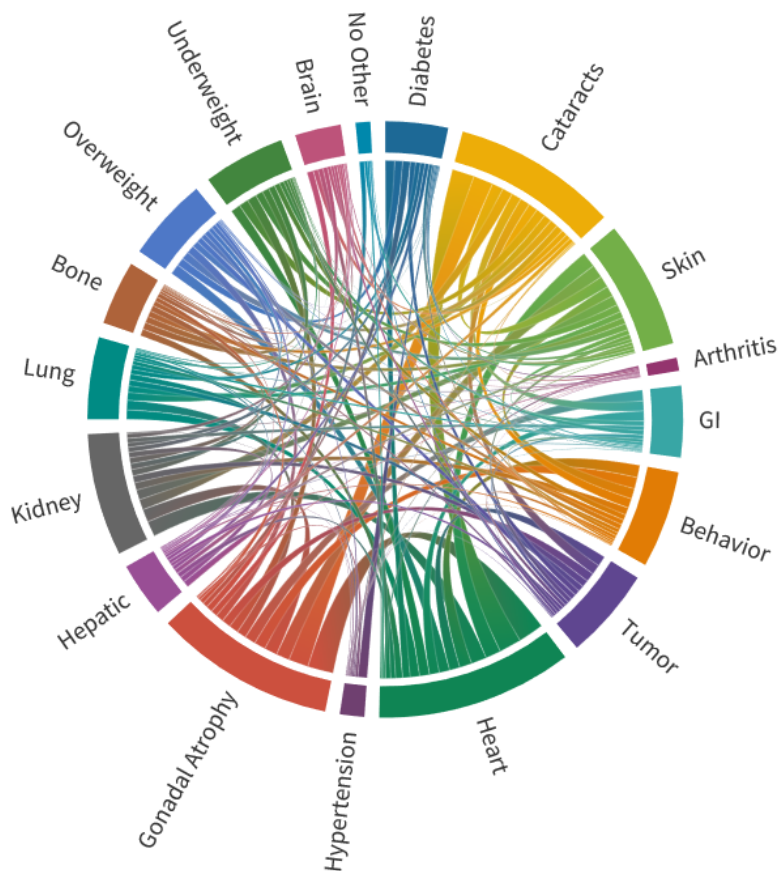


Figure 4. Chord diagram of the proportions of co-existing morbidities in irradiated animals in the Radiation Survivor Cohort. The thickness of each chord reflects the number of animals that have both conditions at the same time (e.g. many animals have both cataracts and gonadal atrophy, which are typical radiation effects)

Pathology

As the project progresses, animals age out of the cohort. In the event that animals die or must be euthanized due to illness, a complete necropsy is done including samples from all major organ systems. Gross lesions are photographed. Major organs are weighed and collected both snap-frozen in liquid nitrogen and fixed in 4% paraformaldehyde (or investigator's method of choice). Histopathologic interpretation of pathologic findings is done by veterinary pathologists. The tissue collection checklist includes over 100 samples, including:

Adrenal glands	Fat (visceral and subcutaneous)	Mammary glands	Skin
Arteries (aorta, coronary, iliac, carotid)	Gonads	Muscle	Small intestine
Bone (humerus, femur, vertebrae)	Hair	Nerves (sciatic, sural)	Spleen
Bone marrow	Heart	Oral mucosa	Stomach
Brain (multiple levels)	Kidney	Ovaries (F)	Teeth
Cervix	Large intestine	Pancreas	Testis (M)
Esophagus	Liver	Pituitary	Thymus
Eyes	Lung	Prostate (M)	Tongue
	Lymph nodes	Salivary gland	Trachea
			Uterus (F)

The Prospective Radiation Cohort (PRC)

The study design for the PRC is shown below. These specific-pathogen-free animals were acquired and quarantined in year 1 and irradiated in October 2016. Imaging and sample collections were done for all projects, and the animals have all been given humane euthanasia at the end of the in-life portion of the study. Complete necropsy examinations were done, including histopathology.

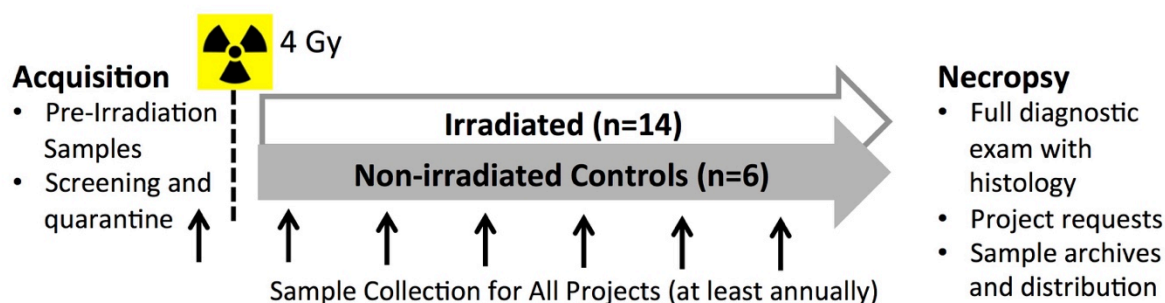


Figure 5: Study design for the Prospective Radiation Cohort (PRC). The in-life portion of this study has been completed.

Prospective Cohort

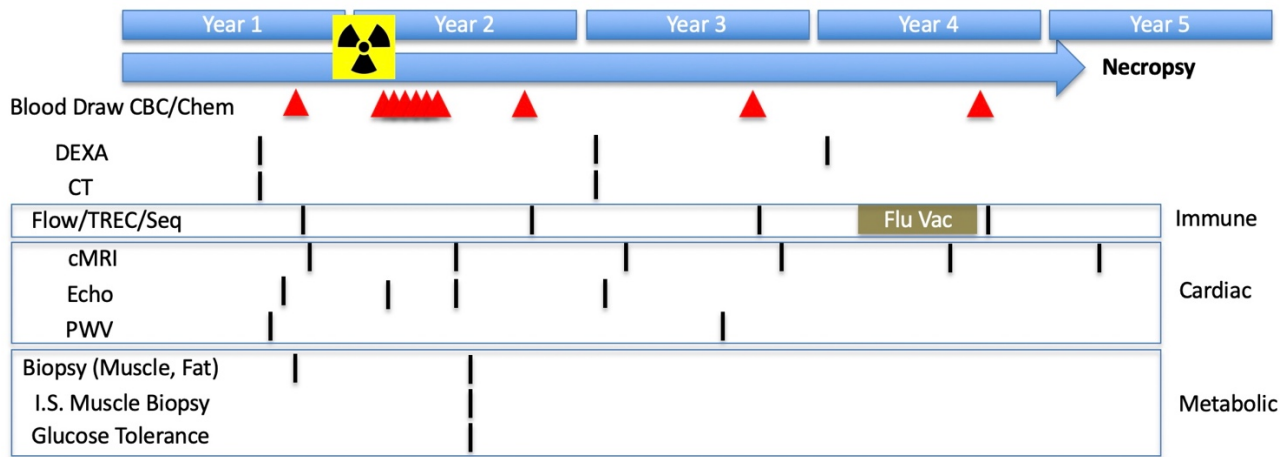
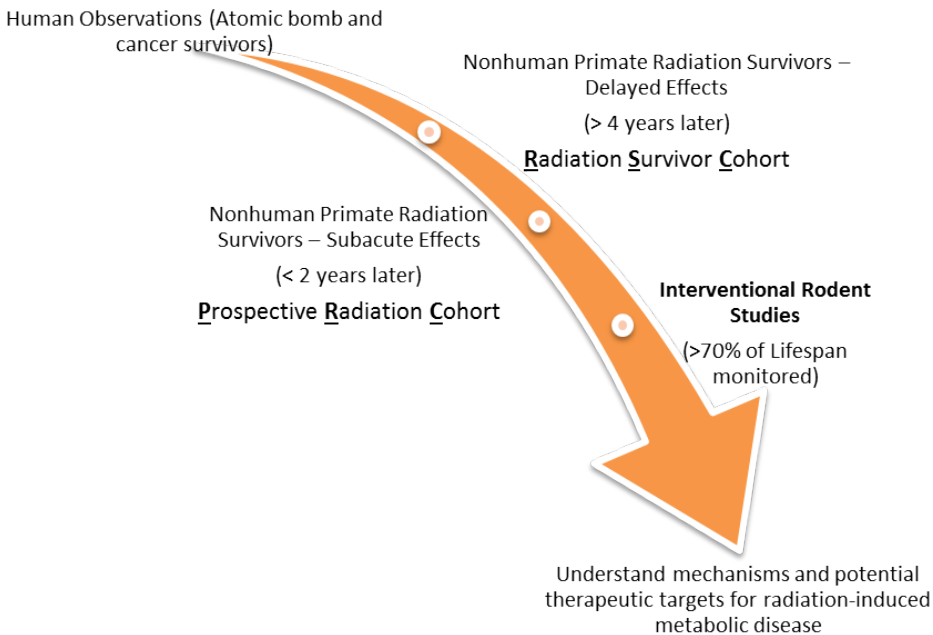


Figure 6: Diagram showing clinical assessments in the Prospective Cohort. The vertical red line indicates the current date. Red triangles indicate blood collection dates. Abbreviations: CBC = complete blood count; Chem = serum chemistry measurements; cMRI = cardiac magnetic resonance imaging; Echo = echocardiography; PWV = pulse wave velocity; I.S. = insulin stimulated.

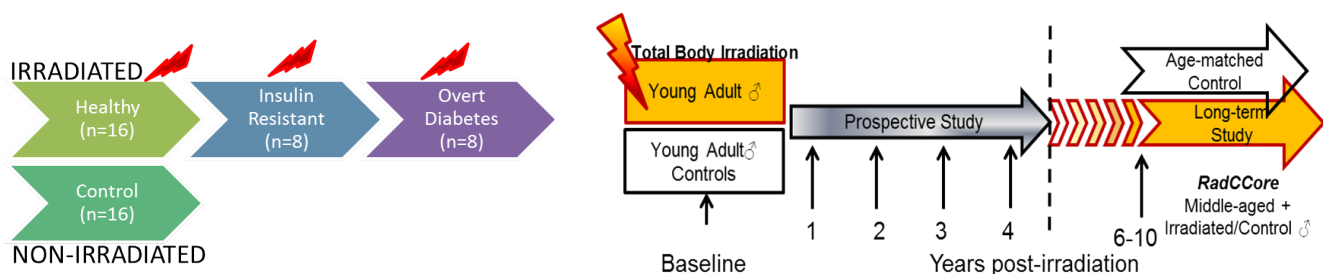
Project 1: Diabetes (Kavanagh)

The overall goal of Project 1 is to identify pathways that track some radiation survivors on a path that results in dysregulated metabolism and increased risk for diabetes. Our studies utilize a unique reverse translational flow whereby diabetes has been reported in survivors of radiation exposures, including our nonhuman primates (NHPs), and we have designed studies to identify the time course, tissue targets, and interventional strategies that will combat diabetogenesis in at-risk individuals. The scope of the research involves using different animal models (rodents and nonhuman primates) to detect and describe the structure and functional differences in key metabolic tissues, adipose and skeletal muscle, which co-occur with radiation and eventual development of metabolic disease,



In accordance with this continuum, this report is divided into sections that address major accomplishments within the sub-studies named the Radiation Survivor Cohort (RSC), the Prospective Radiation Cohort (PRC), and the Interventional Rodent Studies.

A diagram of the monkey cohorts (RSC and PRC) and the assessment points is below to aid in understanding the scope of work.



1) Major activities

1a) Development of the rodent model of radiation-induced metabolic disease

We aimed to generate a rodent model of metabolic disease that resulted from sublethal radiation exposure. Further, we wanted this phenotype to be persistent such that it modeled the monkeys who develop diabetes years after exposure. We achieved our purpose with the combination of low dose and slow exposure to gamma radiation and a human-relevant dietary composition. Mice consumed 5g of a Western-style diet per day (protein = 17.3% of calories, lipids = 34.9% of calories, and carbohydrates = 47.7% of calories with significant levels of simple sugars; constructed in-house at Wake Forest University School of Medicine) and water was available *ad libitum*. We delivered a total dose of seven Gray

in two 3.5 Gray fractions, each delivered at 0.358 Gray/minute with less than 10 minutes between exposures. Radiation was emitted from a Co⁶⁰ source (3 x 6500 Ci; J.L Shepherd & Assoc., Model 484R2, S.N. 7210). Mice were turned from ventral to dorsal recumbency between fractions to ensure even body exposure. We examined the health of mice at < 6 months in our model validation study, and 6 and 12 months in our intervention studies. We were pleased to have zero lethality in this model and have dysregulated glucose metabolism. Example graphs are shown below with fasting glucose elevating early in the course of disease and response to insulin becoming worse also at 3-4 months post exposure (area under the curve p=0.05, right panel)

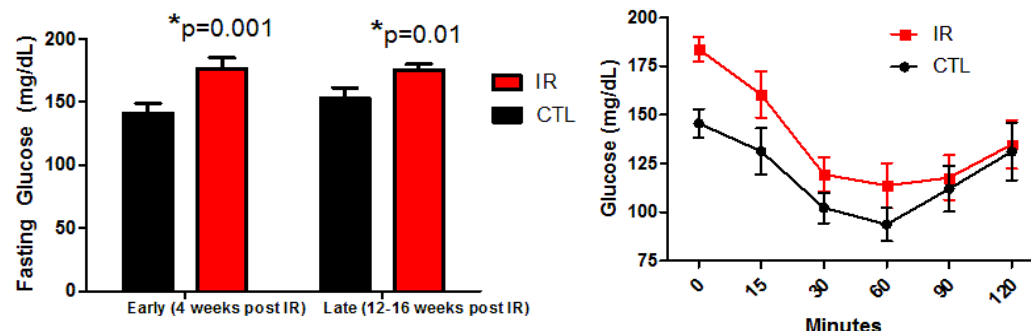


Figure 1.1. Western diet consuming mice exposed to a single sublethal dose of whole body irradiation (IR) have pre-diabetic metabolic disease with higher fasting blood glucose (left panel) and reduced responses to insulin (right panel) as compared to control (CTL).

Another interesting feature that relates to tissue function and the cardiovascular outcomes observed in Project 2 was that physical function in irradiated mice was also significantly compromised ($p < 0.05$, data not shown). These data represent a new rodent model for metabolic disease.

1b) Examination of the Radiation Survivor Monkeys

We selected a subset of the survivor monkeys to do more intensive investigations of which the results are detailed below under the specific objectives. The character of these animals was assessed by imaging for body composition, metabolic testing, skeletal muscle and adipose tissue biopsies, among other phenotypic assessments detailed in the Animal Core. What has consistently presented post-irradiation is loss of mass, specifically fat mass which can be appreciated in the table below where total weight and waist circumference are less than control animals.

		CTL (n=8)	IRRAD (n=16)	p-value (age-adjusted)
Age	yrs	13.1 (0.90)	9.41 (0.72)	0.01
Age at Irradiation	yrs	N/A	4.56 (0.34)	-
Irradiation Dose	Gy	N/A	6.52 (0.33)	-
BW	kg	16.1 (1.90)	8.83 (0.61)	<0.001
Waist	cm	43.3 (9.66)	33.3 (1.52)	0.01

1c) Longitudinal Assessment of the Prospective Radiation Survivor Monkeys

Age-matched monkeys were stratified to control and irradiated groups. Upon arrival they were comparable by key metabolic criteria (weight, glucose, A1c values, and triglycerides) but after acclimating to the Western diet and time between arrival and irradiation, group differences were apparent. All data in the longitudinal assessments were covaried by baseline values to account for individual differences. Based on data observed in the mice where it took 6 weeks for recovery from the irradiation such that they had comparable body weights to control animals, we did not assess the monkeys until 6 months past irradiation and followed them for more than 2 years. This recovery period in a mouse roughly equates to 1.5 monkey-years based on relative lifespans, so we were confident we would capture delayed effects of radiation in survivors.

2) Specific objectives

2a) Understand the time course of radiation-induced metabolic disease

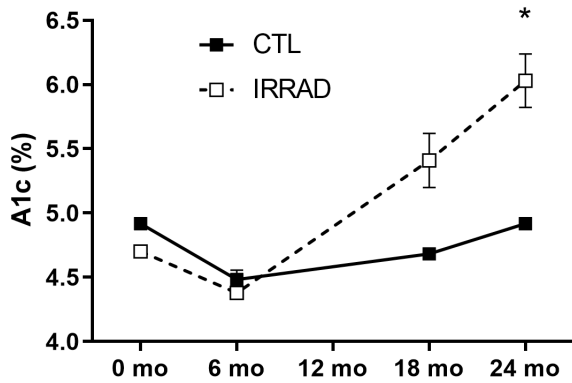


Figure 1.2. Western diet consuming monkeys, who have been exposed to a single dose of 4Gy to their whole body (IRRAD), slowly develop diabetes over the following years, whereas control monkeys (CTL) remain healthy.

The take home message from our collection of controlled animal studies is that radiation exposure reliably and reproducibly induces insulin resistance and diabetes in both nonhuman primates and rodents. In rodents the effect is seen after less than 5% of their lifespan has passed following sublethal doses of whole body exposure. In primates, the radiation survivor cohort had already indicated that overt diabetes does present within years of exposure. The prospective radiation cohort of monkeys filled the gap in knowledge regarding the time course of disease. In these monkeys, it was demonstrated that at least 1 year (approximating 2-3 year human equivalent) of time has to pass before the adverse health trajectory becomes apparent and 2 years (4-6 years human equivalent) for definitive diabetes to present. The consistency in the time course ($\approx 5\%$ of lifespan) in all three studies is an important outcome that substantiates the effect of radiation on key metabolic tissues. All changes in metabolic health occurred without the accumulation of excess adipose tissue, and in the monkeys, poor metabolic health was often seen with less adipose tissue. Graphs from the survivor cohort and prospective cohorts of monkeys and the rodent body composition are shown below. In all cases, irradiation leads to less fat mass and smaller animals. In the prospective cohort of monkeys, bodyweights were equivalent at baseline and irradiation, but lagged in the irradiated animals who remained lighter than control monkeys from 6 months post-exposure onwards. The loss of fat mass preceded the development of measurable metabolic differences, which is what directed us to focus on adipose tissue as a target organ for the delayed effects of radiation.

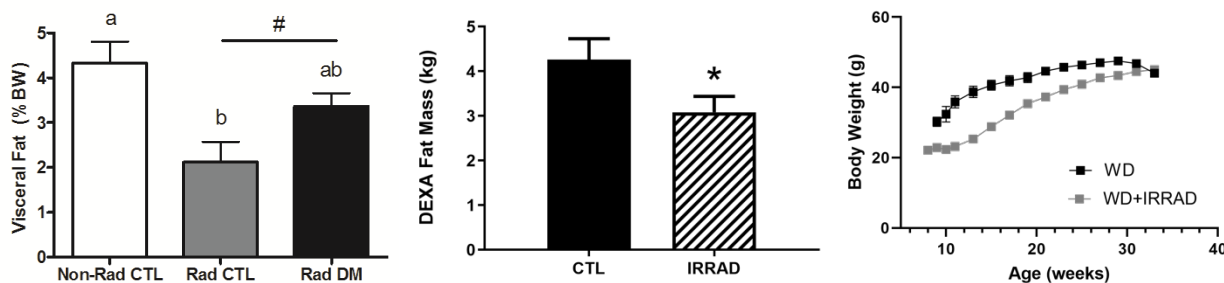


Figure 1.3. Whole body irradiation consistently leads to loss of body fat. In this project, we observed radiation-related loss of fat regardless of their diabetes status (Rad CTL and RadDM) as compared to control animals. The left panel is data from the RSC. This finding was replicated in the PRC monkeys who also had reduced adipose tissue (middle panel) observed prior to the onset of diabetes. Even sublethally irradiated mice (right panel; IRRAD) consuming western diet (WD) are lighter than non-irradiated mice until late adulthood.

2b) Examine changes in tissue proteins of irradiated animals

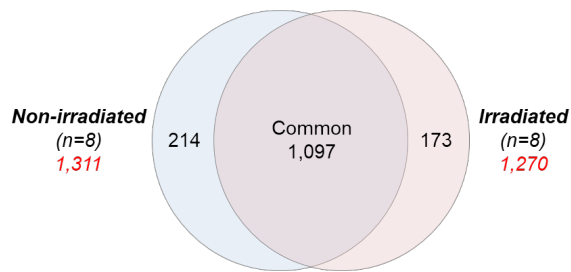


Figure 1.4. Skeletal muscle proteomics analysis from non-irradiated and irradiated monkeys delineated protein groups that were unique to each health state.

We initiated examination of protein shifts with non-targeted proteomics, then evaluation of oxidated proteins. Skeletal muscle samples were selected for proteomics measures on animals that had equivalent metabolic status (A1c, fasting glucose and insulin, and glucose tolerance testing outcome measures; data not shown). This enabled us to focus on specific radiation-related changes in expression. We show that irradiated monkeys had 173 proteins differentially regulated and control animals had 214.

Most proteins that were upregulated included metabolic and mitochondrial-related proteins, which may reflect the relative abundance of these in skeletal muscle. Of particular note was S100-A13 (6-fold higher, $p=0.009$) which is known to interact with transforming growth factor beta-receptor function. Isoforms S100-A16 and s100-A8 were also increased. The transforming growth factor beta-receptor pathway is upregulated following radiation exposure and our experiments reveals a new specific target that could be investigated in the future, and are consistent with improvements observed in our mouse studies following transforming growth factor beta-receptor 1 inhibition. The differential proteins expressed within irradiated tissue indicate stress and inflammation (red bars below, EIF2 and acute phase signaling) and increased need to deal with glucose and lipid fluxes (LXR/RXR/Rac) which is also consistent with subclinical metabolic impairment.

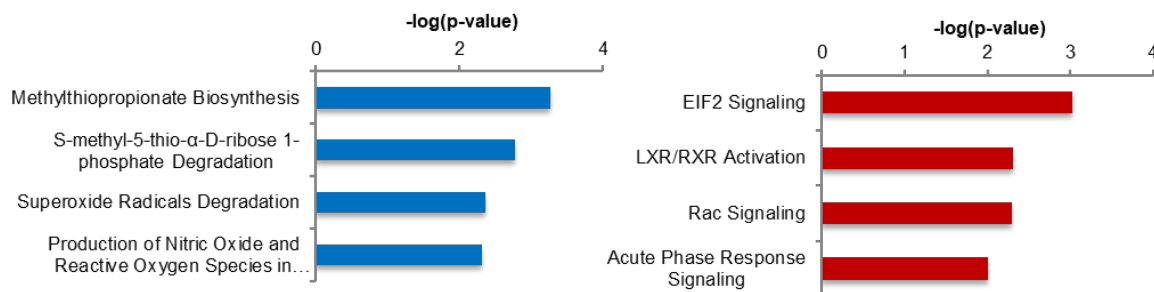
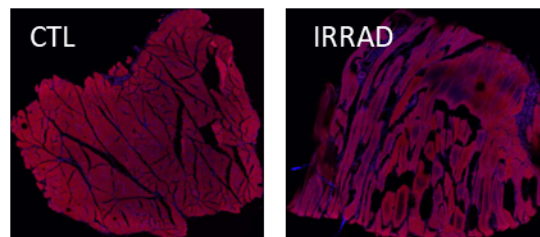


Figure 1.5. Ingenuity pathway analysis on differentially regulated proteins in control non-irradiated monkey skeletal muscle (blue, left panel) shows upregulation of healthy pathways as compared to pathways upregulated in irradiated monkey skeletal muscle (red, right panel). Radiation induces stress and inflammatory pathways, and increases proteins involved in lipid and glucose handling, which is consistent with the insulin resistance observed in these monkeys.

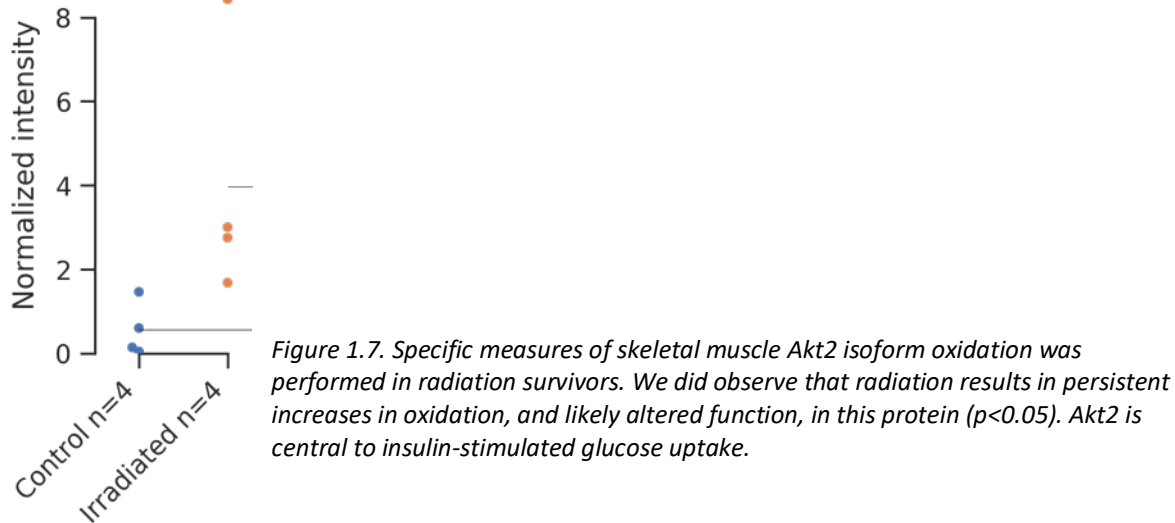
Figure 1.6. Specific redox staining of skeletal muscle proteins in situ did not reveal increases in irradiated (IRRAD) monkeys as compared to control (CTL) monkeys. We also evaluated for DNA oxidation (data not shown) which was comparable. Global increases in oxidative damage were concluded to be a delayed effect of radiation.



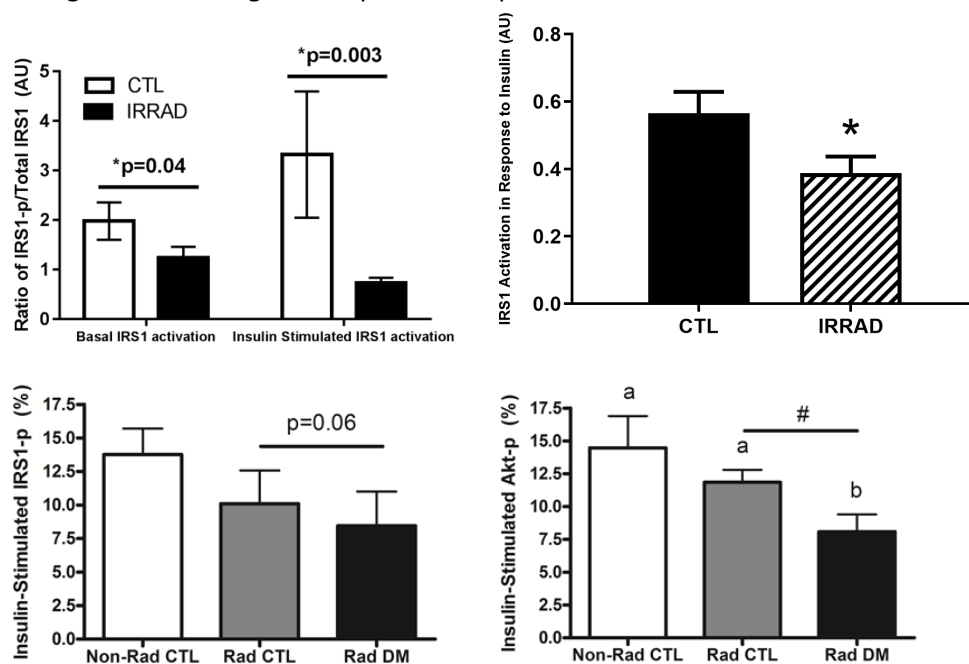
We then evaluated if proteins were oxidated, and thus additionally altering their function or expression via autophagic processes. The ubiquitination pathway was significantly different in irradiated animals. Oxidation was assessed by whole

tissue sections of skeletal muscle stained using a fluorescently labeled oxidant marker. An example is shown below (Figure 1.7), but group differences were not present in section intensities ($p>0.05$).

We then evaluated Akt2, which is a protein central in insulin signaling and growth signaling. Prior in vitro experiments has shown this isoform to be specifically oxidized in response to radiation. In vivo, the ratio of oxidized Akt2 to total Akt2 in skeletal muscle tended to be higher ($p=0.06$) which does support that this target is likely to be in part responsible for worsened insulin signaling and diabetes development.

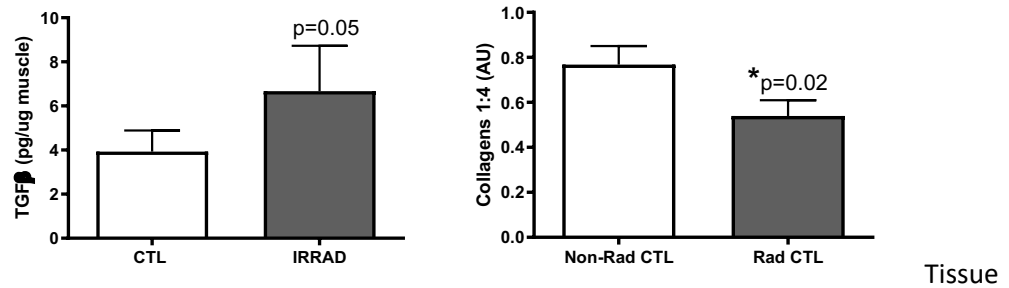


We followed up with physiologically assessing insulin activation in vivo. We assessed irradiated monkey skeletal muscle and adipose tissues for the ability of insulin to activate its receptor on the cell membrane, resulting in phosphorylation of insulin receptor substrate 1 (IRS1). This activation step is upstream of Akt activation. In both tissues, irradiated animals show a deficiency in insulin action. This reduction would be expected to be additive with the Akt2 dysfunction, leading to decreased glucose uptake in response to insulin.



2c) Track tissue remodeling after radiation

Figure 1.9. Excess collagen type 4 was deposited in the skeletal muscle of radiation survivors (Rad CTL; left panel). This deposition is consistent with the increase in transforming growth factor β (TGF β) protein present (right panel).



remodeling occurs in response to radiation, and we wanted to characterize what changes might be related to the reduction in insulin action. In totality, our data did not identify differences in circulating insulin levels or insulin response to glucose stimulation. Therefore, we focused on features that may relate to the efficiency of insulin's interaction with its receptor and its activation. Extracellular matrix is the material between cells that facilitates cell-cell interactions, and is comprised of different collagens and integrins depending on the tissue type. We found in muscle that collagen 4 was overexpressed in irradiated tissues. This leads to thickened basement membranes around myofibers and capillaries, which would be a diffusion barrier to insulin action. The overabundance of collagen is consistent with increased transforming growth factor beta signaling ($p=0.05$) seen in irradiated monkeys skeletal muscle

We examined other extracellular matrix components (collagen 1, 3, fibrinogen) and muscle fiber size, which were comparable between control and irradiated monkeys. Adipose tissue has less extracellular matrix as it needs to be able to expand and contract with nutritional. We examined adipose extracellular matrix and found no differences with irradiation.

Capillaries need to be present and functional for insulin to reach its target cells. In both skeletal muscle and adipose, we did not see a difference in the number of capillaries per unit area in either tissue. This in contrast to rodent studies, and monkey cardiac evaluations after irradiation. We do see that higher transforming growth factor beta is associated lower vascular endothelial growth factor, which stimulates capillary growth, and that higher capillary number specifically protects irradiated animals from developing diabetes (along with vascular functional markers such as nitric oxide metabolites in circulation). Together, these data suggest that in muscle, irradiation leads to greater transforming growth factor beta signaling and excess intercellular matrix collagen 4, which in turn may inhibit vascular remodeling. The heterogeneity in the diabetes progression post-radiation exposure is related to the individual's ability to protect their vascular architecture in muscle.

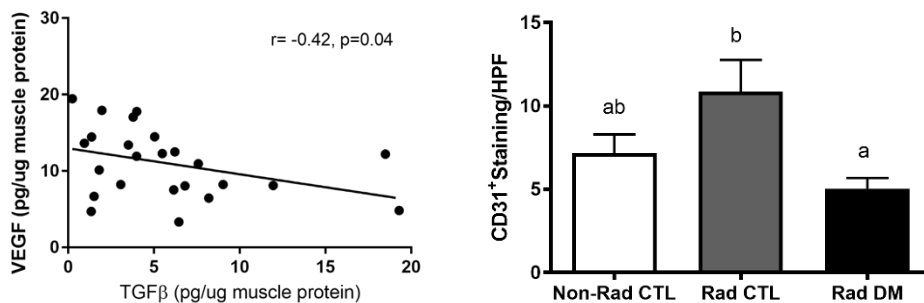


Figure 1.10. Transforming growth factor β (TGF β) protein in muscle is higher in irradiated monkeys (left panel) and is associated with less signaling to remodel the vasculature. Preservation of capillaries (CD31 $^{+}$ cells) identified animals who managed to stave off diabetes as a delayed effect of radiation (right panel).

One mechanism we discovered to also explain the variation in the diabetes-related outcomes within irradiated animals was expression of RhoA protein. RhoA has important roles in smooth muscle and vascular cell proliferation and migration. RhoA lowering reduces fibrosis in diabetes models, and has even been considered as a target for diabetes therapy.

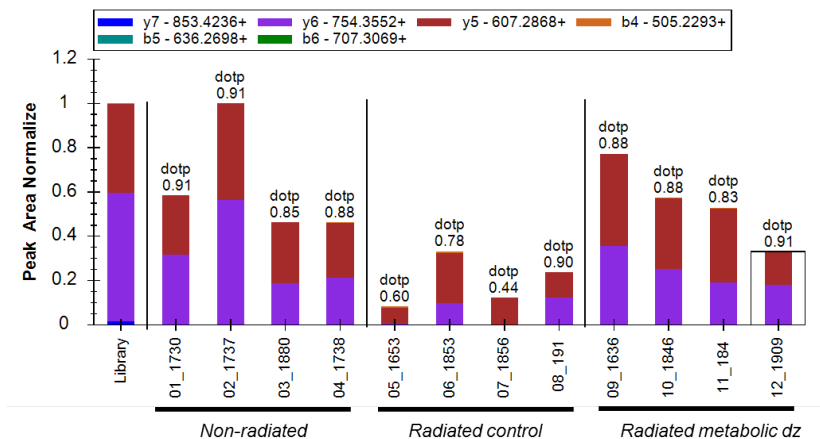


Figure 1.11. Along with vascular maintenance in Figure 10, reducing RhoA protein in muscle after radiation also characterized monkeys able to avoid diabetes after exposure.

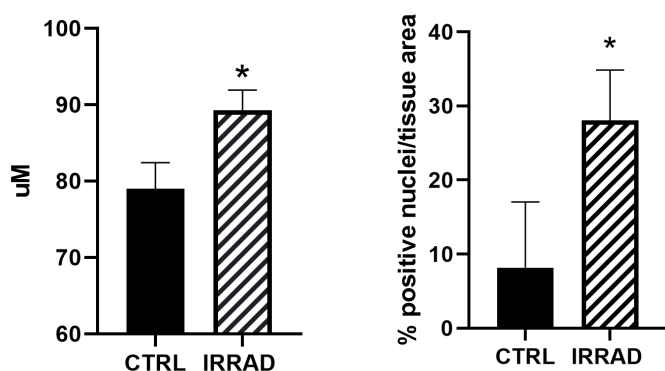


Figure 1.12. Left Panel: Irradiated (IRRAD) adipose tissue had more macrophages present measured as CD68/163 positive stained nuclei on histological sections, which suggests inflammation. Right Panel: Irradiated adipose had larger cell diameters (uM) which is a less healthy phenotype and prone to hypoxic stress and signal inflammation.

n adipose tissue, there were a different set of tissue features that were induced after irradiation. These features related to poor metabolic health outcomes. Primarily, adipose was insulin resistant and had lower mass (shown above); but irradiated individuals had larger adipocytes that existed in a tissue niche that contained more macrophages. The larger cells within less adipose mass is consistent with our in vitro studies showing irradiation leads to fewer adipocyte precursor cells, less differentiation into adipocytes on stimulation and fewer lipid droplets per cell (shown below).

2d) Evaluate therapeutics aimed to target radiation-related tissue remodeling in the rodent model

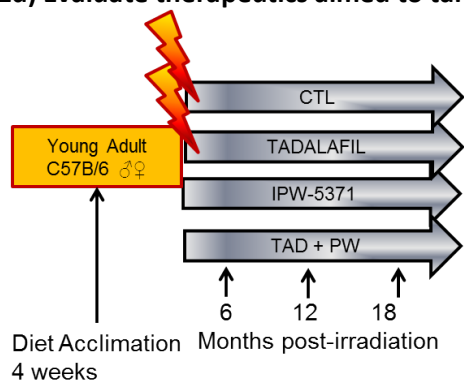


Figure 1.13. The overall study design for our mouse intervention study. We evaluated Western diet fed mice subject to whole body sublethal (7Gy) irradiation. Treatments aimed to reduce fibrosis (IPW-5371) and enhance tissue perfusion (Tadalafil) were continuously administered in the diet from 2 weeks after exposure.

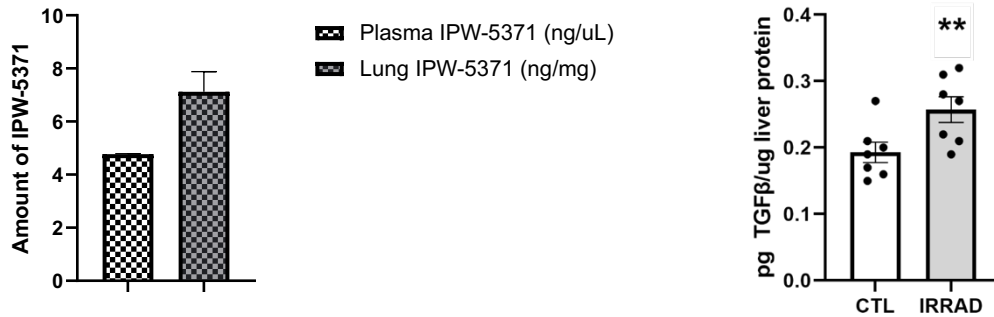


Figure 1.14. In the irradiated rodents, we ensured that therapeutic levels of IPW5371 were achieved in circulation and in tissues (left panel). We also checked that radiation elevated transforming growth factor β (TGF β) to certify that blocking the receptor would be a rational and effective strategy (right panel). Similar results were measured from lung tissue (data not shown).

Our rodent model was examined for the ability of transforming growth factor beta-receptor 1 inhibitors alone or in combination with tadalafil, to reverse the adverse effects on metabolism seen in the months after exposure. We used an experimental therapeutic (Transforming Growth Factor beta [TGF β receptor inhibition; IPW-5371 by Innovation Pathways) as fibrosis is a well-recognized outcome of radiation exposure. A related compound in this drug class is already in Phase 2 clinical trials for pulmonary fibrosis, and IPW-5371 has published efficacy in reducing lung fibrosis following radiation in mice. We also evaluated Tadalafil, a phosphodiesterase-5 inhibitor, marketed as Cialis. This drug was chosen for emerging evidence that it can enhance perfusion, specifically in skeletal muscle. To date, three small clinical trials have demonstrated efficacy and preclinical studies have shown it to reduce heart fibrosis and extracellular matrix accumulation in the context of hyperglycemia. These compounds were studied as single agents and in combination after sub-lethal irradiation.

We were able to successfully deliver the drug agents orally compounded in their Western diet (WD), and achieve therapeutic concentrations. Animals that in the treated groups were of comparable size at study end. Irradiation did increase transforming growth factor signaling in liver, which was chosen for evaluation, as it is central in metabolic regulation of glucose and lipids.

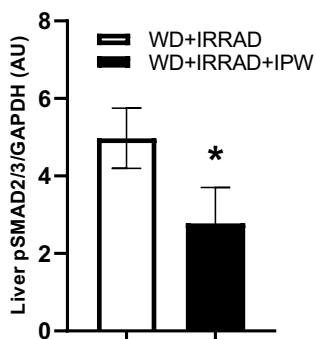


Figure 1.15. In the irradiated rodent (IRRAD) fed western diet (WD), inhibition of transforming growth factor β (TGF β) with IPW5371 treatment following exposure resulted in significantly less activation of the TGF β signaling pathway, measured by the amount of downstream activation of SMAD2 and 3.

The final assessment of our metabolic outcomes indicated that tadalafil had no positive effects alone, or additive effects when given in combination with IPW5371. Therefore, the benefits were due to inhibition of fibrotic signaling. We saw that there was reduced downstream activation of the receptor (phosphorylation of SMAD2 and 3 [pSMAD]) in liver and lung. This is further confirmed by reduction in type 1 collagen in liver and lipid accumulation as triglycerides. The combination of reduced fat and fibrosis is a potent indicator for the treatment of non-alcoholic fatty liver diseases. Blinded observers scored tissues, and scores were significantly lower in IPW5371 treated mice.

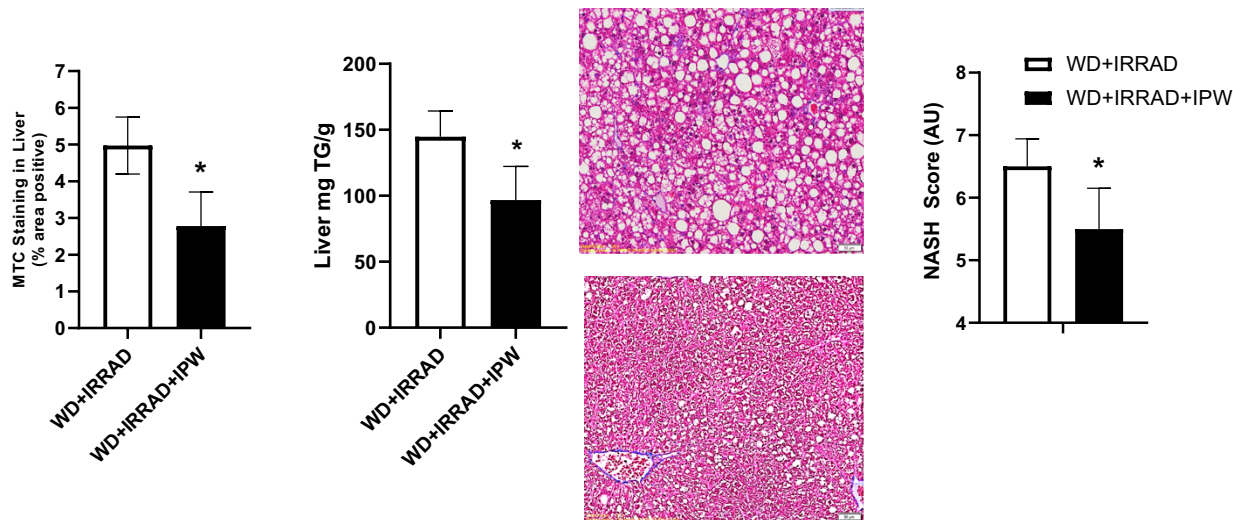


Figure 1.16. At 6 months after irradiation (IRRAD) and 8 months of western diet (WD), liver samples from mice were assessed for fibrosis by Masson's trichrome staining (MTC) and triglyceride (TG) content as key markers of non-alcoholic fatty liver diseases, which is a central part of metabolic disease development in people. IPW5371 significantly prevented fat and fibrosis deposition, which is a new class of drug for consideration in treatment of this disease. Representative MTC liver histology is shown with IPW5371 panel (bottom) having less lipid droplets. NASH scores performed by blinded observers rate IPW5371 liver histology as significantly better than irradiated control mice (right panel).

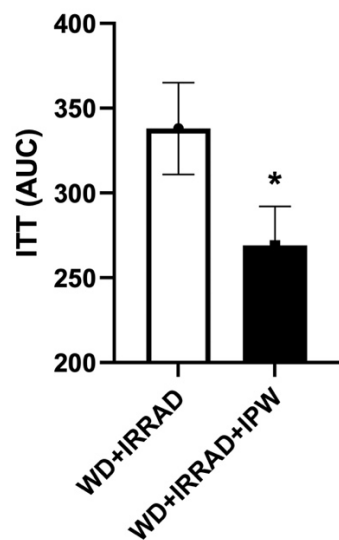


Figure 1.17. Irradiated (IRRAD), Western diet (WD) fed mice treated with the transforming growth factor β (TGF β) inhibitor IPW5371 had significantly better insulin sensitivity at 6 months post-exposure.

Concordant with liver health, we see that the mice treated with IPW5371 also had the best insulin sensitivity, completing the concept that inhibition of fibrotic signaling will improve metabolic health issues that arise as a delayed effect of radiation.

Other significant results that were observed with the inhibition of transforming growth factor beta included normalization of thymic cellularity, particularly CD3⁺ cells, at 6 months post-radiation exposure. This has implications for immunocompetency.

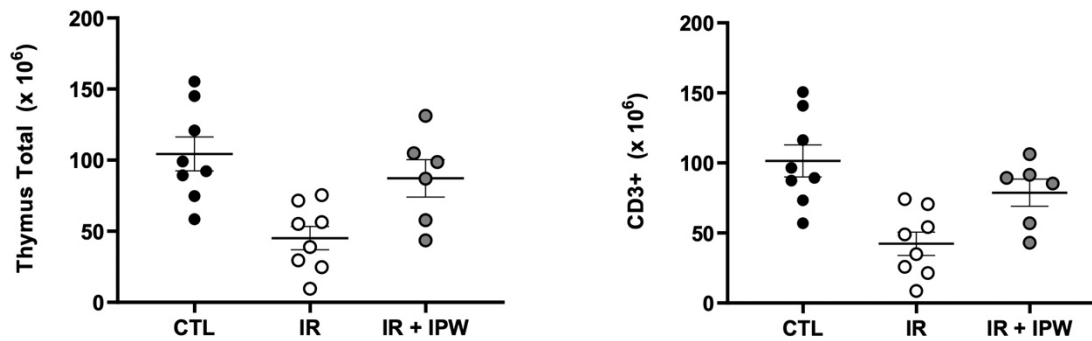


Figure 1.18. Irradiated (IR) had fewer cells, including CD3+ cells, as compared to control (CL) mice at 6 months post-exposure. This effect was reversed by treating IR mice with a transforming growth factor β (TGF β) receptor inhibitor IPW5371 (IPW).

We also learned that there is a potentially dangerous pharmacodynamic interaction between tadalafil and IPW5371. We observed severe cardiovascular pathological lesions when the drugs were combined and the effect had a more pronounced effect in males. Lesions were observed in the heart and great vessels beginning before 2 months of chronic therapy and were characterized as inflammatory with fibroplasia. This pathology was not related to radiation injury as the experiment was repeated in non-irradiated animals and survival was actually worse. We believe the mechanism is related to summation of effects on transforming growth factor beta signaling, and the need for a certain amount to be present to ensure tissue integrity. Attention to dosing in clinical trials and avoidance of co-administration of drugs that also inhibit fibrosis is warranted.

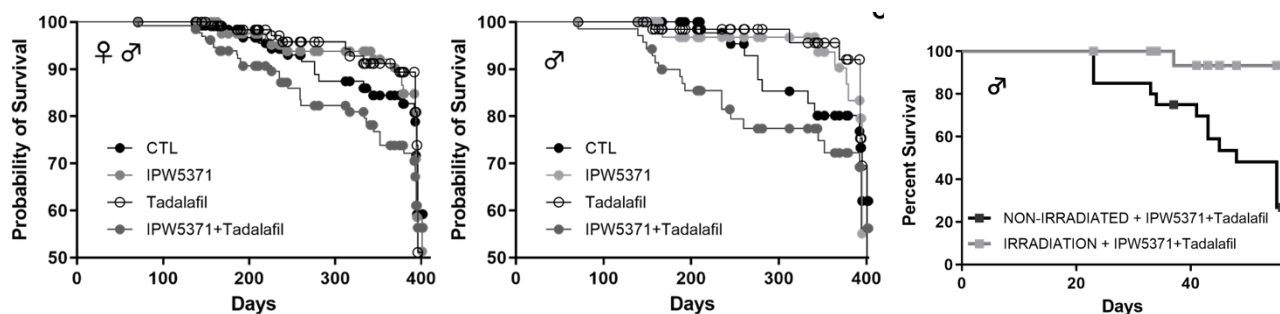


Figure 1.19. During experiments in irradiated, western diet-fed mice treated with potential countermeasures, IPW5371 and tadalafil, we discovered that the combination of treatments resulted in sudden death and worse survival. This was driven by loss in male mice (right panel, $p < 0.01$ for median survival of IPW5371+Tadalafil). The drug interaction was repeated in non-irradiated mice with even more dramatic reductions in survival (bottom panel, $p < 0.05$). Deaths resulted from inflammation and fibroplasia, leading to rupture of cardiovascular tissues.

3) Significant results

In summary, our significant results can be summarized as:

- Radiation reliably induces metabolic disease in people, monkeys and rodents
- Disease develops after exposure and presentation of disease begins after about 5% of lifespan has passed
- Insulin sensitivity (not production nor release) is compromised in peripheral tissues
- Remodeling of tissue structure and proteins implicate insulin signaling pathways
- Reduction of fibrosis and increasing perfusion are likely to maintain health post-irradiation
- Adipose tissue remodeling is inhibited by lack of stem cells
- Tissue senescence is not permanently induced following irradiation

4) Other achievements

4a) Redox proteomics in rodent tissues were not done (noted by red text under Major Task 5 in the SOW table above) as preceding work in nonhuman primates (Major Task 3) did not show strong evidence of persistent oxidation. The lack of

signature in this more translational species led us to examine difference sources of tissue dysfunction. We investigated precursor cell abundance and function in the adipose tissues of irradiated primates, and found them have deficits years past exposure. Further, we examined for evidence of increased *in situ* senescent cells, which is a cellular fate known to be the result of radiation exposure and able induce tissue dysfunction. In multiple tissues and both rodents and monkeys, we did not find this type of cellular damage. These findings steer the research community towards therapeutics that regenerate precursor cells, rather than tackle oxidation or senescence, as a strategy to mitigate the delayed effects of radiation. We believe this is a key outcome from this project that will influence the field in the future. These data are currently under review (Ruggiero....Kavanagh, *IJROBP*, 2022).

Key results from this latest redox proteomics study can be summarized in the abstract copied below:

Purpose: The pathogenesis of many age-related diseases is linked to cellular senescence, a state of inflammation-inducing, irreversible cell cycle arrest. The consequences and mechanisms of age-associated cellular senescence are often studied using *in vivo* models of radiation exposure. However, it is unknown whether radiation induces persistent senescence, like that observed in ageing.

Methods and Materials: We performed analogous studies in mice and monkeys, where young mice and rhesus macaques received sub-lethal doses of ionizing radiation and were observed for 10-15% of their expected lifespan. Assessments of 8-hydroxy-2'-deoxyguanosine (8-OHdG), senescence-associated beta-galactosidase (SA β -gal), and p16^{Ink4a} and p21 were performed on mitotic and post-mitotic tissues - liver and adipose tissue - six months and three years post-exposure for the mice and monkeys, respectively.

Results: No differences in 8-OHdG, SA- β gal staining, or p16^{Ink4a} or p21 gene or protein expression were found in mouse and monkey liver or adipose tissue. Despite no evidence of senescence, progenitor cell dysfunction persisted after radiation exposure, as indicated by lower *in situ* CD34⁺ adipose cells ($p=0.03$), and deficient adipose stromal vascular cell proliferation ($p<0.05$) and differentiation ($p=0.04$) *ex vivo*.

Conclusions: Our investigation cautions that employing radiation to study senescence-related processes should be limited to the acute post-exposure period, less than 5% of expected lifespan, and that stem cell damage likely underpins the dysfunction associated with delayed effects of radiation.

In this manuscript we show that precursor cell density is reduced. We tag CD34 gene expression *in situ* as a general precursor for cells that eventually differentiate into endothelial cells and preadipocytes. In the top left panel, the irradiated animals clearly have reduced density of these precursor cells ($p=0.03$) and the functional consequences of this deficiency is shown in the poor proliferation curves that result from growth of adipose stromal vascular cells (the niche which contains CD34 positive precursors). Further, these cells were unable to functionally differentiate into adipocytes. This is shown in the bottom panel where few irradiated cells were able to produce lipid, the key characteristic of an adipocyte, and when quantified – the number of droplets made per cell and total cell area was smaller than healthy control animals. This demonstration of adipose dysfunction is consistent with our findings above that irradiation leads to altered adipose biology in that fat does not normally expand yet is insulin resistant and diabetes ultimately does result years after exposure.

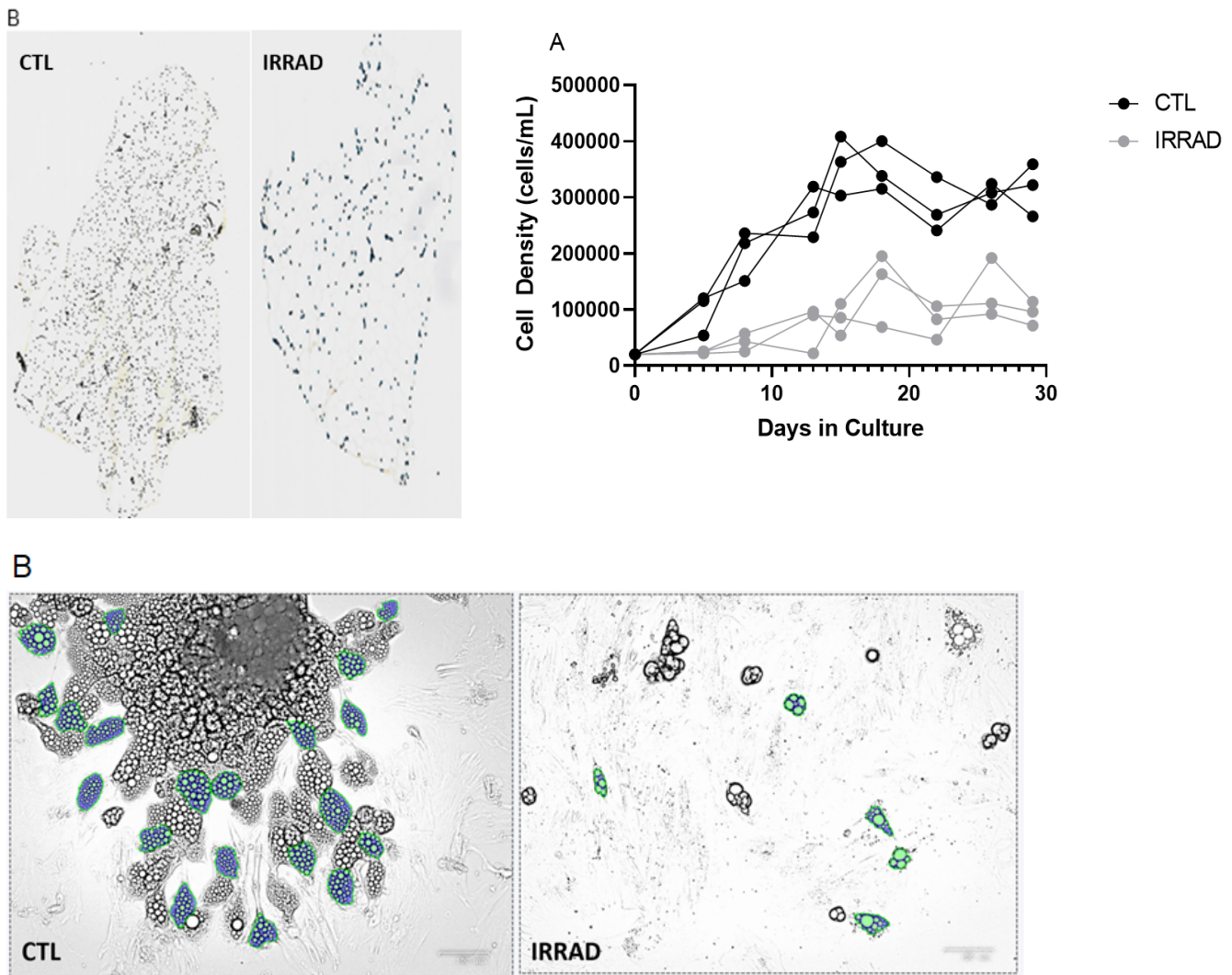


Figure 1.20. Radiation exposure (IRRAD) induces adipose tissue dysfunction in monkeys. We observe a significant reduction in the density of CD34+ precursor cells in adipose (top left panel, example). This lack of precursors resulted in a lower ability of adipose stromal vascular cells to proliferate in culture (top right panel). Further, these irradiated stromal vascular cells were resistant to differentiation into new adipocytes, producing fewer cells with fewer lipid droplets (bottom panel, example).

4b) Longitudinal tracking of irradiated monkeys; implication of the microbiome

In trying to understand the causes of insulin resistance, inflammation is a unifying pathway in which intracellular signaling can be reduced per unit of insulin binding to its' extracellular receptor. As noted in above-listed objectives, Akt isoform oxidation is one mechanism we explored for reduced insulin-mediated signaling. Inflammation can induce inhibitory phosphorylation of Akt and insulin-receptor substrates. We evaluated subclinical 'sterile' endotoxemia, seen in geriatric and obese people, as a potential mechanism. Bacteria in the intestinal microbiome can translocate and circulate to remote tissues, inciting inflammation and insulin resistance. We did not see evidence of radiation inducing permanent changes in the gut's microbiome nor levels of bacteria translocating into circulation. However we learned from this cohort of animals that blood pressure was a determining factor in the competency of the intestine to be a barrier in keeping the microbiome in the gut lumen. These data have been reviewed and a revised manuscript has been submitted (Vemuri..Kavanagh, Am J Physiol Heart Circ, 2022).

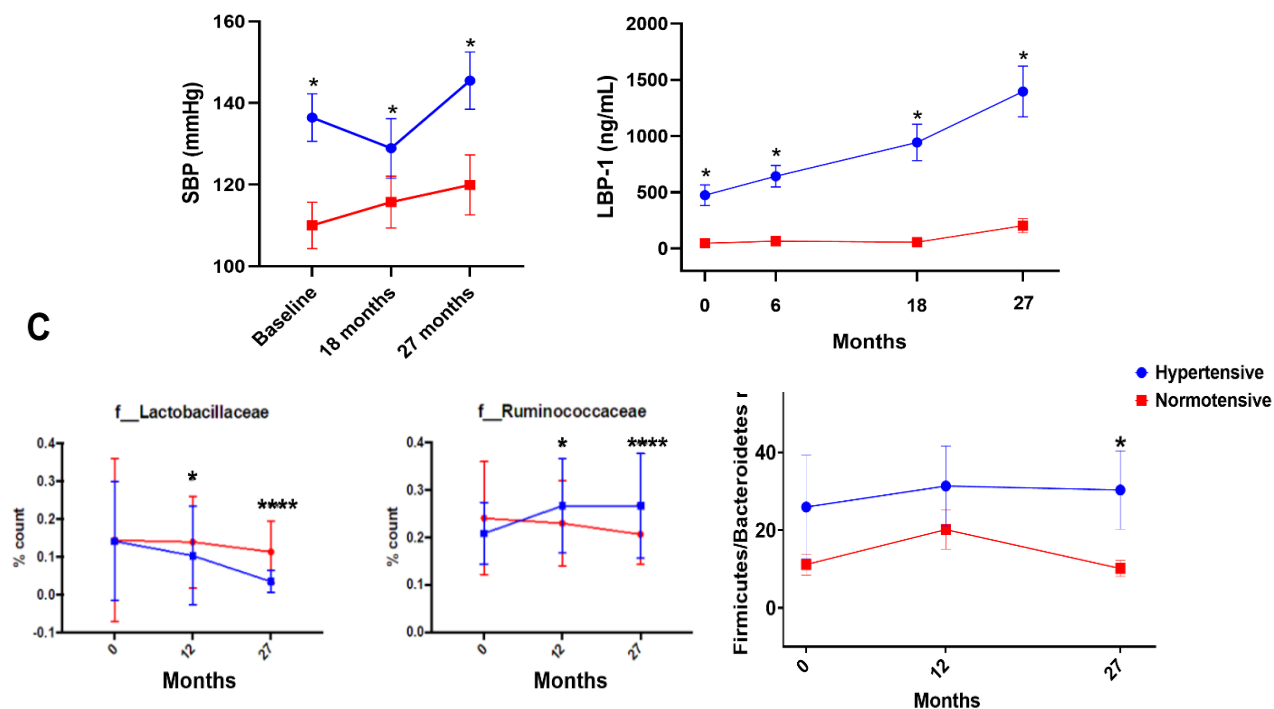


Figure 1.21. We used the Prospective Radiation Cohort (PRC) to examine the effect of hypertension on intestinal function. Radiation exposure did not have effects on blood pressure, intestinal barrier function (measured by lipopolysaccharide binding protein 1 [LBP1]) or the microbiome. However hypertension (systolic blood pressure [SBP] >130mmHg) was stable and present in half of the PRC, and these animals had elevated LBP1 which worsened over time. During the course of studying delayed effects of radiation, hypertensive animals progressively developed a less healthy microbiome, evidenced in the bottom 3 panels by less beneficial Lactobacillae, more pathogenic Ruminococcae and a worsened ratio of the Firmicutes to Bacteroidetes phylum.

These data from our prospective radiation cohort are summarized above. These demonstrate that hypertension is a stable physiological feature, however the gut barrier (LBP-1) deteriorates over time coincidentally with worsened microbiome features. At the end of study (27 months) the hypertensive animals had a lack of Lactobacilli, a beneficial microbe, excess Ruminococci, a pathogenic microbe, and worse Firmicutes: Bacteroidetes ratio at the phylum level. On the basis of our microbiome assessments and health signatures that align with radiation resilience in rodents, we are currently funded to examine the baseline microbiome signatures that may confer resilience to the acute and delayed effects of radiations exposure (supplement to U01AI50578).

4c) Use of the radiation survivor cohort as a monkey model of accelerated aging.

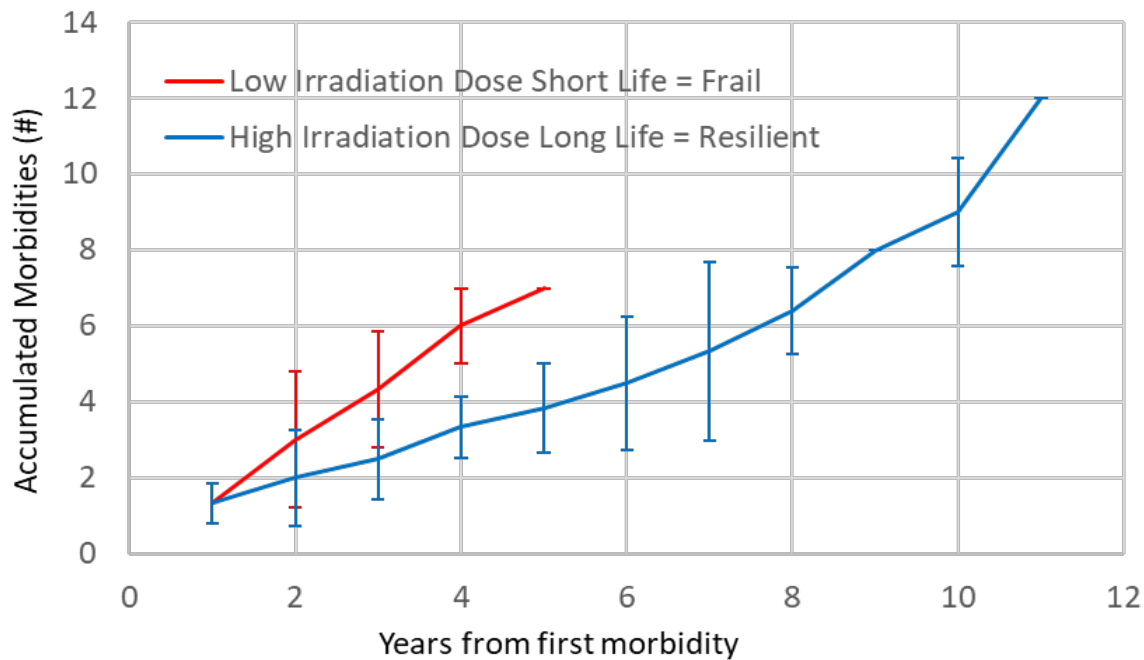


Figure 22. The Radiation Survivor Cohort of monkeys accumulate co-morbidities such as diabetes as a result of radiation exposure and aging. Some animals are more susceptible to poor health trajectories, as shown by differences in the accumulation rate of comorbidities in frail and resilient animals. These data support the use of the radiation survivor monkey as a model of aging.

Diabetes is the most frequently observed significant co-morbidity, of which age and insulin resistance are the primary risk factors. The survivor cohort monkey has been characterized in this project as having dysfunction skeletal muscle and adipose tissue, ultimately leading to increased prevalence and incidence of overt diabetes. This is consistent with human aging, however the health trajectory of each individual differs widely, leading to a concept of biological age versus chronological age. With the synergy available from studies with this program, we have been able to generate health profiles over time to find animals that are resilient and those that are more frail, and accumulate more health deficits as they age (aka greater biological aging). An example of age-matched individuals (n=6/group) with divergent health trajectories is shown below. This concept has resulted in submission of an administrative supplement to NIA (PI Cline, Co-I, Kavanagh, Quillen, Schaaf and Justice – all Wake Forest University School of Medicine).

Project 2: Cardiovascular Disease (Register)

Radiation induced heart disease (RIHD) is a known side effect of total body and chest irradiation. Early structural and functional indices which may predict future development of RIHD would be useful in the clinical setting. In addition, there is a need for identification of new cellular and molecular biomarkers of RIHD. Circulating cells and biomolecules may provide important clues into the mechanisms underlying RIHD. Macrophages are important in situ tissue level determinants of organ pathologies, markers of extracellular matrix turnover will provide clues into organ specific and systemic matrix remodeling, while small noncoding RNAs (microRNAs, miRNAs) may provide tissue specific markers of responses to radiation exposure and eventual disruption of organ cellular and molecular composition and function impairment. The NHP model is more likely to experience the gamut of cardiac responses to irradiation than rodent models, due to differences in anatomy including the presence of epicardial coronary arteries. Previously and newly irradiated animals provide a unique controlled setting in which to evaluate RIHD in a model similar to humans. We evaluated cardiac functional, cellular, and molecular phenotypes to determine the global effects of radiation on the heart and to try to identify targetable mechanisms underlying the condition. Elucidation of cellular and molecular phenotypes associated with RIHD and identification of targetable molecules are first steps towards developing approaches to prevent or delay RIHD.

1) Major Activities

- a) Collaborations with Duke investigators on cross-project cell collections, processing, and data analysis, manuscript development, and future grant applications
- b) Cardiac tissue analyses: Processing of myocardial and coronary artery tissue blocks (RSC, PRC, & past Project with mitigator) for assessment of coronary artery atherosclerosis (CAA) and cell types using immunohistochemical analyses, stained sections for which we developed new protocols for semi-automated atherosclerosis assessments. Myocardium from PRC subjects was processed for scanning electron microscopic analyses, images acquired and initial assessments conducted, further qualitative and quantitative analyses are planned.
- c) Cardiac Ultrasound Analysis Software: Upgraded the computer and software for customized output to accommodate the smaller NHP heart sizes, purchased and maintained TomTec US image analysis software and hardware and trained professional and technical staff in its use for off instrument quantitative echocardiographic assessments.
- d) Cardiac Ultrasound Data collected from the PRC and RSC studies were been completed and compiled, data from the RSC underwent extensive analysis in combination with other phenotypes. Ultrasound images from years 01-04 of the program in the RSC are 100% completed and a new manuscript has been placed on a preprint server and submitted for publication.
- e) Cardiac MRI studies were established in preliminary pilot work and acquired longitudinally in the 16 PRC subjects. Images were assessed for cardiac structure and function, including strain analyses using newly purchased software (SEGMENT) from Medviso AB Imaging. Attempts to analyze cardiac perfusion imaging data were confounded by insufficient resolution of MRI images and technical limitations of the manufacturer protocols on the 3T MR instrument.
- f) Biomarkers: Cardiac biomarkers: Assessed plasma NT-proBNP and multiplex cytokines/chemokines/biomarkers in samples from a previous study in which we have cardiac phenotypes including fibrosis and global gene expression. Shipped plasma samples from a prior study to an outside collaborating miRNA analytical group (Chromologic LLC, Monrovia CA) for miRNA analysis which was not completed due to technical problems related to miRNA isolation. Other biomarker work was conducted using multiplex panels to evaluate effects of radiation and relationships with cardiac phenotypes. All materials for final analyses of serum/plasma miRNA and key markers of ECM turnover were acquired and are now being used for completion of aims in RSC and PRC.
- g) Monthly DOD Conference calls, separate collaborative meetings between investigators, and annual project retreats.
- h) Work continues on a daughter grant "Effects of Ionizing Radiation (IR) on Myocardial DNA Methylation Profiles in Relation to Cardiomyopathy in a Nonhuman Primate Model" from the 2018 Peer Reviewed Medical Research Program for a Discovery Award to expand this work into a new area (epigenetic regulation through DNA methylation). DNA methylation arrays have been completed and are undergoing analysis.

2) Specific Objectives

Completions of necropsies in RSC subjects undergoing euthanasia. Ongoing collections of samples and images from the RSC. Analysis of tissues, images, and biofluids for the PRC. Analysis of myocardium and data from RSC. Ongoing development of multiple manuscripts from the experiment.

3) Significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative)

3.1 Molecular Characterization of Myocardium from Deceased RSC Subjects

Subjects

Male rhesus macaques (n=41) were obtained from the Armed Forces Radiobiology Research Institute, the University of Maryland, the University of Illinois at Chicago, the Lovelace Respiratory Research Institute, and the Michael E. Keeling Center for Comparative Medicine and Research. Irradiated macaques were ~3-10 years of age at time of irradiation and received 5.0-8.5 Gy total body gamma irradiation. Other details are shown in **Table 2.1**.

Sample Preparation: Sections of left ventricle were obtained from animals that had died or were euthanized due to morbidity. At necropsy, subjects were flushed with saline, and sections of myocardium were removed and flash frozen for later analysis. Tissue was partially pulverized under liquid N2 and further homogenized with a bead mill. Total RNA was isolated and purified using a Zymo Quick DNA/RNA Miniprep Plus Kit to retain miRNAs.

Table 2.1. Subject Details	
Controls (n=15)	
Age at death (Years, Mean ± SD)	10.59 ± 3.56
Irradiated (n=23)	
Age at death (Years, Mean ± SD)	11.15 ± 4.68
Irradiation age (Years, Mean ± SD)	4.66 ± 2.18
Years since irradiation (Years, Mean ± SD)	6.48 ± 3.84
Radiation dose (Gy, Mean ± SD)	7.10 ± 0.60

RNAseq Methods: cDNA Library Preparation and Sequencing Methods Total RNA was used to prepare cDNA libraries with the SMARTert Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (TaKaRa Bio USA Inc., Mountain View, CA). Briefly, 8 ng of total RNA was subjected to fragmentation followed by reverse-transcription. Illumina sequencing adaptors were attached during PCR amplification and the double-stranded cDNA was purified using AMPure XP magnetic beads. Finally, the cDNA was subjected to rRNA depletion and the stranded libraries were pre-amplified with PCR. The library size distribution was validated and quality inspected on an Agilent 2100 Bioanalyzer using a High Sensitivity DNA chip (Agilent Technologies). The quantity of each cDNA library was measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). Libraries were sequenced to a read depth of .35M reads per sample using 1 x 75 bp single-end (SE) sequencing (Illumina, San Diego, CA) on the Illumina NextSeq 500. **miRNAseq Methods:** miRNAseq libraries were generated using a Trilink Biotechnologies CleanTag® Small RNA Library Prep Kit.

mRNA analysis: Raw sequences were examined for quality using FASTQC. Both Phred scores (probability a base was called correctly) and GC content were observed on a per base and per sequence level to look for abnormalities and trim the reads accordingly. After quality control of the reads was conducted, alignment was performed using STAR two pass alignment. Reads were aligned to the *Macaca mulatta* assembly (Mmul_10) and scored on how well they corresponded to the reference genome and whether or not they map to multiple positions across the genome. Low scoring reads, usually short and poor quality reads, were not retained (Mapping Quality Score < 2). Post-alignment, reads were quantified at the gene level using the program featureCounts, and DESeq2 was used to transform gene counts and estimate fold-change values for differentially expressed genes. Genes with an average read count below 5 were dropped to remove noisy and lowly expressed genes. Samples with low RNA Integrity Numbers (RIN) scores (RIN < 6) were removed from the analysis. The normalized values generated from the DESeq2 transformation were used to compute a principal component analysis of the expression values to identify batch effects and outliers.

Approaches to further analysis were recently revised. Differential expression analysis was conducted in DESeq2 where the gene expression values were evaluated as the outcome in a negative binomial generalized linear model. Whether or

not the non-human primate had been irradiated was the predictor and the age at death was included as a covariate. A Benjamini-Hochberg false discovery rate (FDR) was applied to the unadjusted p-value to account for multiple comparisons. A genomic control was implemented to adjust for inflation and help make results more reproducible. Genes that met a genomic adjusted p-value threshold of 0.001 (the point of deviation on the corrected pp-plots) and demonstrated a fold-change > 1.3 were reported (**Table 2.2**). A second tier of results that met a FDR p-value < 0.01 (not adjusted for inflation, only multiple comparisons) and demonstrated a fold-change > 1.3 were retained to provide additional information for pathway analyses.

Results were analyzed in IPA, STRING, and MCODE to find biological pathways enriched between irradiated and non-irradiated samples (Figure 1, Table 4, Table 5). STRING was used to obtain protein-protein interactions for all genes that met a FDR p-value < 0.01 (not adjusted for inflation, only multiple comparisons) and demonstrated a fold-change > 1.3. STRING was applied to find only “High confidence” protein-protein interactions with options for “textmining” and “neighborhood” disabled. MCODE was applied to the remaining interactions to obtain a set of highly interconnected gene clusters. Genes meeting the genomic adjusted p-value threshold < 0.001 remained the primary focal points in the pathway analyses, but the second tier of genes were used to improve performance of the pathway analysis tools and obtain better evaluations of pathway enrichment. MCODE clusters that did not consist of more than 15 genes or that did not contain at least one gene meeting the genomic adjusted p-value threshold < 0.001 were removed and the biological functions of each of the remaining clusters were identified through the Reactome.

miRNA analysis: Raw sequences were examined for quality using FASTQC. Both Phred scores (i.e., the probability a base was correctly identified) and GC content were observed on a per base and per sequence level to evaluate for abnormalities and to trim reads accordingly. After quality control, alignment and feature quantification steps were implemented with a modified QuickMIRSeq pipeline. Sequence information for *Macaca mulatta* (Mmul_8.0.1) was retrieved from Ensemble (release 90) and sequence information for *Homo sapiens* (GRCh38/hg38) was retrieved from Ensemble (release 99). Sequence information for known mature miRNA was retrieved from miRBase and extracted for the genomes of both species. At the time, only miRNA information was available for Mmul_8.0.1, which is the reason for using this genome build rather than the updated build Mmul_10 that was used for the RNA-seq analysis. Bowtie index libraries were constructed for alignment with Bowtie and miRNA quantification was computed within QuickMIRSeq. QuickMIRSeq collapses identical reads into unique reads and then jointly maps the unique reads across multiple samples; this process includes remapping the mismatched miRNA reads with the reference genome to reduce false hits, and it also accounts for strand information to provide more accurate read mapping. Read counts were obtained for both *Macaca mulatta* and *Homo sapiens*. miRNAs were filtered if more than 60% of the samples were zero or if the average read count was < 2 across all samples.

Read counts were normalized using DESeq2’s median of ratios method and a principal component analysis was performed with the normalized values to observe for outlier samples and to evaluate the degree of similarity between the *Macaca mulatta* and *Homo sapiens* alignments. Due to a high degree of similarity between the two alignments and more recent updates to the human genome along with better annotation, downstream analysis was conducted on the alignment to the human miRNAs. The differential expression analysis was computed using DESeq2’s negative binomial regression model. Differential expression analysis modeled gene expression as the outcome and irradiation status as the predictor with age at death included as a covariate. PP plots were examined for inflation and a Benjamini-Hochberg false discovery rate (FDR) procedure was implemented to adjust for multiple comparisons.

mRNA-miRNA correlation analysis: To correlate mRNA and miRNA expression values, the DESeq2 normalized read counts were retained for all miRNA and mRNA meeting their respective quality control criteria. Only the mRNAs that met a genomic adjusted p-value threshold of 0.001 (the point of deviation on the corrected pp-plots) and demonstrated a fold-change > 1.3 were correlated with all miRNAs to find which miRNAs demonstrated an inverse relationship with the significantly differentially expressed mRNAs. A histogram of all of the correlation values was made to observe the distribution of the correlations between miRNAs and mRNAs. Any miRNA-mRNA correlation coefficients < -0.4 were retained as potentially interesting miRNA-mRNA relationships.

Results Summary

- Samples:
 - 43 samples sequenced, 3 samples with low RIN scores dropped from analysis
 - 15 controls, 23 irradiated (**Table 2.1**)
- mRNA
 - 32,871 transcripts detected with at least 1 read count
 - 17,037 transcripts with average read count > 5 retained for DE analysis
 - 626 met a DESeq2 FDR < 0.01 (not adjusted for inflation) and FC > 1.3
 - 18 genes met genomic-corrected p-value < 0.001 and FC > 1.3 (**Table 2.2**)
- miRNA
 - Mmul_8.0.1 alignment – 795 detected with at least 1 read count
 - Mmul_8.0.1 alignment – 468 meeting criteria (< 60% samples are zero, average read count > 2)
 - Mmul_8.0.1 alignment – no miRNAs met FDR < 0.05 and fold-change > 1.3
 - GRCh38/hg38 alignment – 1,293 detected with at least 1 read count
 - GRCh38/hg38 alignment – 536 meeting criteria (< 60% samples are zero, average read count > 2)
 - Proceed to DE analysis with GRCh38/hg38
 - GRCh38/hg38 alignment – no miRNAs met FDR < 0.05 and fold-change > 1.3
 - GRCh38/hg38 alignment – 26 miRNAs met raw p-value < 0.05 and fold-change > 1.3 (**Table 2.3**)
- miRNA-mRNA
 - 10 relationships with correlation coefficient < -0.4 (**Table 2.6**)
- Major pathways (**Figure 1, Table 2.4**) and upstream regulators (**Table 2.5**)

Table 2.2. Differentially expressed genes with genomic adjusted p-value < 0.001 and fold-change > 1.3. The top differentially expressed genes listed with their log₂(fold-change), mean expression level by group, unadjusted p-value, unadjusted false discovery rate (FDR), and genomic adjusted p-value.

Table 2:		Normalized Expression Level (95% CI)		p-value	FDR	Genomic adjusted p- value
Gene	log ₂ (FC)	Control	Irradiated			
	-					
RET	1.87	159 (120:198)	42 (33:198)	5.81x10 ⁻¹⁴	9.89x10 ⁻¹⁰	1.60x10 ⁻⁰⁵
PAPPA	1.62	115 (96:135)	353 (267:135)	2.02x10 ⁻¹²	1.72x10 ⁻⁰⁸	5.37x10 ⁻⁰⁵
	-					
CNTN5	2.39	232 (102:361)	48 (29:361)	4.10x10 ⁻¹¹	1.99x10 ⁻⁰⁷	1.50x10 ⁻⁰⁴
SERPINA3	4.68	571 (244:898)	16769 (-465:898)	5.15x10 ⁻¹¹	1.99x10 ⁻⁰⁷	1.63x10 ⁻⁰⁴
PLAC8	2.88	11 (7:15)	83 (48:15)	5.83x10 ⁻¹¹	1.99x10 ⁻⁰⁷	1.70x10 ⁻⁰⁴
CDKL5	0.38	509 (491:527)	660 (625:527)	1.53x10 ⁻¹⁰	3.92x10 ⁻⁰⁷	2.36x10 ⁻⁰⁴
CP	2.95	196 (73:320)	1331 (809:320)	1.61x10 ⁻¹⁰	3.92x10 ⁻⁰⁷	2.41x10 ⁻⁰⁴
SELP	1.93	65 (51:79)	251 (164:79)	3.08x10 ⁻¹⁰	6.56x10 ⁻⁰⁷	3.01x10 ⁻⁰⁴
	-					
C13H2orf40	1.13	280 (228:332)	126 (105:332)	5.04x10 ⁻¹⁰	9.53x10 ⁻⁰⁷	3.56x10 ⁻⁰⁴
S100A8	3.67	33 (5:60)	393 (172:60)	5.65x10 ⁻¹⁰	9.62x10 ⁻⁰⁷	3.70x10 ⁻⁰⁴
SLCO2A1	1.25	40 (32:49)	98 (77:49)	7.38x10 ⁻¹⁰	1.14x10 ⁻⁰⁶	4.06x10 ⁻⁰⁴
GRK5	0.71	798 (716:881)	1302 (1162:881)	1.09x10 ⁻⁰⁹	1.49x10 ⁻⁰⁶	4.64x10 ⁻⁰⁴
CRLF1	2.53	7 (2:12)	38 (24:12)	1.14x10 ⁻⁰⁹	1.49x10 ⁻⁰⁶	4.71x10 ⁻⁰⁴
	-					
ANGPTL7	2.90	2111 (1478:2744)	292 (117:2744)	1.87x10 ⁻⁰⁹	2.27x10 ⁻⁰⁶	5.59x10 ⁻⁰⁴
SMAD6	1.05	154 (133:175)	319 (262:175)	2.42x10 ⁻⁰⁹	2.74x10 ⁻⁰⁶	6.11x10 ⁻⁰⁴
C14H11orf87	2.15	162 (115:208)	717 (382:208)	3.55x10 ⁻⁰⁹	3.79x10 ⁻⁰⁶	6.98x10 ⁻⁰⁴
VWF	1.29	2326 (1913:2739)	5669 (4195:2739)	4.10x10 ⁻⁰⁹	4.10x10 ⁻⁰⁶	7.33x10 ⁻⁰⁴
		135				
NRG3	1.89	(103:168)	514 (372:657)	8.55x10 ⁻⁰⁹	8.09x10 ⁻⁰⁶	9.46x10 ⁻⁰⁴

Table 2.3. Differentially expressed miRNAs p-value < 0.05 and fold-change > 1.3. The top differentially expressed miRNAs listed with their log₂(fold-change), mean expression level by group, and unadjusted p-value.

Table 2.3		Normalized Expression Level (95% CI)		
miRNA	log₂(FoldChange)	Control	Irradiated	p-value
hsa-miR-1468-5p	-0.70	13.2 (6.9:9.5)	8.2 (9.1:9.5)	1.91x10 ⁻⁰³
hsa-miR-663a	1.78	2.5 (4:12.2)	8.1 (1.2:12.2)	2.13x10 ⁻⁰³
hsa-miR-122-5p	2.41	32.7 (-32.4:422.1)	194.8 (-0.1:422.1)	5.00x10 ⁻⁰³
hsa-miR-1248	0.99	10.6 (15.2:27)	21.1 (5.8:27)	5.15x10 ⁻⁰³
hsa-miR-619-5p	1.36	4.7 (7.8:15.4)	11.6 (2.3:15.4)	5.29x10 ⁻⁰³
hsa-miR-4485-3p	1.20	1474.2 (2156.8:5352.3)	3754.5 (942:5352.3)	5.66x10 ⁻⁰³
hsa-miR-4286	-0.53	34.5 (20.3:28.2)	24.3 (27.5:28.2)	7.02x10 ⁻⁰³
hsa-let-7f-1-3p	-0.82	4.1 (1.8:3.3)	2.6 (3.1:3.3)	9.00x10 ⁻⁰³
hsa-miR-5697	0.66	4.2 (5.7:8.1)	6.9 (2.9:8.1)	1.15x10 ⁻⁰²
hsa-miR-5095	1.87	0.6 (0.8:2.8)	1.8 (0.3:2.8)	1.25x10 ⁻⁰²
hsa-let-7g-3p	-0.60	9.9 (5.2:8.1)	6.7 (7.8:8.1)	1.36x10 ⁻⁰²
hsa-miR-1273f	1.64	0.9 (1.4:4.1)	2.7 (0.3:4.1)	1.42x10 ⁻⁰²
hsa-miR-495-3p	-0.66	7.7 (3.6:6.1)	4.9 (5.8:6.1)	1.66x10 ⁻⁰²
hsa-miR-1273g-3p	1.24	10.7 (14.7:38.1)	26.4 (5.4:38.1)	1.87x10 ⁻⁰²
hsa-miR-422a	1.20	1 (1.6:3.3)	2.4 (0.3:3.3)	1.91x10 ⁻⁰²
hsa-miR-7641	0.93	11.2 (14.8:27.5)	21.1 (6.5:27.5)	2.06x10 ⁻⁰²
hsa-miR-877-5p	-0.66	48.8 (24.1:36.9)	30.5 (17.2:36.9)	2.17x10 ⁻⁰²
hsa-miR-4454	0.58	79.9 (94.2:143.4)	118.8 (47.4:143.4)	2.18x10 ⁻⁰²
hsa-miR-3591-5p	-0.53	138.9 (75:117.3)	96.2 (106.9:117.3)	2.21x10 ⁻⁰²
hsa-miR-375	1.22	2 (2.7:7.1)	4.9 (0.7:7.1)	2.24x10 ⁻⁰²
hsa-miR-5096	1.05	4.3 (5.7:11.5)	8.6 (2.4:11.5)	2.52x10 ⁻⁰²
hsa-miR-205-5p	1.88	2.1 (1.1:13.9)	7.5 (0.8:13.9)	2.79x10 ⁻⁰²
hsa-miR-1973	1.07	8.4 (11.1:23.7)	17.4 (4.4:23.7)	3.36x10 ⁻⁰²
hsa-miR-412-5p	-0.50	13.2 (7.3:11.2)	9.3 (9.9:11.2)	3.66x10 ⁻⁰²
hsa-miR-6501-5p	-0.82	3.7 (1.2:2.9)	2 (2.2:2.9)	3.67x10 ⁻⁰²
hsa-miR-203a-3p	0.78	3.8 (4.6:8.9)	6.8 (2.4:8.9)	3.88x10 ⁻⁰²

Table 2.4. IPA canonical pathways with $-\log(p\text{-value}) > 6$. IPA canonical pathways are listed with their respective ratio, z-score, and $-\log(p\text{-value})$. The ratio represents the proportion of the total genes from the biological pathway captured by the input list of differentially expressed genes. The z-score represents a measure of the predicted direction of the pathway activity. Generally, a positive z-score indicates an overall increase of the pathway activity in the irradiated samples.

Ingenuity Canonical Pathways	$-\log(p\text{-value})$	Ratio	z-score
Hepatic Fibrosis / Hepatic Stellate Cell Activation	12.90	0.14	-
Granulocyte Adhesion and Diapedesis	12.90	0.14	-
Osteoarthritis Pathway	9.57	0.11	1.342
Agranulocyte Adhesion and Diapedesis	9.40	0.11	-
Phagosome Formation	9.05	0.14	-
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	7.24	0.08	-
IL-10 Signaling	7.12	0.16	-
Complement System	6.97	0.24	1.342
Hepatic Fibrosis Signaling Pathway	6.74	0.07	2.041
LXR/RXR Activation	6.02	0.11	-1.069

Table 2.5. IPA upstream regulators with predicted activation states. IPA upstream regulators are listed with their respective $\log_2(\text{FoldChange})$, predicted activation state, activation z-score, and p-value. The activation z-score is computed to infer the activation states of predicted transcriptional regulators relative to the provided gene expression profile.

Upstream Regulator	$\log_2(\text{FoldChange})$	Predicted Activation State	Activation z-score	Overlap p-value
NOS2	-1.808	Activated	2.051	5.46×10^{-06}
STAT5A	0.520	Activated	2.120	3.53×10^{-06}
PTPN6	0.704	Inhibited	-2.006	8.99×10^{-07}
TGM2	0.743	Activated	3.109	1.56×10^{-08}
STAT3	0.786	Activated	3.597	3.15×10^{-14}
C5AR1	1.016	Activated	2.778	3.38×10^{-06}
TLR2	1.205	Activated	3.706	3.64×10^{-08}
TIMP1	1.699	Activated	2.236	1.41×10^{-03}
SELP	1.927	Activated	2.219	1.09×10^{-03}
CXCL8	2.080	Activated	2.653	4.50×10^{-05}
LIF	2.583	Activated	2.909	5.69×10^{-05}
OSM	2.888	Activated	4.419	1.69×10^{-12}
S100A9	3.233	Activated	3.214	6.13×10^{-11}
IL6	3.521	Activated	3.668	1.39×10^{-15}
S100A8	3.674	Activated	2.904	3.23×10^{-11}

Table 2.6. mRNA-miRNA correlating with a correlation coefficient < -0.4. mRNAs are listed with the corresponding miRNAs that are negatively correlated with expression.

mRNA	miRNA	Correlation Coefficient
ANGPTL7	hsa-miR-378a-5p	-0.523
ANGPTL7	hsa-miR-378a-3p	-0.502
PAPPA	hsa-miR-502-5p	-0.460
SMAD6	hsa-let-7g-3p	-0.443
GRK5	hsa-let-7f-1-3p	-0.439
RET	hsa-miR-378a-5p	-0.438
ANGPTL7	hsa-miR-378e	-0.436
PAPPA	hsa-miR-532-5p	-0.406
PLAC8	hsa-miR-92b-5p	-0.403
PAPPA	hsa-miR-500a-5p	-0.400

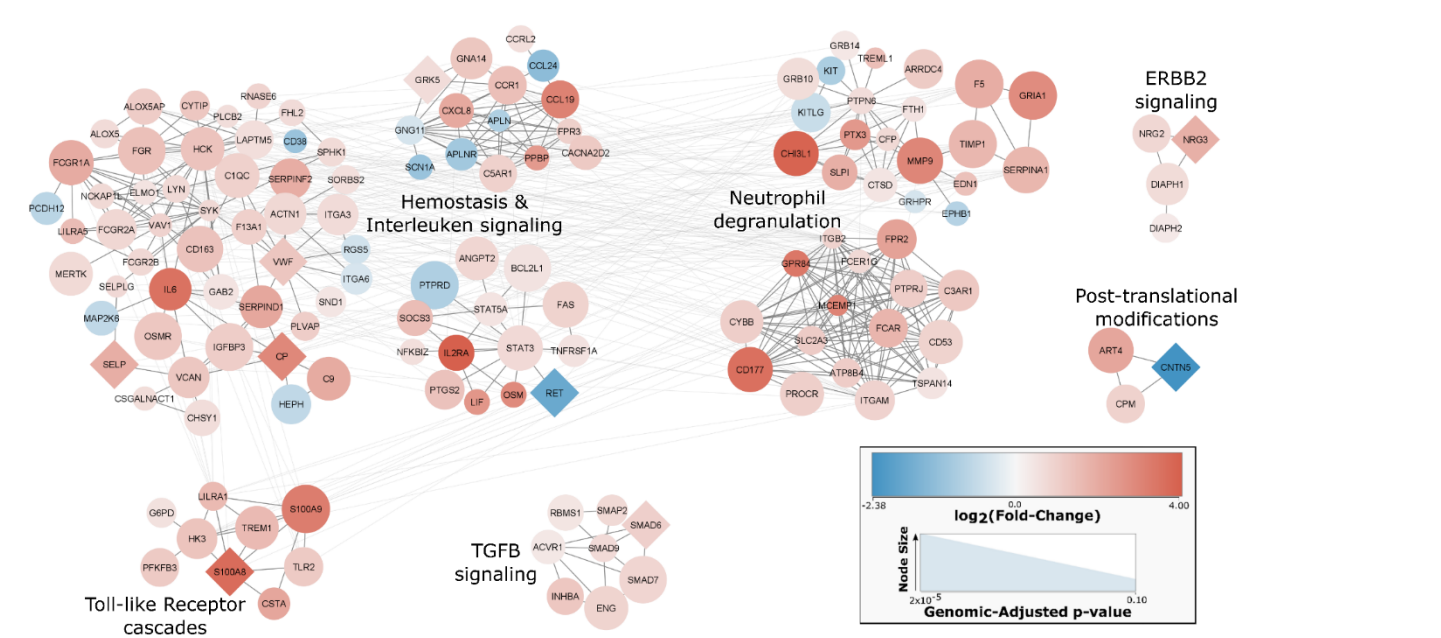


Figure 2.1. Protein–protein interaction network of differentially expressed genes significantly altered between irradiated and control samples. Protein interactions were obtained from STRING’s protein interaction database. MCODE was used to find tightly connected clusters of interactions that are labeled according to function defined in the REACTOME and KEGG. Mean difference in expression values is visualized by the blue-to-red color scale. Blue indicates lower expression in the irradiated samples, while red indicates an increase in expression in the irradiated samples. The size of the node reflects statistical significance and diamonds represent genes that have a genomic-adjusted p-value < 0.001.

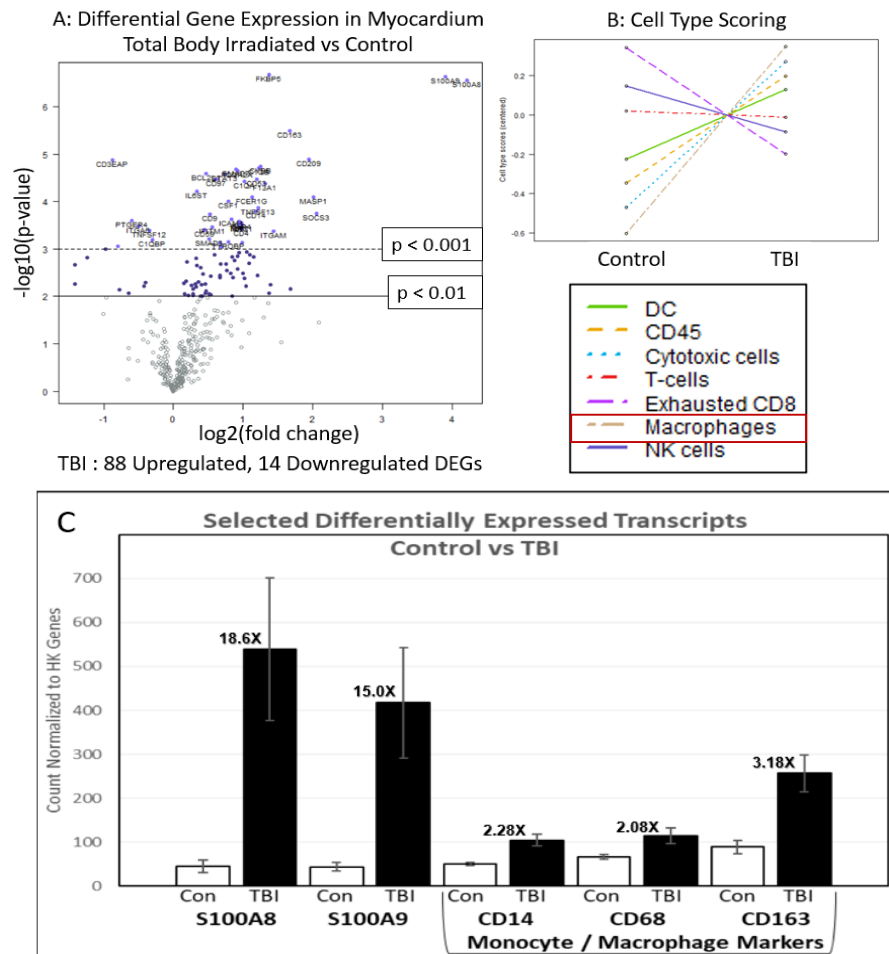
Summary of Myocardial RNAseq and miRNAseq Analyses: Key pathways have been identified which provide insights into processes underlying DEARE on cardiac structure and function. A manuscript draft has been developed and additional analyses are underway to expand these findings.

nCounter XT Gene Expression Assay: Purified RNA samples were diluted with RNase free water to a final concentration of 10 ng/μL. Samples were hybridized with a Nanostring NHP Immunology Reporter CodeSet Panel and incubated at 65°C for 16 hours after which temperature ramped down to 4°C until analyses. Hybridized samples were read using an nCounter SPRINT Profiler Instrument. **Statistical Analysis:** Analysis was performed using nCounter Advanced Analysis Software (version 2.0.115) and Statistica (version 13.1).

Results: At $p=0.05$, 125 transcripts were higher and 23 genes were lower in TBI myocardium relative to control (**Figure 2A**). The top ten upregulated transcripts were: S100A8, S100A9, C6, SOCS3, MASP1, CD209, FOS, CD163, ITGAM, and CCL2. Numerous transcripts for macrophage markers were significantly higher in the TBI heart, including CD14, CD68, CD163. Upregulated genes associated with M1 (classically activated, proinflammatory) macrophage phenotype included MAPK3, TLR1 and FKBP5. Upregulated genes associated with the M2 (alternatively activated, profibrotic) phenotype included CD209, C1QB, SMAD7, MASP1, MRC1, CYBB, IL13RA1, CEBPB and CSF1. Amongst all upregulated genes, S100A8 and S100A9 showed the greatest increases in TBI (**Figure 2.2A, C**). The top ten downregulated transcripts were: CD38, KIT, CCL24, THY1, DPP4, DEFb1, CD3EAP, MYL2, MAP2K6, and OAS2. **Figure 2.2B** shows relative differences in markers associated with specific immune cell types between control and TBI. Genes related to macrophages were the showed the greatest increase in TBI myocardium, followed by signatures representative of cytotoxic cells, CD45+ cells (lymphocyte common antigen (LCA) expressed on all leucocytes) and dendritic cells. Differential content of transcripts for the monocyte marker CD14 and key macrophage markers CD68, CD163 are shown in **Figure 2.2C**.

Figure 2.2: Immunology Related Targeted Myocardial Gene Expression

Analyses: **A:** Volcano plot of the statistical significance vs. fold difference between control and TBI myocardial genes. **B:** Plot showing relative differences in cell type specific gene expression signatures between control and TBI groups. **C:** Selected differentially expressed transcripts in control vs TBI.



Ongoing miRNA analyses: We recently acquired miRNA profiles on the myocardial RNA using Nanostring miRNA analysis technology which is currently being analyzed and which will be compared to the miRNAseq data described above. In addition, serum samples from both the RSC and the PRC are currently being prepared for miRNA analyses using this approach.

3.2 Cardiac Functional, Structural, and Cellular Alterations in Rhesus Macaque Survivors of Ionizing Radiation Exposure (manuscript posted in online preprint server and submitted to journal)

Abstract:

Rationale: Radiation induced heart disease (RIHD) is a significant concern for survivors of ionizing radiation exposures through accidental, malicious, or medical means.

Objective: The purpose of this study was to investigate the effects of total body irradiation (TBI) on the heart in male rhesus macaques, a translational non-human primate (NHP) model.

Methods and Results: Echocardiography was performed on survivors of a single dose (6.4-8.5 Gy) of TBI (n=34) and non-irradiated controls (n=26) divided into longer (LT IRR) and shorter term (ST IRR) survivors and controls. LT IRR had increased transmitral early filling velocities (E), decreased early mitral annular descent velocities (e'), higher E/e' ratios and decreased early (e') to late (a') mitral annular descent velocity ratios (e'/a') compared to LT CTL (all p≤0.05), indicating diastolic dysfunction. Apical 4 chamber-derived stroke volume, cardiac output, and end-diastolic volume were also lower in LT IRR than controls (all p ≤ 0.05). ST IRR animals had similar alterations in LV diastolic function but not in cardiac volumetric measures. Histochemical and immunohistochemical analyses of sections of LV, IVS, and RV from deceased irradiated animals (n=17) exposed to a single dose (6.9-8.05) Gy TBI and deceased non-irradiated controls (n=12) demonstrated that IRR animals had increased LV fibrosis decreased LV and IVS capillary density, increased pan-cardiac fibroblast staining area, increased pan-cardiac macrophage staining, increased LV and IVS M2 macrophages, and increased pan-cardiac M1 macrophages (all p<0.05).

Conclusions: LV diastolic dysfunction due to radiation exposure may be a physiological consequence of decreased capillary density, activation and expansion of fibroblasts, and increased accumulation of pro-fibrotic and pro-inflammatory macrophages, all of which lead to increased myocardial stiffness due to fibrosis. Collectively, these findings provide insight on the delayed effects of acute ionizing radiation exposure and indicate that therapies targeting macrophage regulation of fibrosis may mitigate radiation induced heart disease.

RESULTS

Echocardiographic phenotyping:

Two groups of animals were chosen from our available dataset of 60 NHP for the current study of echocardiographic phenotypes, 1) an older, longer term survivor group of 14 mature adult (12.6 to 20.3 year old) long-term survivors (LT IRR) exposed to a single TBI dose of 6.5 to 8.4 Gy approximately 9.7 to 11.6 years earlier, which were compared to 15 age-matched, nonirradiated controls (LT CTL)(**Table 2.7**), and younger, shorter term survivor group (ST IRR) of 20 young adult (4.7 to 6.0 year old) exposed to a single TBI dose of 6.4-8.5 Gy approximately 2.16 to 2.96 years earlier, which were compared to 10 age-matched, nonirradiated controls (ST CTL) (**Table 2.7**).

Table 2.7: Animal demographics of Previously Irradiated Long Term, Short Term, and Deceased Male Rhesus Macaque Survivors (LT IRR, ST IRR, Deceased IRR) and Controls (LT CTL, ST CTL, Deceased CTL)

	LT IRR (N=14)	LT CTL (N=15)	P	ST IRR (N=20)	ST CTL (N=10)	P	Deceased IRR (N=17)	Deceased CTL (N=12)	P
Age (Years)	14.96 (0.63)	14.15 (0.28)	0.25	5.47 (0.08)	5.25 (0.33)	0.40	12.14 (0.96)	9.33 (0.92)	0.47
Years Since Irradiation	10.48 (0.18)	---	---	2.51 (0.07)	---	---	5.80 (0.55)	---	---
Dose (Gy)	7.40 (0.16)	---	---	7.01 (0.14)	---	---	6.93 (0.13)	---	---
Body Weight (kg)	11.31 (1.11)	14.62 (1.25)	0.06	9.22 (0.56)	8.77 (0.35)	0.86	13.25 (0.85)	10.90 (0.54)	0.04^a
Body Surface Area (m ²)	0.48 (0.03)	0.55 (0.03)	0.16	0.42 (0.29)	0.41 (0.01)	0.97	0.54 (0.02)	0.47 (0.02)	0.06

Data presented as mean (SEM)

Echocardiographic Phenotypes of Long Term Survivors

Structural and functional differences between LT IRR and LT CTL animals were assessed using B mode, M mode, and pulsed wave and tissue Doppler echocardiography. LT IRR animals differed from LT CTL in several diastolic function measures including increased transmitral early filling velocities (E) and E wave deceleration slope and decreased early mitral annular descent velocities (e') that resulted in higher E/ e' ratios and decreased early (e') to late (a') mitral annular descent velocity ratios (e'/a') (all $p \leq 0.05$) (**Figure 2.3**). The remaining diastolic function measures of transmitral late filling velocity (A), a' , and E/A ratio were not different between groups. Stroke volume (biplane and apical 4 chamber views), cardiac output (apical 4 chamber view), and end diastolic volume (apical 4 chamber views), were lower in LT IRR than LT CTLs (all $p \leq 0.05$) and biplane measures of cardiac output and end diastolic volume tended to be lower as well (both $p=0.07$) (**Figure 2.3**). M mode measures of cardiac structure corroborated our previous findings of decreased left ventricular diameters at end systole (LVIDs) and diastole (LVIDd) in LT IRR animals compared to LT CTLs, and also showed tendencies for decreased LV posterior wall thickness at end systole (LVPWs) ($p=0.07$) and diastole (LVPWd) ($p=0.06$). No differences were observed in interventricular septal thicknesses at end systole (IVSs) or end diastole (IVSd) (both $p > 0.5$). Parasternal long axis measurement of the LV outflow tract diameter (LVOT) showed that LT IRR animals had significantly narrower outflow tract diameters compared to LT CTL ($p=0.01$). No differences in heart rate, blood pressures (systolic, diastolic, and mean), ejection fraction, fractional shortening, or left atrium volume and size measures; aortic root to left atrium ratio (AO/LA) and left atrial end systolic volume (biplane) (LA Vol BP) were found.

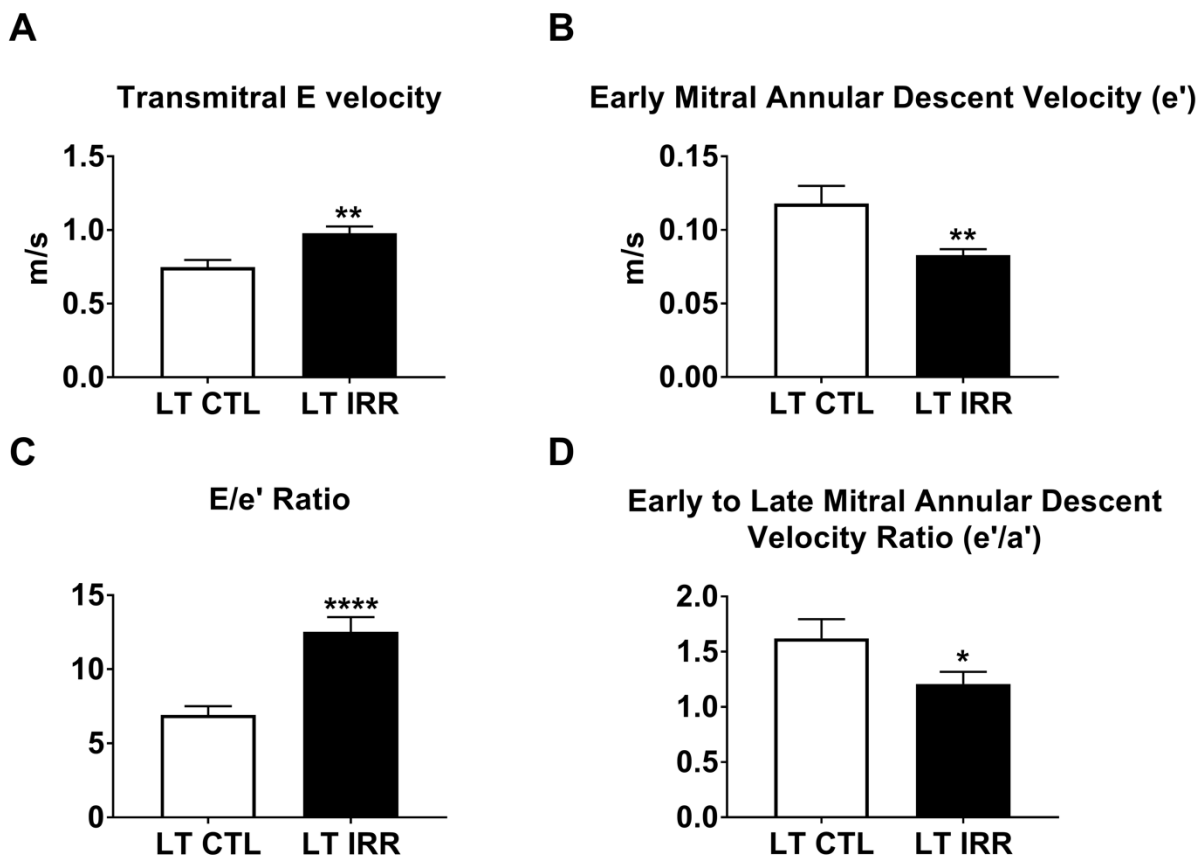


Figure 2.3 Echocardiographic Diastolic Function in Long Term Irradiated (LT IRR) and Long Term Controls (LT CTL): LT IRR animals had significantly increased transmitral early filling peak velocity (E) (Panel A), decreased early mitral annular descent velocity (e') (Panel B), increased E/ e' ratio (Panel C) and decreased early to late mitral annular descent velocity ratio (e'/a') (Panel D) compared to LT CTL animals. Data are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

Echocardiographic Phenotypes of Short Term Survivors

Structural and functional differences between ST IRR and ST CTL animals were evaluated similarly as LT IRR vs LT CTLs. Like LT IRR animals, ST IRR animals also had increased E/e' ratios and decreased e' and e'/a' ratios compared to ST CTLs (all $p \leq 0.05$) (**Figure 2.4**), while E , A , E/A ratio, and a' were not significantly different between groups. ST IRR and ST CTL did not differ in cardiac output, stroke volume, end diastolic volumes, heart rate, blood pressures (systolic diastolic, and mean), ejection fraction, fractional shortening, IVSd, ESV, LVIDs, LVIDd, AO/LA, or LA Vol BP.

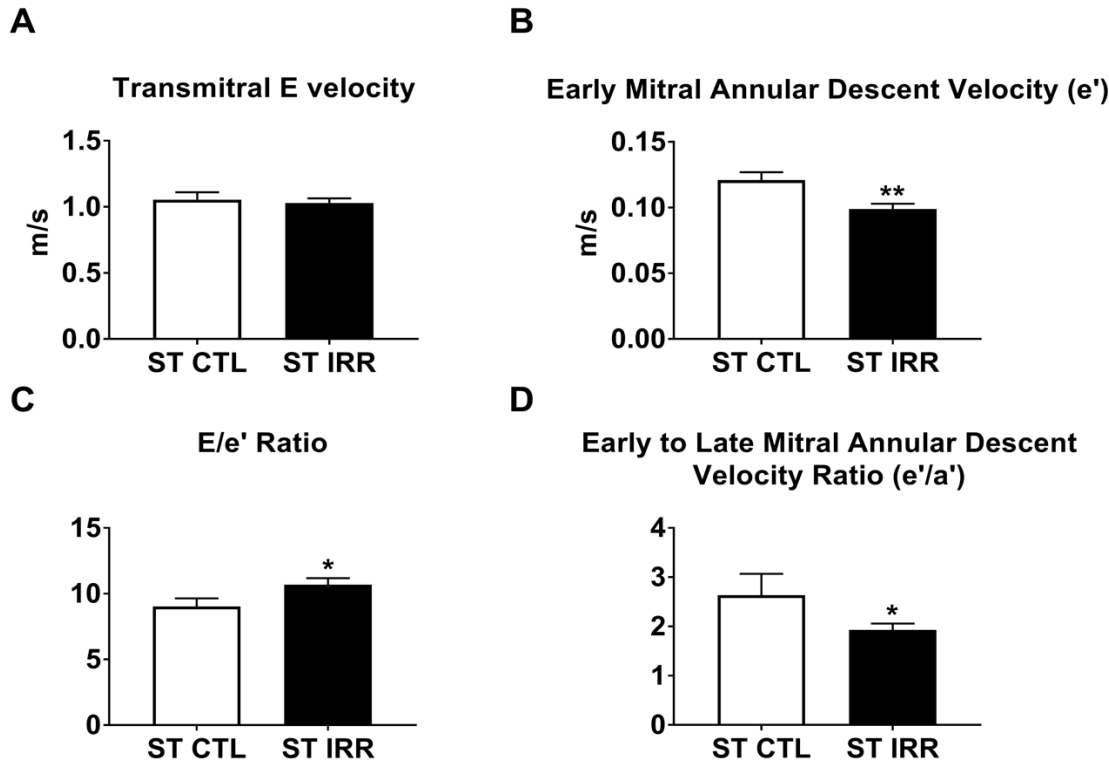


Figure 2.4 Echocardiographic Diastolic Function in Short Term Irradiated (ST IRR) and Short Term Controls (ST CTL): ST IRR animals had no differences in transmitral early filling peak velocity (E) (Panel A), but decreased early mitral annular descent velocity (e') (Panel B), increased E/e' ratio (Panel C) and decreased early to late mitral annular descent velocity ratio (e'/a') (Panel D) compared to ST CTL animals. Data are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$

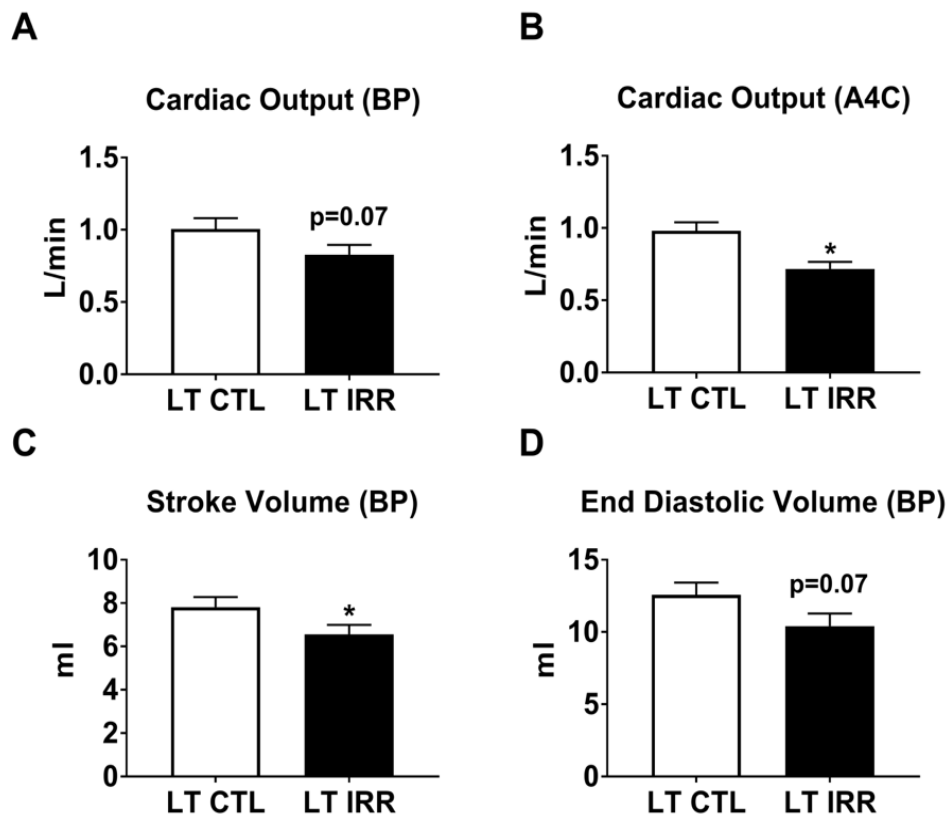


Figure 5 Echocardiographic Cardiac Volume Measures in Long Term Irradiated (LT IRR) and Long Term Controls (LT CTL): LT IRR animals tended to have decreased cardiac output calculated by method of disks measurements using the perpendicularly oriented apical 2 chamber (A2C) and apical 4 chamber (A4C) views resulting biplane measurement (BP) (Panel A). LT IRR animals did have significantly lower cardiac output when only the A4C chamber view was used (Panel B). Additionally, LT IRR animals had decreased stroke volume (BP measurement) and a tendency for decreased end diastolic volume (BP measurement). Data are mean $\pm$ SEM. * $p < 0.05$

Histological and Immunohistochemical Staining of the Left Ventricular, Interventricular Septal, and Right Ventricular Myocardium

Sections of LV, IVS, and RV from 17 deceased previously irradiated animals (Deceased IRR) and 12 deceased controls (Deceased CTL) were evaluated for structural and cellular components using histochemical and immunohistochemical stains for fibrosis (Masson's trichrome), capillary density (CD31), fibroblasts (S100A4), macrophages (CD68 and CD163) and M1 or M2 macrophages (pSTAT1/CD163 and c-Maf/CD163 respectively). IRR animals had significantly greater positive trichrome staining of the LV ($p<0.05$), and no significant difference in the RV or IVS compared to CTL animals. (Figure 2.6) Capillary density was reduced in the IRR group in the LV ($p<0.05$) and IVS ($p<0.01$), but not the RV. (Figure 2.6).

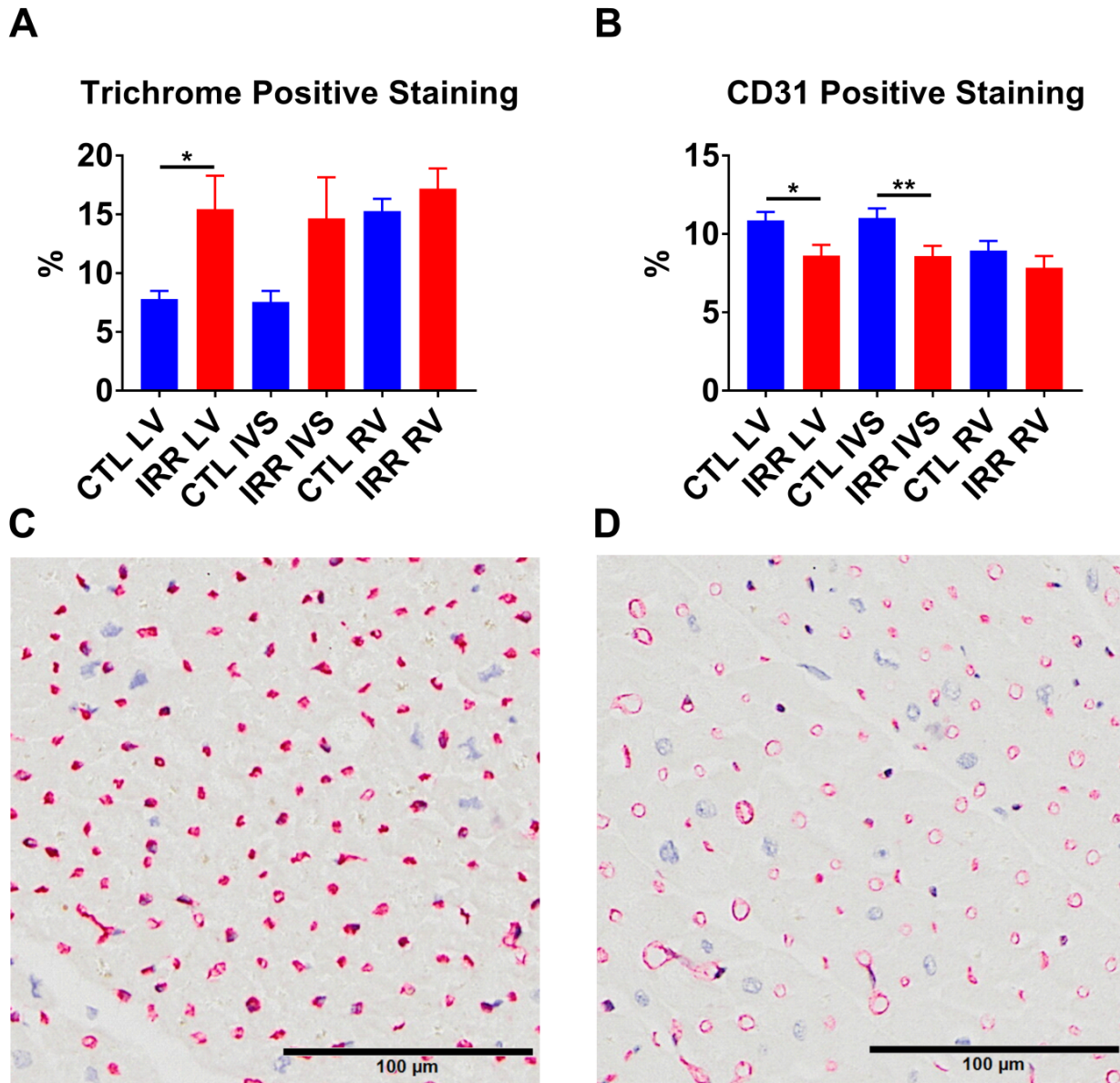


Figure 2.6 Fibrosis and Capillary Density Immunostaining in Deceased Irradiated (IRR) and Control (CTL) Animals: IRR animals had significantly increased percent positive trichrome staining of the left ventricle (LV) but not the interventricular septum (IVS) or right ventricle (RV) compared to CTL. IRR animals also had significantly decreased CD31 percent positive staining in the LV and IVS, but not RV compared to CTL (Panel B). Panels C and D are representative CD31 stained sections of LV mid-myocardium from a CTL and IRR animal respectively, demonstrating increased CD31 staining in CTLs. * $p<0.05$, ** $p<0.01$

IRR myocardium exhibited increased S100A4⁺ area in all cardiac sections (LV $p<0.01$, IVS $p<0.001$, and RV $p<0.05$) compared to CTL (**Figure 2.7**). In addition, approximately 100 fold less CD68⁺ macrophage staining area (LV mean= 8900 μm^2 , IVS mean=6134 μm^2 , RV =3484 μm^2) was observed relative to CD163⁺ macrophage staining area (LV mean= 905232 μm^2 , IVS mean= 720474 μm^2 , RV mean= 339053 μm^2) across all animals, and CD163 staining frequently co-localized with CD68 staining (**Figure 2.7**). Based on these findings we subsequently used CD163 as a pan-macrophage marker and used nuclear staining pSTAT1 or c-Maf to indicate M1 or M2 like macrophages respectively. Deceased IRR animals had increased CD163⁺ macrophage staining in the LV ($p<0.05$), IVS ($p<0.01$), and RV ($p<0.05$), increased c-Maf⁺/CD163⁺ macrophages in the LV ($p<0.05$) and IVS ($p<0.01$), but not RV, and increased pSTAT1⁺/CD163⁺ macrophages in the LV ($p<0.01$), IVS ($p<0.01$), and RV ($p<0.05$). (Figure 2.8) Finally, we compared the c-Maf⁺/CD163⁺ (M2) to pSTAT1⁺/CD163⁺ (M1) ratio and found that IRR animals had a significant decrease in the M2/M1 ratio across all assessed regions of the heart (all $p<0.05$). (Figure 2.8)

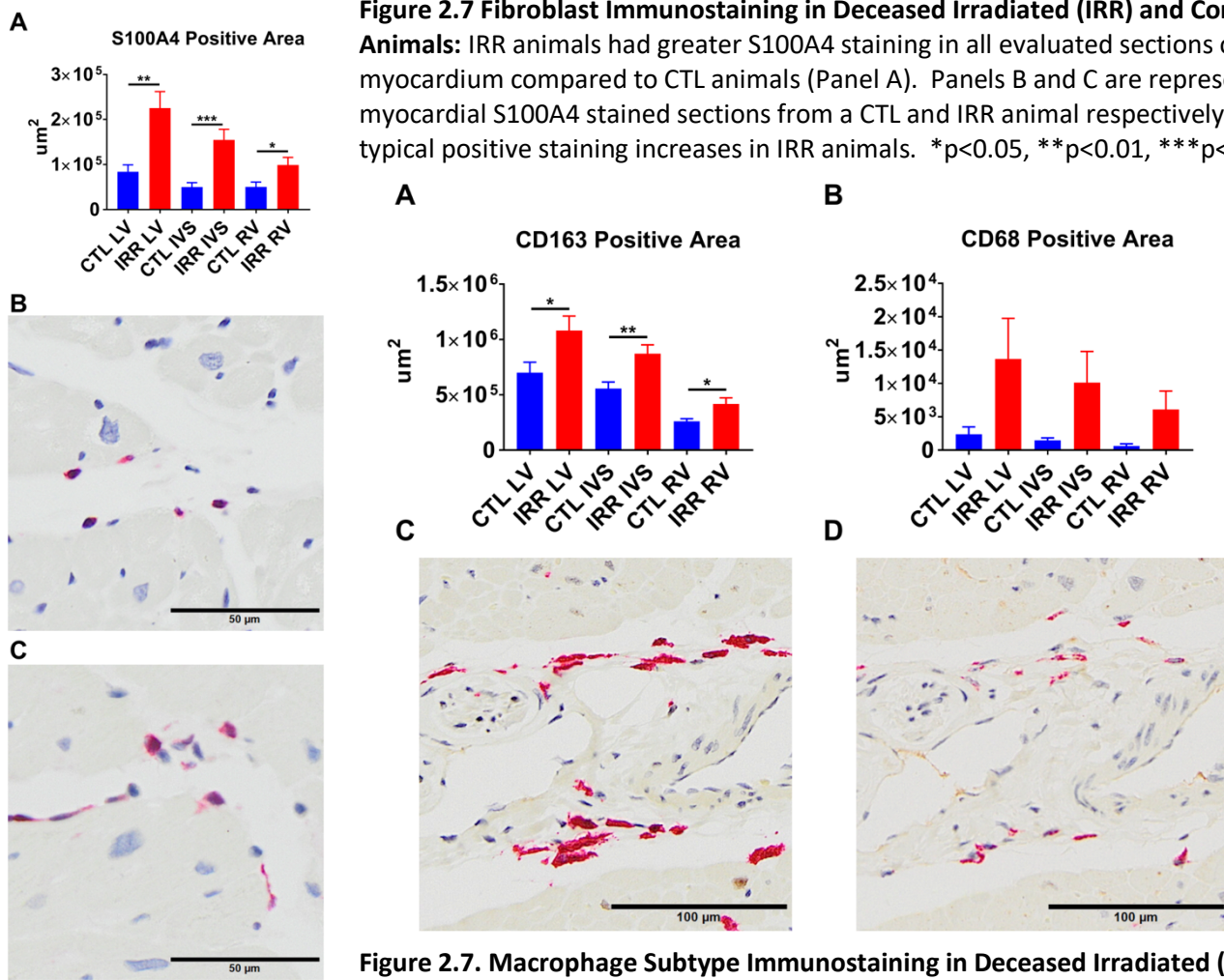


Figure 2.7 Fibroblast Immunostaining in Deceased Irradiated (IRR) and Control (CTL) Animals: IRR animals had greater S100A4 staining in all evaluated sections of myocardium compared to CTL animals (Panel A). Panels B and C are representative mid-myocardial S100A4 stained sections from a CTL and IRR animal respectively illustrating typical positive staining increases in IRR animals. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Figure 2.7. Macrophage Subtype Immunostaining in Deceased Irradiated (IRR) and Control (CTL) Animals: IRR animals had increased normalized CD163 positive staining area in the left ventricle (LV), interventricular septum (IVS), and right ventricle (RV) compared to controls (CTL) (Panel A). In contrast no differences were observed in CD68 staining which was highly variable across both groups (Panel B). Panels C and D are CD163 and CD68 stains respectively from the same animal in the same location illustrating that CD68⁺ macrophages were also often CD163⁺. * $p<0.05$, ** $p<0.01$

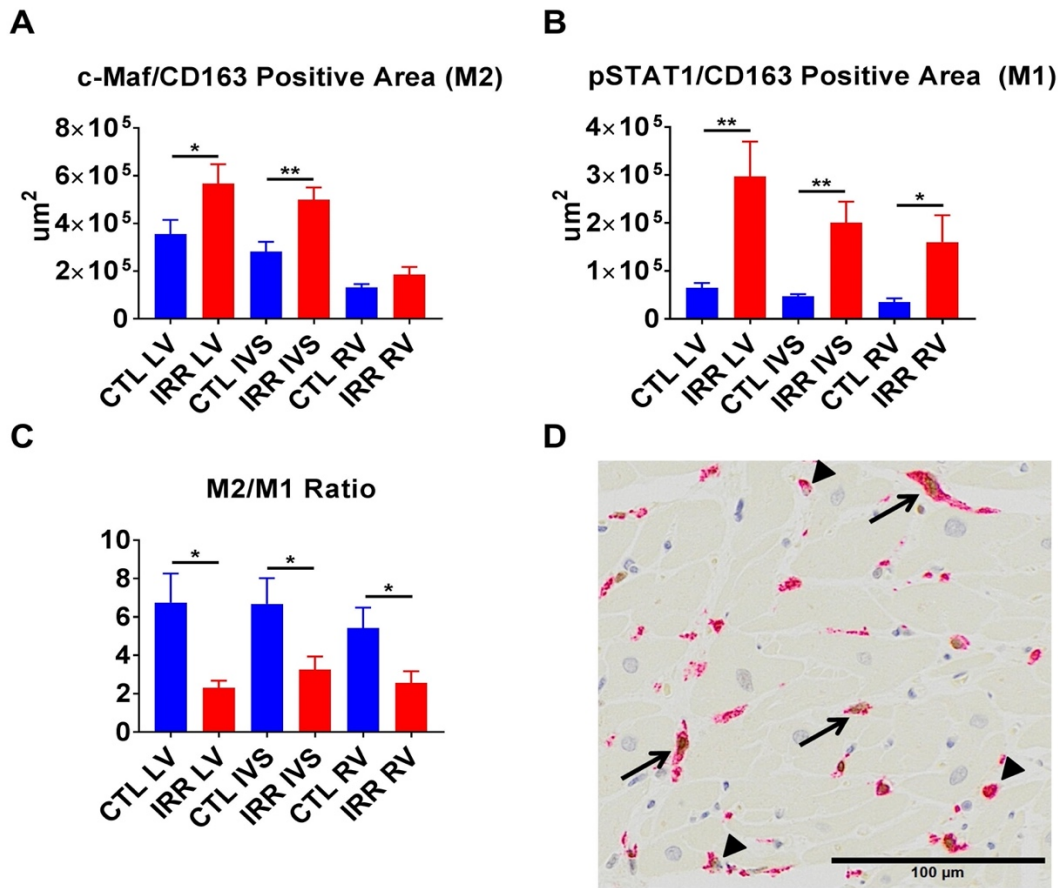
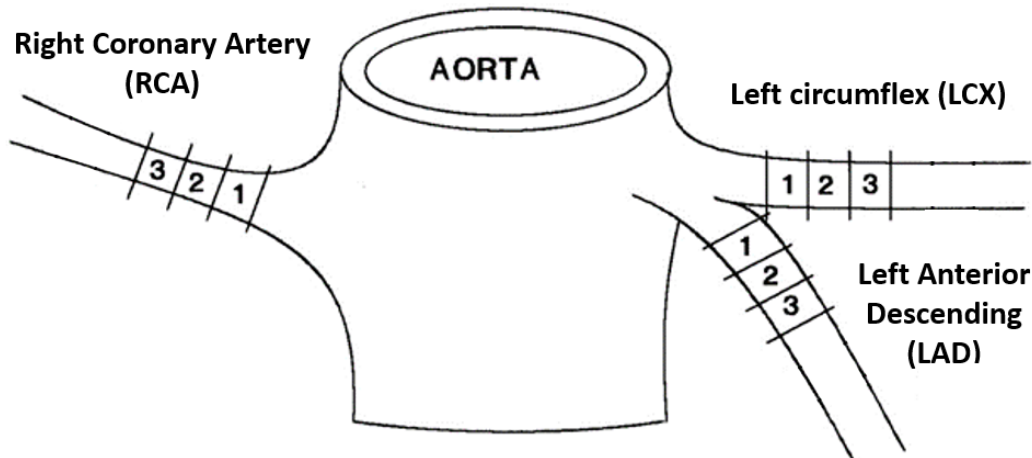


Figure 2.8. Macrophage Polarization in Irradiated (IRR) and Control (CTL) Myocardium: IRR animals had increased c-Maf⁺/CD163⁺ staining (M2 polarization) in the left ventricle (LV), interventricular septum (IVS), but not right ventricle (RV) compared to controls (CTL) (Panel A). Increased pSTAT1⁺/CD163⁺ staining (M1 polarization) was noted in all sections (LV, IVS, and RV) in IRR animals compared to CTL (Panel B). This translated to a decrease in the M2/M1 macrophage ratio across all sections in IRR animals (Panel C). Panel D is a representative dual stained image indicating dual stained c-Maf⁺/CD163⁺ macrophages (arrows) and single stained CD163⁺ macrophages (arrowheads) from the LV of an irradiated animal. **p*<0.05, ***p*<0.01

Conclusions: LV diastolic dysfunction due to radiation exposure may be a physiological consequence of decreased capillary density, activation and expansion of fibroblasts, and increased accumulation of pro-fibrotic and pro-inflammatory macrophages, all of which lead to increased myocardial stiffness due to fibrosis. Collectively, these findings provide insight on the delayed effects of acute ionizing radiation exposure and indicate that therapies targeting macrophage regulation of fibrosis may mitigate radiation induced heart disease.

3.3 Coronary Artery Atherosclerosis in RSC: Hearts obtained from RSC and control rhesus monkeys which have gone to necropsy were evaluated for atherosclerosis extent using standard methods and approaches developed at Wake Forest. Briefly, three blocks from each of the main epicardial coronary arteries (left circumflex, left anterior descending and right epicardial arteries (shown in **Figure 2.9**) were sectioned and then stained with Verhoeff-van Gieson stain in order to facilitate identification of the internal and external elastic lamella (stained black), cell nuclei (stained black), and collagen (stained red) and the measurement of atherosclerosis (intimal area). Intimal area and other parameters (maximal intimal thickness, maximal medial thickness, internal elastic lamella length) were measured using algorithms and protocols developed in the Register lab for Visiopharm. Images of the unfiltered raw data from each of the coronary blocks are shown in **Figure 2.10**. Statistical analysis of these data are underway, and will require correction for amount of time while consuming the moderately atherogenic diet, age, and other key parameters. Note: controls are animals that have been euthanized due to illness and are not true age and time matched healthy controls.

Sources of Coronary Artery Blocks



Adapted from Register, Appt, Clarkson [Methods Mol Biol.](#) 2016;1366:517-532.

Figure 2.9: Illustration of the sources of the coronary arteries evaluated in the RSC.

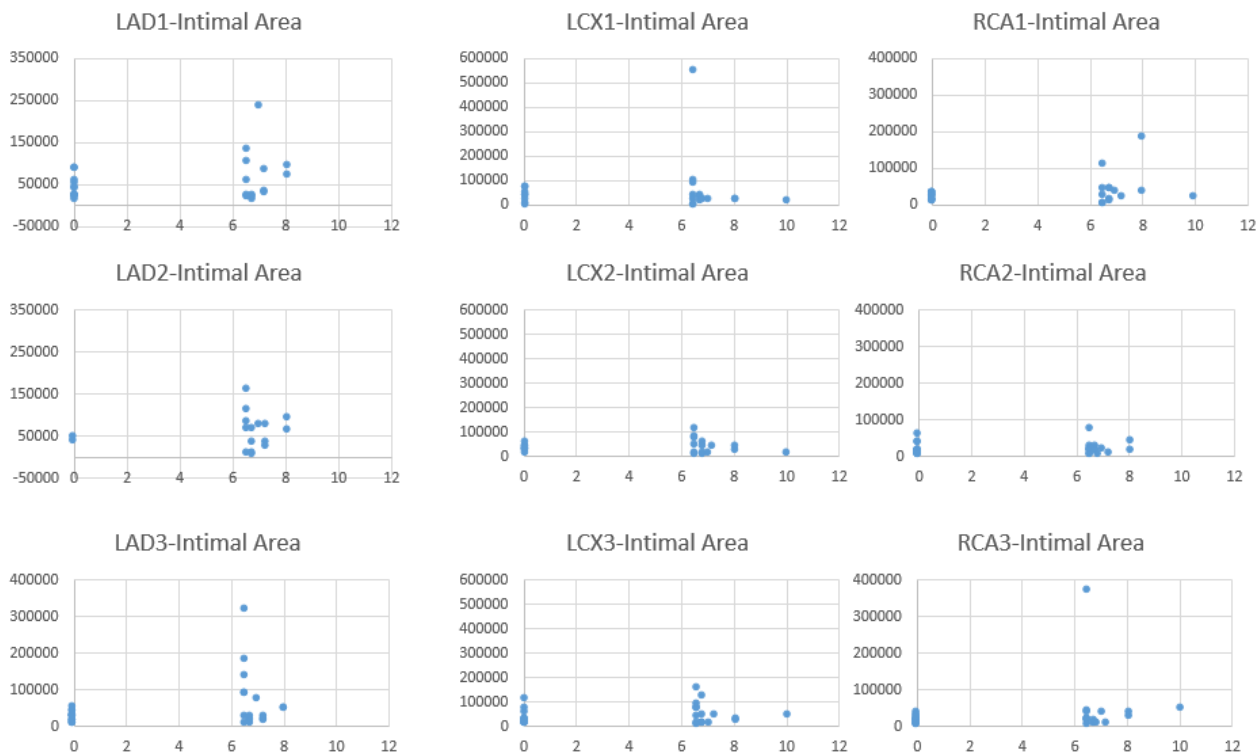


Figure 2.10: Raw data of atherosclerosis (intimal area in μm^2) from the 9 blocks of coronary arteries illustrated in Figure 2.9.

4) Other Achievements

Nothing to report

Project 3: Immune Injury (Sempowski/Chen)

T cells are rapidly depleted in the periphery by radiation exposure, and little is known about how broadly reactive peripheral T cells can be properly reconstituted after radiation injury. Project 3 therefore had two aims: 1) To define the roles of thymopoiesis and peripheral expansion in long-term T cell reconstitution after radiation-induced injury based on radiation dose using both murine and NHP models of radiation injury. 2) To determine if therapeutic agents identified in our previous studies (eg. IL-7) were able to promote or accelerate T cell immunity in murine models after radiation injury.

1) Major Activities

- a) Assessed immune phenotype in the long-term NHP survivor cohort. Developing additional manuscripts for submission.
- b) Characterized how T cells were recovered in peripheral blood and different lymphoid organs after different doses of radiation exposure.
- c) Determined the roles of thymopoiesis and peripheral expansion in overall T cell recovery using several different models.
- d) Studied how radioresistant T cells recovered from radiation injury at both cell and molecular levels using state of art technologies including single cell RNA sequencing.
- e) Established a novel T cell reporter model that allows monitoring T cell reconstitution at different lymphoid organs in live animals using bioluminescent imaging.
- f) Studied the effects of several potential mitigator (e.g. IGF-1, long-acting IL-7) on phenotypic and functional immune cell recovery after radiation injury.
- g) Delineated the roles of thymopoiesis and peripheral expansion in overall T cell recovery after treatment with a long-acting IL-7.

2) Specific Objectives

Major Task 1: NHP Studies

- Milestone 1.1. Completed phenotypic analyses in long-term cohort
- Milestone 1.2. Completed molecular analyses in long-term cohort
- Milestone 1.3. Completed phenotypic analysis in prospective cohort
- Milestone 1.4. Completed molecular analysis in prospective cohort
- Milestone 1.5. Determined primary immune response to influenza and pneumovax vaccines

Major Task 2: Murine Studies

- Milestone 2.2. Determined the roles of thymopoiesis and peripheral expansion in overall T cell recovery in the classic radiation injury model.
- Milestone 2.3. Determined the roles of thymopoiesis and peripheral expansion in overall T cell recovery in a second radiation injury model.

Major Task 3&4: Mitigator Studies

- Milestone 3.1&4.1. Determined the effect of HGH/IGF-1/IL-7 on phenotypic T cell reconstitution.
- Milestone 3.2&4.2. Determined the effect of HGH/IGF-1/IL-7 on functional T cell recovery.
- Milestone 3.3&4.3. Determined the mechanism by which IL-7 promote T cell reconstitution.

SA 1: Define the roles of thymopoiesis and peripheral expansion in overall T cell reconstitution after radiation-induced injury based on radiation dose.

Major Task 1: NHP Studies - Radiation Survivor Cohort

Blood was drawn annually from 221 animals in the radiation survivor cohort for up to five years depending on when each animal entered the cohort. The blood was transferred from Wake Forest University by courier to Duke for PBMC and plasma separation the day after collection. Fresh PBMC were immunophenotyped via flow cytometry, and plasma

plus any remaining PBMC were cryopreserved. Representative flow cytometry gating strategies are shown in **Fig. 3.1**. sjTREC in CD4⁺ and CD8⁺ T cells isolated from cryopreserved PBMC were also quantified via quantitative PCR [1].

Of the 221 subjects studied, 185 were previously irradiated (126 male and 59 female) and 36 were never irradiated (33 male, 3 female). The irradiated animals were exposed to between 1.1 Gy and 8.5 Gy TBI (mean: 6.12 Gy). Irradiated animals were grouped by the approximate lethal dose (LD) of the exposure (<5.5Gy equals <LD10, 5.5Gy - <7.65Gy equals LD10-LD50, 6.75Gy - <8Gy equals LD50-LD90, ≥8Gy equals >LD90). Animals were irradiated at between 2.3 and 15.5 years of age and blood was drawn between 0.5 and 14.3 years after exposure. A total of 706 blood samples were transferred to Duke. NB Some draws did not yield sufficient PBMC for analysis of all populations of interest, some data were eliminated due to a reagent recall, and additional markers were added to the staining panels after the first year of the project.

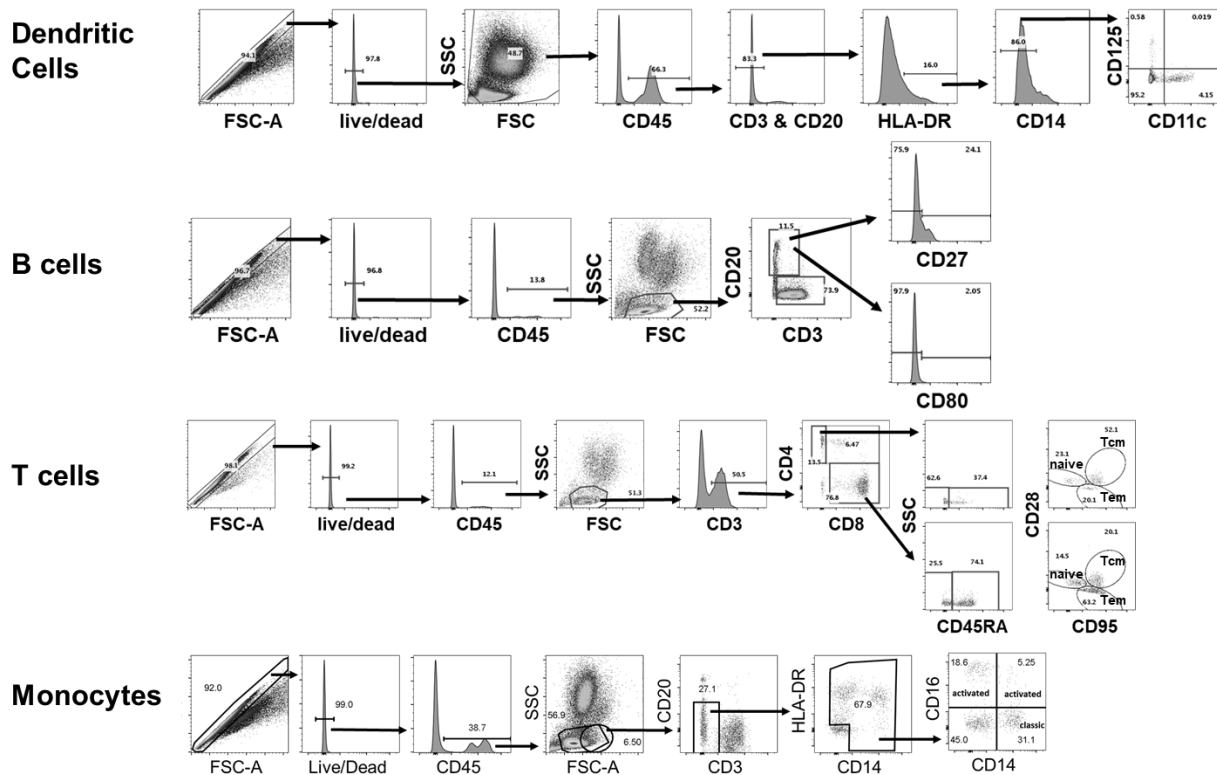


Figure 3.1. PBMC immunophenotyping gating strategies used for prospective and/or survivor cohorts.

Three statistical analyses were performed on the immune metrics by Janet Tooze at Wake Forest University. Means were adjusted for sex and repeated measures and then evaluated to determine if radiation exposure expressed 1) as a binary (yes/no) or 2) categorical variable grouped by dose (none, <LD10, LD10-LD50, LD50-90, >LD90) correlated with the immune metric being measured and whether or not there was an interaction between radiation exposure and age (as a categorical variable). Finally, the time from irradiation was then calculated, adjusted for age at time of collection (as a continuous variable), repeated measures and sex, and then 3) the adjusted means evaluated to determine if the effect of TBI on the immune metric changed the longer ago the exposure occurred. The preliminary results of these statistical analyses are reported below.

Radiation Exposure Causes Subtle, Long-term Changes to Monocyte Proportions

Monocytes respond to tissue injury and are important for initiation, propagation and dampening of immune responses. In both humans and NHP, monocytes can be classified as classical (CD14⁺⁺, CD16⁻), intermediate (CD14⁺⁺, CD16⁺) and non-classical (CD14^{+/low}, CD16⁺⁺). The percentage of classical, intermediate and non-classical populations in the live CD45⁺ monocyte FSC/SSC gate were grouped by irradiation status and the means plotted against age as a categorical variable after adjustment for sex and repeated measures (**Fig. 3.2A-C, Table 3.1**). In non-irradiated animals, the proportion of intermediate monocytes dropped sharply prior to adolescence and then remained stable, while classical monocytes peaked at early adulthood (age 6-7) and then declined. Conversely, the proportion of non-classical

monocytes gradually increased as animals aged (**Fig. 3.2A-C**). Irradiated animals tended to have a lower percentage of classical monocytes at most ages, and there was a strong interaction effect between irradiation status and age ($p=0.006$). The effect was most significant at animals aged 6-7 years ($p=0.001$) (**Table 3.1**), and was significantly dose-dependent being most pronounced in the highest dose ($\geq$ LD90) group (**Fig. 3.2D,G**). Young irradiated animals (age 3-4 years old) also exhibited a striking reduction in the percentage of intermediate monocytes ($p=0.046$) that was evident in all dose groups (**Fig. 3.2B,E**). Interestingly, older animals (>12 years old) had a concomitant increase in non-classical monocytes regardless of dose (**Fig. 3.2F**).

Monocyte distributions were tested for time-effects of irradiation (with correction for age effects) by calculating the time since irradiation and adjusting the results for sex, repeated measures and age at time of collection (**Fig. 3.2G-I, Table 3.2**). The percentages of both classical and intermediate monocytes gradually increased in the years after irradiation, and five years after irradiation there was a clear stratification of effects on monocytes by dose, with the percentage of both classical and intermediate monocytes increasing as dose increased (over LD90>LD50-90>LD10-50>sub-LD10) ($p=0.027$ for classical and $p=0.003$ for intermediates). These findings indicate that TBI causes subtle effects on monocytes that remain evident at least five years after irradiation.

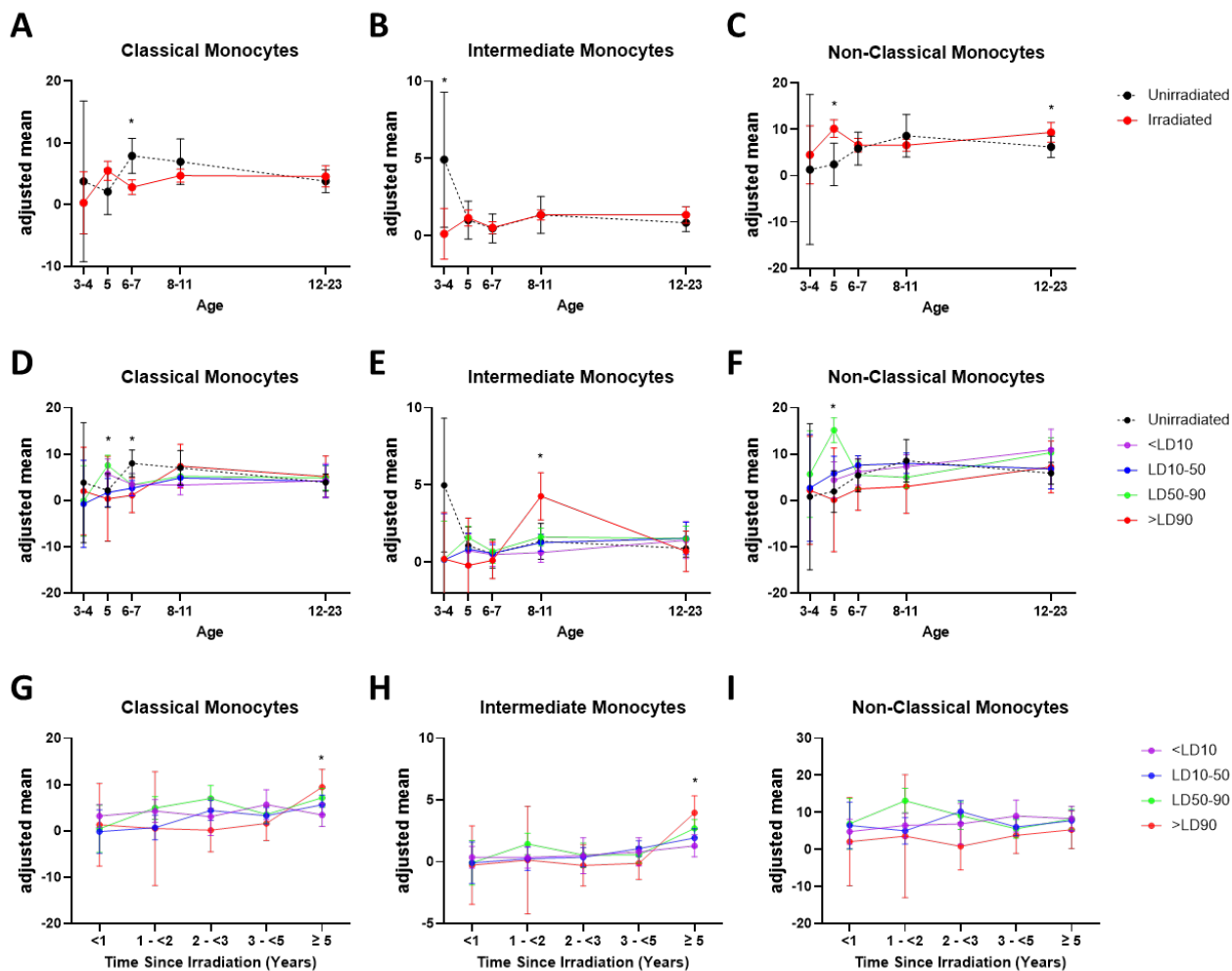


Figure 3.2. Long-term effects of TBI on monocyte proportions. The percentages of classical, intermediate and non-classical monocytes in the PBMC of radiation survivor NHPs were determined annually. Results were grouped by (A-C) irradiation status or (D-F) dose, means were adjusted for sex and repeated measures and then plotted against age. (G-I) Means for irradiated animals were then further adjusted for age at sample collection and plotted against time since irradiation. Data show adjusted means with 95% confidence interval. Stars indicate $p < 0.05$ for significant differences between adjusted means.

Table 3.1. Effects of irradiation status, dose and interactions with age in monocyte populations

Monocyte Population (as percentage of cells in CD45+ monocyte FSC/SSC gate)*	N	Grouping Type	Main Effect	Interaction of Age and Type	Age (years)				
					3-4	5	6-7	8-11	12-23
classical	565	Irradiated (Yes/No)	0.408	0.006	0.627	0.094	0.001	0.250	0.521
		Grouped by Dose	0.674	0.053	0.933	0.013	0.017	0.320	0.966
intermediate	565	Irradiated (Yes/No)	0.116	0.266	0.046	0.842	0.938	0.998	0.187
		Grouped by Dose	0.421	0.081	0.252	0.547	0.952	0.001	0.559
non-classical	565	Irradiated (Yes/No)	0.199	0.065	0.718	0.003	0.690	0.406	0.042
		Grouped by Dose	0.134	<0.001	0.943	<0.001	0.311	0.201	0.133

* Monocytes in PBMC from NHP in the radiation survivor cohort were immunophenotyped annually. Data were adjusted for sex and repeated measures and then modelled for effects of irradiation as a binary variable (Yes/No) or by dose category and tested for interaction between these variables and age. Adjusted means were then tested for significant differences within each age group. $p < 0.05$ in bold.

Table 3.2. Effects of dose and interactions with time since irradiation in monocyte populations

Monocyte Population (as percentage of cells in CD45+ monocyte FSC/SSC gate)*	N	Dose Effect	Interaction of Dose and Time Since Irradiation	Time Since Irradiation (years)				
				<1	1 - <2	2 - <3	3 - <5	≥5
classical	453	0.225	0.038	0.532	0.087	0.065	0.439	0.027
intermediate	453	0.703	0.076	0.929	0.203	0.852	0.373	0.003
non-classical	453	0.144	0.140	0.864	0.004	0.059	0.450	0.739

* Monocytes in PBMC from NHP in the radiation survivor cohort were immunophenotyped annually. Data were adjusted for sex, age at collection, and repeated measures and then modelled for correlation with dose grouping and tested for interaction effects between dose and time since irradiation. Adjusted means were then tested for significant dose differences within each time category. $p < 0.05$ in bold.

Irradiation at a Young Age Causes Transient Increases in the Percentage of myeloid DC

We next investigated age- and irradiation- changes in dendritic cell (DC) populations within the PBMC. Both human and macaque DCs can be defined as CD3⁺CD20⁺HLD-DR⁺CD14⁺ and then divided into distinct populations by expression of CD11c and CD123. In humans there are two major DC populations: CD11c⁺CD123^{lo} conventional / myeloid DC (mDC) and CD11c⁺CD123^{hi} plasmacytoid DC (pDC). There is also a minor less differentiated subset that expresses neither marker. Macaque DC can also be grouped as mDC and pDC using these definitions, although the CD11c⁺CD123⁺ less differentiated population typically forms a larger proportion of the DC in comparison to humans.

PBMC DC populations were expressed as a percentage of the live CD45⁺ PBMC, grouped by irradiation status, adjusted for sex and repeated measures and plotted against age (**Fig. 3.3A-C**). The pDC population showed no differences between irradiated and unirradiated animals (**Fig. 3.3A, Table 3.3**). Irradiated animals (age 3-4 years) showed a large increase in the proportion of CD45⁺ cells that were mDC ($p < 0.001$), with the mDC population being highly sensitive to irradiation status and exhibiting a strong interaction effect between age and irradiation. This effect changed depending

on the dose the animals received; and was most evident in the animals that received LD50-90 and lowest in the animals that received LD10-50 (Fig. 3.3B, Table 3.3). No animals in the 3- to 4-year age group received a dose <LD10 (Fig. 3.1A) and so the impact of low dose TBI could not be evaluated. After correcting the data for age at sampling, the dose dependent effect of irradiation was only significant in animals within two years of irradiation (Fig. 3.3E, Table 3.4). The percentage of the PBMC which were less-differentiated DC dropped as animals got older regardless of irradiation status (Fig. 3.3C), although there was a dose- and age- dependent interaction whereby young adult macaques (aged 5 to 7 years) irradiated at >LD10 had higher proportion of this subset in comparison to age-matched controls (Fig. 3.3C). After correction for age at time of collection, there was a highly significant ($p < 0.001$) interacting effect of aging and dose on this population, with dose affecting the less-differentiated DC population percentage up to three years after exposure (Fig. 3.3E, Table 3.4).

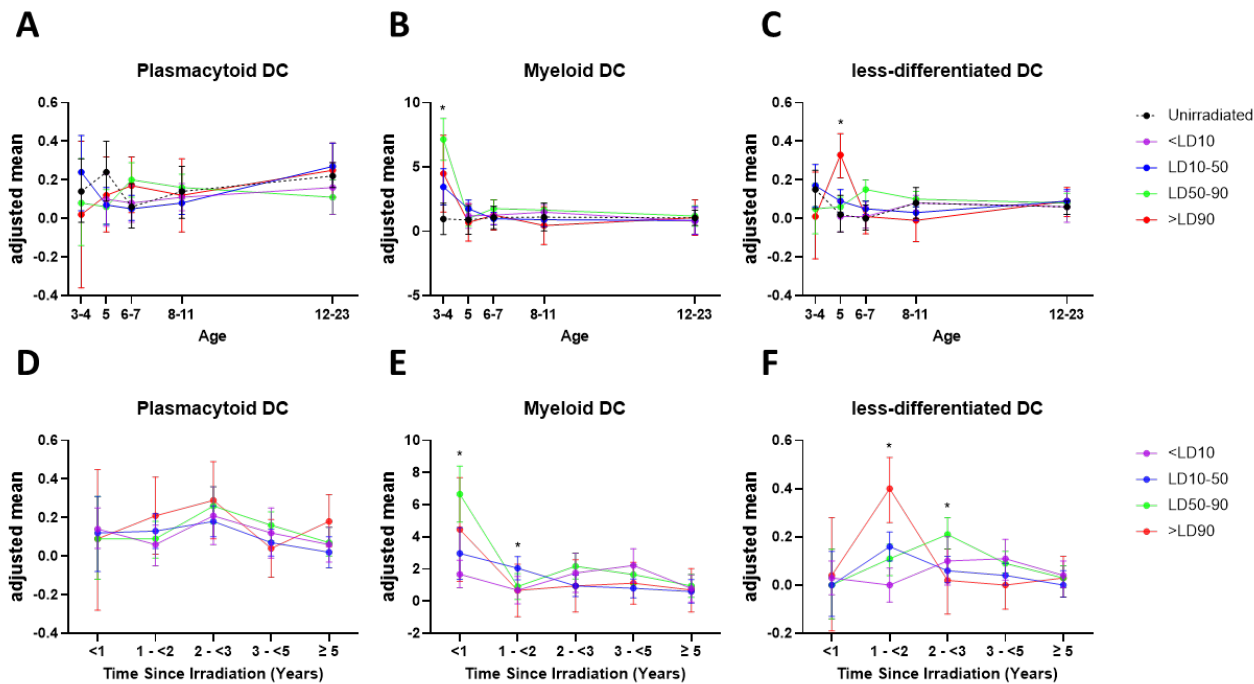


Figure 3.3. Long-term effects of TBI on dendritic cells (DC). The percentages of plasmacytoid, myeloid and non-differentiated DC in PBMC of the radiation survivor NHPs were determined annually. Results were grouped by (A-C) dose and then means were adjusted for sex and repeated measures and plotted against age. (D-F) Means for irradiated animals were then further adjusted to correct for age at sample collection and plotted against time since irradiation. Plots show adjusted means with 95% confidence interval. Stars indicate $p < 0.05$ for significant differences between adjusted means.

Table 3.3. Effects of irradiation status and dose on DC and interactions with age in DC

Dendritic Cell Population (as percentage of CD45+ PBMC)	N	Grouping Type	Main Effect	Interaction of Age and Type?	Age (years)				
					3-4	5	6-7	8-11	12-23
Myeloid DC	669	Irradiated (Yes/No)	0.002	<0.001	<0.001	0.536	0.733	0.769	0.879
		Grouped by Dose	<0.001	<0.001	<0.001	0.460	0.458	0.279	0.975
Plasmacytoid DC	669	Irradiated (Yes/No)	0.290	0.431	0.890	0.051	0.531	0.731	0.373
		Grouped by Dose	0.689	0.176	0.667	0.352	0.067	0.543	0.150
Less-differentiated DC	669	Irradiated (Yes/No)	0.349	0.596	0.568	0.244	0.120	0.728	0.333
		Grouped by Dose	0.182	<0.001	0.373	<0.001	0.001	0.055	0.851

* Dendritic cells in PBMC from the radiation survivor cohort were immunophenotyped annually. Data were adjusted for sex and repeated measures and then modelled for effects of irradiation as a binary variable (Yes/No) or by dose category and tested for interaction between these variables and age. Adjusted means were then tested for significant differences within each age group. $p < 0.05$ in bold.

TABLE 3.4. Effects of dose and interactions with time since irradiation in DC

Dendritic Cell Population (as percentage of CD45+ PBMC)	N	Dose Effect	Interaction of Dose and Time Since Irradiation	Time Since Irradiation (years)				
				<1	1 - <2	2 - <3	3 - <5	≥5
Myeloid DC	537	0.007	<0.001	<0.001	0.038	0.091	0.075	0.899
Plasmacytoid DC	537	0.679	0.737	0.962	0.486	0.482	0.315	0.175
Less-differentiated DC	537	0.265	<0.001	0.975	<0.001	0.002	0.157	0.688

*Dendritic cells in PBMC from irradiated animals in the survivor cohort were immunophenotyped annually. Data were adjusted for sex, age at collection, and repeated measures and then modelled for correlation with dose grouping and tested for interaction effects between dose and time since irradiation. Adjusted means were then tested for significant dose differences within each time category. $p < 0.05$ in bold.

The proportion of B cells in the live CD45+ lymphocyte gate showed a strong interaction effect between age and both irradiation status ($p=0.003$) and dose ($p<0.001$), with the most significant impact being observed in young (3-4 years old) animals irradiated at $>LD90$ (**Table 3.5, Fig. 3.4A**). While radiation exposure status had no significant overall long-term effect on the percentage of activated B cells (CD80+) when evaluated as a binary variable, there was short-term (<2 years after irradiation) increase in activated cells in young animals that received $<LD50$ (**Fig. 3.4B, 3.4F**). In contrast, there was a highly significant interacting effect of age and TBI on the percentage of CD27+ memory B cells ($p=0.005$) (**Table 3.5, Fig. 3.4C**). This was highly dose-dependent in animals aged under 6 (**Table 3.5, Fig. 3.4C**).

B cell surface expression of MHC Class II receptor HLA-DR dropped as animals aged (**Fig. 3.4D**). This decrease was mildly accelerated in young animals that were previously irradiated, and there was an interaction effect on HLA-DR expression between age and TBI status ($p=0.047$). There were highly significant interacting effects on B cell HLA-DR expression between dose and age ($p<0.001$) (**Table 3.5**) and between dose and time since irradiation (**Table 3.6**), with the dose effects remaining significant for at least three years after TBI after correcting for age and sex (**Table 3.6, Fig. 3.4H**). The full impact of irradiation on B-cell surface HLA-DR expression did not occur until at least 1-2 years after exposure ($<LD10$ and $LD50-90$ groups) or 3 years ($LD10-50$ and $LD>90$), and the $<LD10$ group showed the greatest recovery 5 years or more after irradiation after adjusting for age at sampling and sex (**Table 3.8, Fig. 3.4H**).

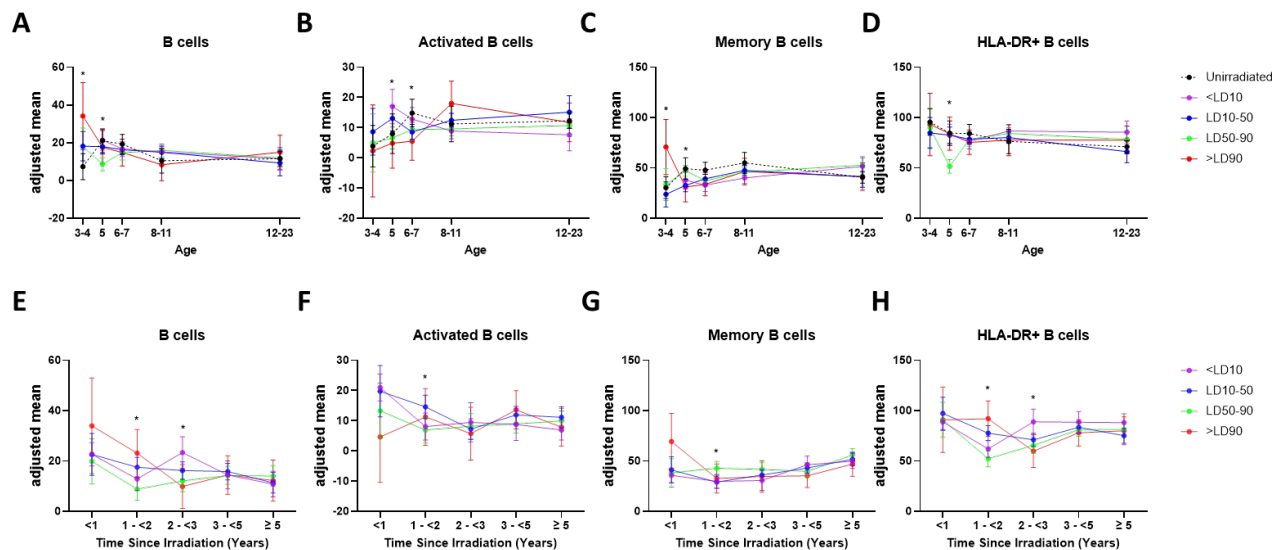


Figure 3.4. Long-term effect of TBI on B cells. B cells in the PBMC of the radiation survivor cohort were immunophenotyped annually. Results were grouped by (A-D) dose and then means were adjusted for sex and repeated measures and plotted against age. (E-H) Means for irradiated animals were then further adjusted to correct for age at sample collection and plotted against time since irradiation. Data show adjusted means with 95% confidence interval. Stars indicate $p < 0.05$ for significant differences between adjusted means.

TABLE 3.5. Effects of irradiation status, dose and interactions with age in B cells

Population	N	Grouping Type	Main Effect	Interaction of Age and Type?	Age (years)				
					3-4	5	6-7	8-11	12-23
B cells (% of all lymphocytes)	64	Irradiated (Yes/No)	0.399	0.003	0.007	0.069	0.215	0.188	0.961
	8	Grouped by Dose	0.352	<0.001	0.018	<0.001	0.693	0.367	0.903
CD27+ Memory B cells (% of B cells)	63	Irradiated (Yes/No)	0.144	0.005	0.821	0.095	0.015	0.063	0.028
	6	Grouped by Dose	0.071	<0.001	0.025	0.004	0.054	0.111	0.055
CD80+ Activated B cells (% of B cells)	63	Irradiated (Yes/No)	0.748	0.524	0.599	0.462	0.050	0.791	0.490
	4	Grouped by Dose	0.195	0.027	0.792	0.013	0.045	0.086	0.376
HLA-DR+ B cells (% of B cells)	65	Irradiated (Yes/No)	0.254	0.047	0.323	0.027	0.192	0.313	0.167
	0	Grouped by Dose	0.062	<0.001	0.768	<0.001	0.718	0.413	0.079

* B cells in PBMC from the radiation survivor cohort were immunophenotyped annually. Data were adjusted for sex and repeated measures and then modelled for effects of irradiation as a binary variable (Yes/No) or by dose category and tested for interaction between these variables and age. Adjusted means were then tested for significant differences within each age group. $p < 0.05$ in bold.

TABLE 3.6. Effects of dose and interactions with time since irradiation in B cells

Population	N	Dose Effect	Interaction of Dose and Time Since Irradiation	Time Since Irradiation (years)				
				<1	1 - <2	2 - <3	3 - <5	≥5
B cells (% of all lymphocytes)	52 7	0.259	0.009	0.617	0.004	0.018	0.926	0.679
CD27+ Memory B cells (% of B cells)	51 9	0.304	0.047	0.131	0.009	0.289	0.385	0.363
CD80+ Activated B cells (% of B cells)	51 8	0.058	0.204	0.117	0.033	0.892	0.351	0.255
HLA-DR+ B cells (% of B cells)	52 7	0.057	<0.001	0.826	<0.001	0.012	0.589	0.122

* B cells in PBMC from the radiation survivor cohort were immunophenotyped annually. Data were adjusted for sex, age at collection, and repeated measures and then modelled for correlation with dose grouping and tested for interaction effects between dose and time since irradiation. Adjusted means were then tested for significant dose differences within each time category. $p < 0.05$ in bold.

TBI at a young age causes a transient, dose-dependent increase in thymic output

T cells are phenotypically and functionally classified as CD4+ or CD8+. NHP and humans, but not mice, also have minor population of circulating CD4+CD8+ double positive cells whose development and function is not well understood. Aging led to reduction in the proportion of total T cells within the PBMC, and this loss was accelerated by irradiation regardless of age or dose (**Fig. 3.5A,E, Table 3.7**). All animals in the radiation survivor cohort showed a skewing in CD4/8 balance from CD8 to CD4 T cells as animals aged, and this skewing was accelerated in the LD50-90 dose group (**Fig. 3.5B-C, F-G, Table 3.7**). Curiously, irradiated animals also showed a sustained, non-age dependent increase in the proportion of CD4+CD8+ double-positive T cells ($p=0.017$) (**Fig. 3.5D, H**).

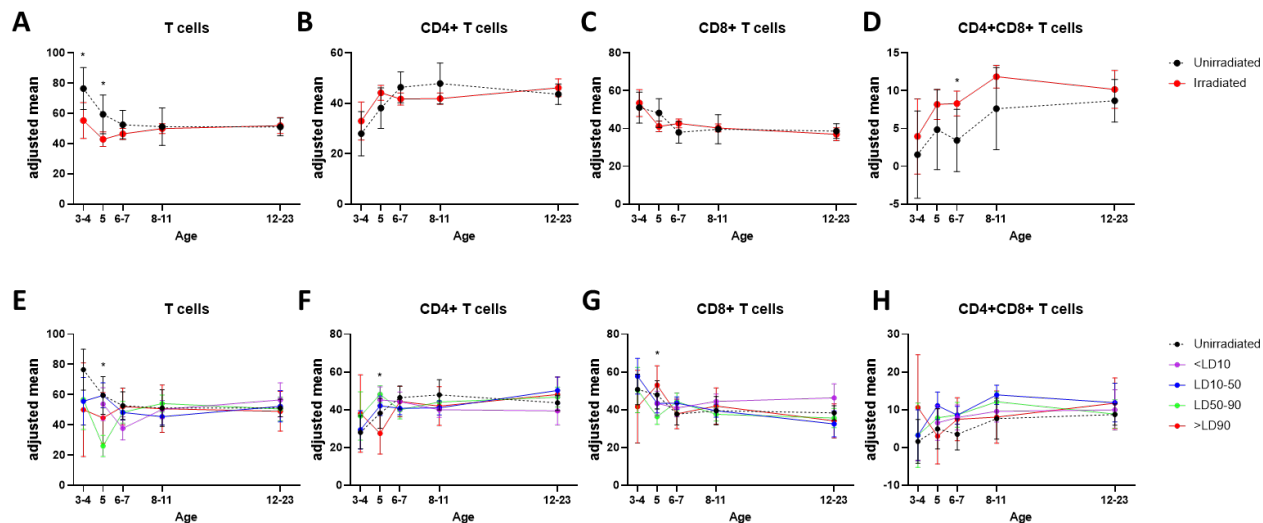


Figure 3.5. Long-term effect of TBI on major T cell populations. T cells in the PBMC of the radiation survivor were immunophenotyped annually. Results were grouped by (A-D) irradiation status or (E-H) dose and then means were adjusted for sex and repeated measures and plotted against age. Data show adjusted means with 95% confidence interval. Stars indicate $p < 0.05$ for significant differences between adjusted means.

CD8 and CD4 T cells can be further classified as naïve (CD28+CD95-), effector memory (Tem) (CD28-CD95+) and central memory cells (Tcm) (CD28+CD95+). As observed in other rhesus cohorts [2], the CD8 T cell population skewed from a naïve to Tem phenotype as animals aged (**Fig. 3.6A-C**). Irradiation at a young age had a dramatic impact on this balance,

leading to a sharp reduction in the percentage of CD8 Tcm and naïve T cells and an increase in Tem. The Tem and Tcm effects were significant regardless of age ($p=0.011$ and $p=0.016$ respectively), and there were significant interacting effects between age and dose (**Fig. 3.6E-G, Table 3.8**). After adjusting for sex, repeated measures and age at collection, irradiation dose had a highly significant impact on the proportions of all three populations, and there were strong interacting effects between dose and time since irradiation ($p<0.001$ for all three populations). This effect was most apparent within the first year of irradiation, where the decrease in naïve cells and increase in Tem was directly proportional to dose received ($p<0.001$ for each population). The dose-dependent effects of irradiation on the CD8 T cell balance were significant for at least two years after irradiation (**Fig. 3.6I-K, Table 3.8**).

In contrast to the CD8 T cells, the CD4 population in non-irradiated animals skewed away from a Tem phenotype as animals aged (**Fig. 3.7A-C**). Irradiation had a striking impact on CD4 T cells, with TBI leading to a dramatic increase in the percentage of naïve CD4+ T cells and a concomitant reduction in Tcm until around 7 years of age (**Fig. 3.7A-C, E-G Table 3.7**). These effects were highly significant regardless of age (all $p<0.001$), and there were also significant interacting effects between age and dose on these balances. After adjusting for sex, repeated measures and age at collection, there were dose-dependent effects on the CD4 T cell balance for at least three years after the initial exposure (**Fig. 3.7I-K, Table 3.8**), with the highest dose ($>LD90$) animals exhibiting the largest reduction in CD4 naïve T cells for at least five years after irradiation (**Fig. 3.7I**). Young animals receiving LD10 to LD90 showed the greatest increase in naïve CD4 T cells (**Fig. 3.7E**), and animals in these dose groups also exhibited a faster recovery in the naïve CD4 T cell population after irradiation, within 1-2 years of TBI, when compared to the $<LD10$ and $>LD90$ groups (**Fig. 3.7I**). This may reflect a rheostatic response wherein low dose TBI is insufficient to trigger aggressive homeostatic regeneration by thymopoiesis or peripheral expansion while the physiological damage caused by doses over LD90 precludes recovery for at least a few years after exposure.

Similar to humans, macaque memory T cell populations can be further delineated by CCR7 expression, with populations defined as Tcm (CD95+CD28+CCR7+), Tem (CD95+CD28-CCR7-) plus a transitional Tcm population (CD95+CD28+CCR7-). When defined in this manner, CD4 and CD8 Tcm and Tem showed similar results to those described above. The proportion of both CD4+ and CD8+ transitional populations animals regardless of age ($p<0.001$ $p=0.024$ respectively; **Table 3.9**), and these differences were especially acute in animals under seven years old (**Fig. 3.6D and 7D**). Strikingly, after correction for age and sex these differences remained highly dose-dependent and there were significant interacting effects between dose and time since irradiation ($p=0.001$ and $p=0.004$ respectively; **Fig. 3.6H, 3.6L, 3.7H, 3.7L Table 3.7, 3.8**). Collectively, these data indicate that young irradiated animals show a multiyear destabilization in T cell naïve/Tcm/Tem subset balance that in many cases is similar to the effect of aging.

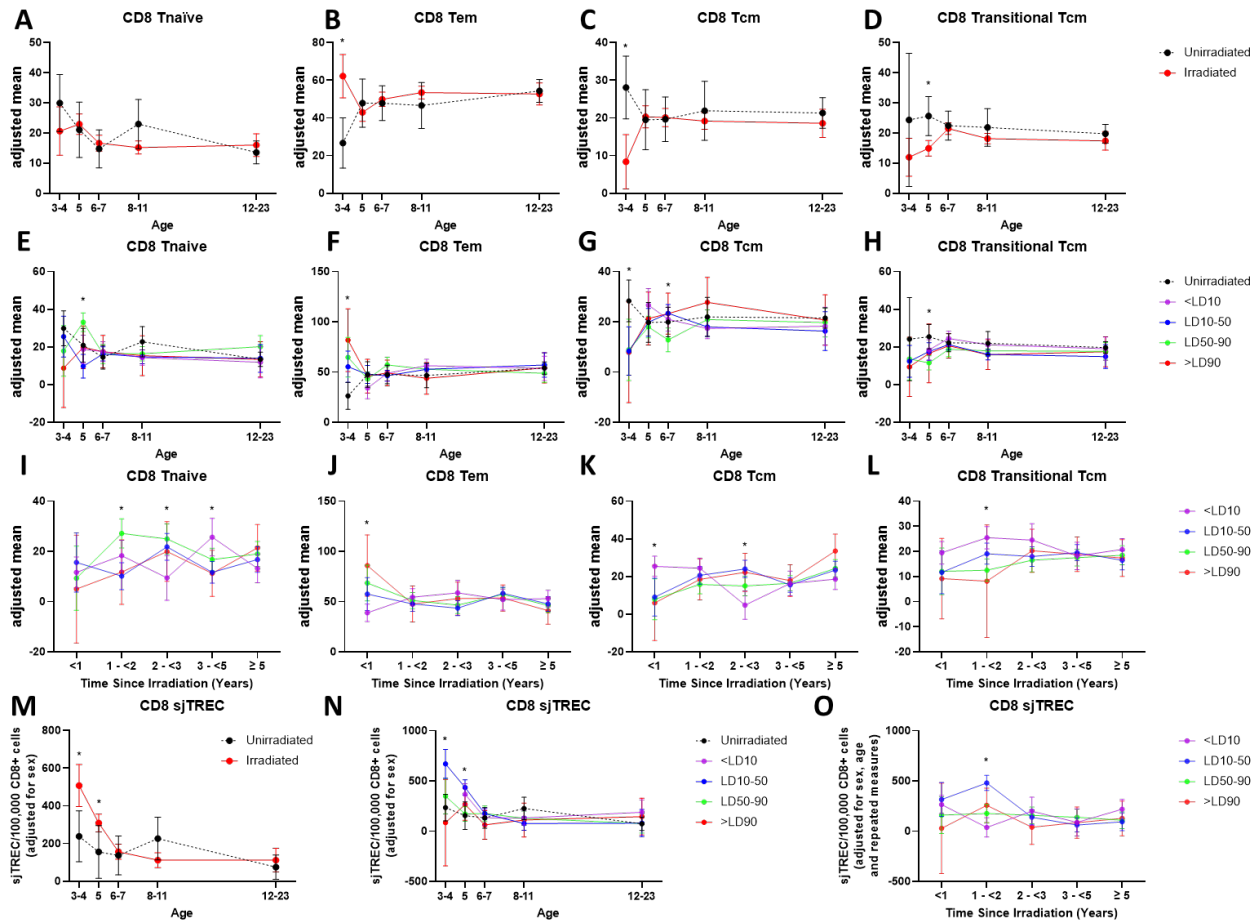


Figure 3.6. Long-term effect of TBI on CD4 T cell subsets. CD4 T cells in the PBMC of the radiation survivor cohort were immunophenotyped and assayed for sjTREC content annually. Results were grouped by (A-D, M) irradiation status or (E-H, N) dose and then means were adjusted for sex and repeated measures and plotted against age. (I-L, O) Means for irradiated animals were then further adjusted to correct for age at sample collection and plotted against time since irradiation. Data show adjusted means with 95% confidence interval. Stars indicate $p < 0.05$ for significant differences between adjusted means. Data show adjusted means with 95% confidence interval. Stars indicate $p < 0.05$ for significant differences between adjusted means.

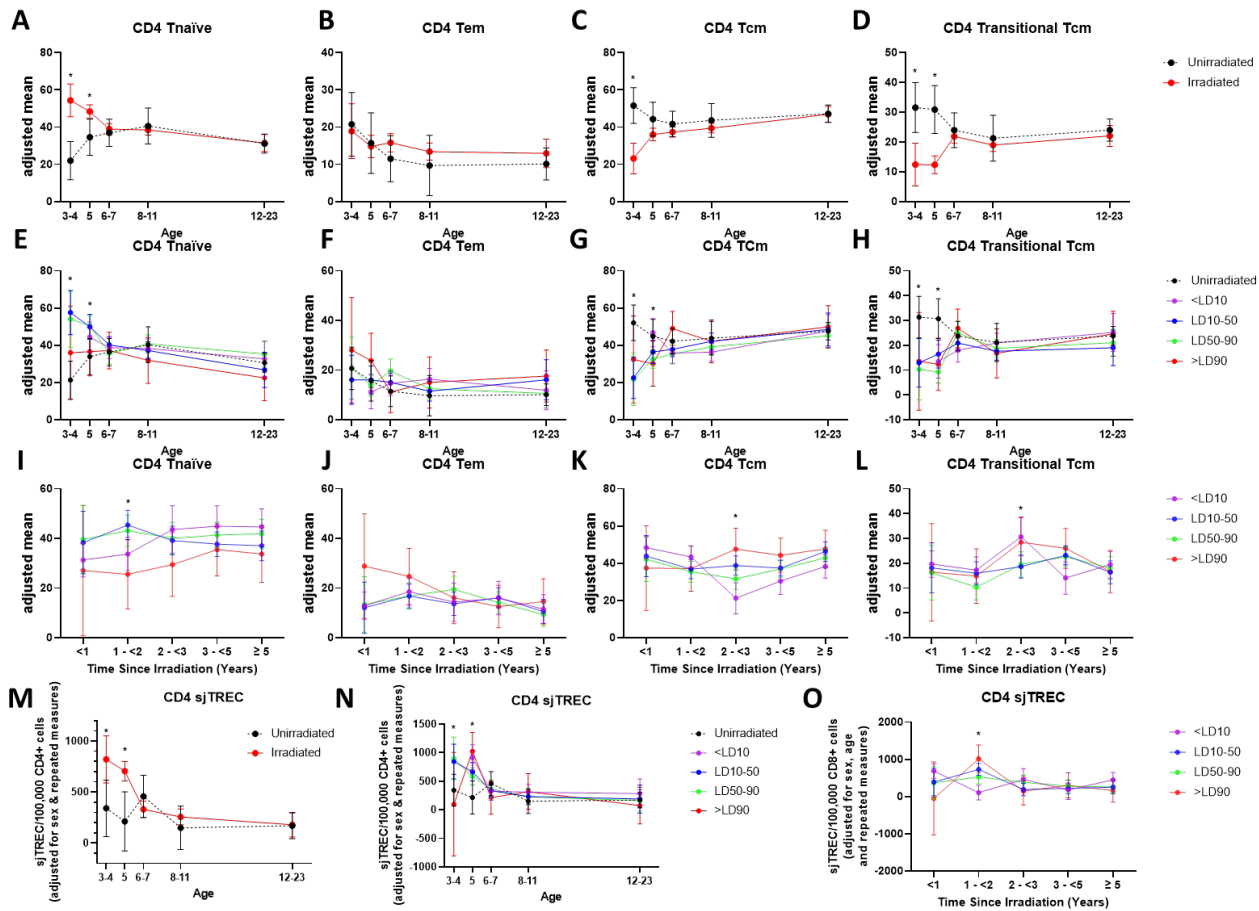


Figure 3.7. Long-term effect of TBI on CD8 T cell subsets. CD8 T cells in the PBMC of radiation survivor NHPs were immunophenotyped and assayed for sjTREC content annually. Results were grouped by (A-D, M) irradiation status or (E-H, N) dose and then means were adjusted for sex and repeated measures and plotted against age. (I-L, O) Means for irradiated animals were then further adjusted to correct for age at sample collection and plotted against time since irradiation. Data show adjusted means with 95% confidence interval. Stars indicate $p < 0.05$ for significant differences between adjusted means. Data show adjusted means with 95% confidence interval. Stars indicate $p < 0.05$ for significant differences between adjusted means.

Table 3.7. Effects of irradiation status, dose and interactions with age in T cells

Population	N	Grouping Type	Main Effect	Interaction of Age and Type?	Age (years)				
					3-4	5	6-7	8-11	12-23
T cells (% of all lymphocytes)	651	Irradiated (Yes/No)	0.002	0.076	0.023	0.019	0.225	0.845	0.831
		Grouped by Dose	0.030	<0.001	0.125	<0.001	0.082	0.316	0.912
CD4+CD8+ T cells (% of all T cells)	633	Irradiated (Yes/No)	0.017	0.805	0.531	0.243	0.028	0.142	0.415
		Grouped by Dose	0.088	0.870	0.715	0.190	0.282	0.078	0.778
CD4+ T cells (% of all T cells)	655	Irradiated (Yes/No)	0.761	0.100	0.389	0.163	0.157	0.166	0.336
		Grouped by Dose	0.710	0.028	0.619	0.007	0.338	0.355	0.242
CD8+ T cells (% of all T cells)	633	Irradiated (Yes/No)	0.922	0.171	0.662	0.083	0.129	0.863	0.520
		Grouped by Dose	0.206	0.018	0.438	0.008	0.251	0.150	0.080
CD8 Tem (% of CD8 T cells)	607	Irradiated (Yes/No)	0.011	0.003	<0.001	0.478	0.683	0.289	0.697
		Grouped by Dose	0.077	0.012	<0.001	0.361	0.231	0.484	0.834
CD8 Tcm (% of CD8 T cells)	607	Irradiated (Yes/No)	0.016	0.022	<0.001	0.854	0.883	0.510	0.313
		Grouped by Dose	0.075	0.009	0.006	0.312	0.010	0.266	0.784
CD8 Tnaive (% of CD8 T cells)	608	Irradiated (Yes/No)	0.241	0.175	0.139	0.707	0.581	0.072	0.356
		Grouped by Dose	0.071	0.004	0.219	<0.001	0.961	0.434	0.358
CD4 Tem (% of CD4 T cells)	621	Irradiated (Yes/No)	0.440	0.778	0.747	0.837	0.194	0.385	0.319
		Grouped by Dose	0.639	0.362	0.752	0.389	0.224	0.455	0.532
CD4 Tcm (% of CD4 T cells)	620	Irradiated (Yes/No)	<0.001	0.003	<0.001	0.095	0.242	0.383	0.921
		Grouped by Dose	0.001	0.004	<0.001	0.011	0.074	0.403	0.945
CD4 Tnaive (% of CD4 T cells)	621	Irradiated (Yes/No)	<0.001	<0.001	<0.001	0.009	0.614	0.690	0.931
		Grouped by Dose	0.001	0.008	<0.001	0.020	0.736	0.669	0.365
CD4 Transitional Tcm (% of CD4 T cells)	620	Irradiated (Yes/No)	<0.001	0.001	<0.001	<0.001	0.509	0.570	0.439
		Grouped by Dose	<0.001	0.011	0.009	<0.001	0.155	0.748	0.671
CD8 Transitional Tcm (% of CD8 T cells)	572	Irradiated (Yes/No)	0.024	0.195	0.291	0.003	0.699	0.265	0.275
		Grouped by Dose	0.069	0.641	0.751	0.007	0.349	0.132	0.706

* T cells in PBMC from NHP in the radiation survivor cohort were immunophenotyped annually. Data were adjusted for sex and repeated measures and then modelled for effects of irradiation as a binary variable (Yes/No) or by dose category and tested for interaction between these variables and age. Adjusted means were then tested for significant differences within each age group. $p < 0.05$ in bold.

Table 3.8. Effects of dose and interactions with time since irradiation in T cells

Population	N	Dose Effect	Interaction of Dose and Time Since Irradiation	Time Since Irradiation (years)				
				<1	1 - <2	2 - <3	3 - <5	≥5
T cells (% of all lymphocytes)	528	0.002	0.076	0.023	0.019	0.225	0.845	0.831
CD4+CD8+ T cells (% of all T cells)	512	0.030	<0.001	0.125	<0.001	0.082	0.316	0.912
CD4+ T cells (% of all T cells)	532	0.017	0.805	0.531	0.243	0.028	0.142	0.415
CD8+ T cells (% of all T cells)	512	0.088	0.870	0.715	0.190	0.282	0.078	0.778
CD4 Tem (% of CD4 T cells)	499	<0.001	<0.001	<0.001	0.492	<0.001	0.175	0.782
CD4 Tcm (% of CD4 T cells)	498	0.002	<0.001	0.005	<0.001	0.001	0.159	0.896
CD4 Tnaive (% of CD4 T cells)	499	0.431	0.009	0.074	0.029	0.010	0.372	0.569
CD8 Tem (% of CD8 T cells)	487	0.016	<0.001	<0.001	0.008	0.005	0.307	0.954
CD8 Tcm (% of CD8 T cells)	487	<0.001	<0.001	<0.001	0.076	0.639	0.813	0.559
CD8 Tnaive (% of CD8 T cells)	488	<0.001	<0.001	<0.001	0.024	0.309	0.533	0.191
CD4 Transitional Tcm (% of CD4 T cells)	498	0.012	0.001	0.586	<0.001	0.992	0.454	0.001
CD8 Transitional Tcm (% of CD8 T cells)	463	0.001	0.004	<0.001	0.011	0.074	0.403	0.945

* T cells in PBMC from NHP in the radiation survivor cohort were immunophenotyped annually. Data were adjusted for sex, age at collection, and repeated measures and then modelled for correlation with dose grouping and tested for interaction effects between dose and time since irradiation. Adjusted means were then tested for significant dose differences within each time category. $p < 0.05$ in bold.

The resurgence in T cells in irradiated animals in the years after acute radiation induced lymphopenia may be mediated by thymopoiesis and/or peripheral expansion. This is an important distinction as the generation and selection of novel T cell antigen receptors (TCR) only occurs within the thymus, and so only thymopoiesis enhances TCR diversity. We therefore quantified sjTREC in CD8+ and CD4+ T cells isolated from cryopreserved PBMC collected during the annual bleeds of the radiation survivor cohort. After adjusting for sex and repeated measures, there was a highly significant impact of irradiation on sjTREC numbers in both CD8+ ($p=0.025$) and CD4+ ($p=0.03$) T cells and strong interacting effects of age with irradiation and dose in both populations (**Fig. 3.6M, 7M, Table 3.9**). These changes were most significant in animals age 5 years and under. After adjusting the data for age at collection and sex, there was a highly significant ($p<0.001$) interaction effect of dose and years since irradiation on sjTREC numbers in both T cell populations (**Table 3.12**). Animals 1-2 years out from irradiation exhibited clear dose-dependency on sjTREC numbers ($p<0.001$), and for both CD4 and CD8 cells animals that received doses lower than LD10 showed the lowest numbers of sjTREC 1-2 years after TBI (**Fig. 3.6O, 3.7O**). Collectively, these results indicate that thymopoiesis contributes to peripheral T cell reconstitution in young animals for around one to two years after TBI in a dose-dependent manner. In contrast, the sjTREC numbers and naïve T cell populations were similar in older irradiated and unirradiated animals were similar, suggesting thymic output does not drive such extensive repopulation in older animals.

Table 3.9. Effects of irradiation status, dose and interactions with age on thymopoiesis

sjTREC per 100,000:	N	Grouping Type	Main Effect	Interaction of Age and Type?	Age (years)				
					3-4	5	6-7	8-11	12-23
CD4+ cells	608	Irradiated (Yes/No)	0.003	0.002	0.009	0.002	0.243	0.346	0.911
		Grouped by Dose	0.036	0.014	0.025	<0.001	0.711	0.760	0.897
CD8+ cells	576	Irradiated (Yes/No)	0.025	0.003	0.003	0.041	0.721	0.060	0.401
		Grouped by Dose	0.002	<0.001	<0.001	<0.001	0.574	0.25	0.547

sjTREC were quantified in CD4+ and CD8+ cells T cells isolated annually from the PBMC of radiation survivor cohort NHP. Data were adjusted for sex and repeated measures and then modelled for effects of irradiation as a binary variable (Yes/No) or by dose category and tested for interaction between these variables and age. Adjusted means were then tested for significant differences within each age group. $p < 0.05$ in bold.

Table 3.10. Effects of dose and interactions with time since irradiation on thymopoiesis

sjTREC per 100,000:	N	Dose Effect	Interaction of Dose and Time Since Irradiation	Time Since Irradiation (years)				
				<1	1 - <2	2 - <3	3 - <5	≥5
CD4+ cells	479	0.975	<0.001	0.183	<0.001	0.216	0.900	0.281
CD8+ cells	451	0.131	<0.001	0.454	<0.001	0.541	0.537	0.191

* sjTREC were quantified in CD4+ and CD8+ cells T cells isolated annually from the PBMC of radiation survivor cohort NHP. Data were adjusted for sex, age at collection, and repeated measures and then modelled for correlation with dose grouping and tested for interaction effects between dose and time since irradiation. Adjusted means were then tested for significant dose differences within each time category. $p < 0.05$ in bold.

Major Task 1: NHP Studies - Prospective Cohort

Immunological findings using the prospective cohort were recently published in Macintyre et al. (Adv Radiat Oncol. 2021) and are summarized below.

Animals in the prospective cohort were immunophenotyped multiple times using the same approach as for the survivor cohort, above (**Fig. 3.1**). The percentage of cells classified as mDC and pDC decreased over time (mDC: $p=0.0117$; pDC $p=0.0008$) (**Fig. 3.8, B**), there was no significant impact of irradiation or interaction between time and irradiation. The frequency of circulating B cells fell significantly as time passed ($p<0.0001$) (**Fig. 3.8C**), and radiation exposure did not have a significant effect on this frequency. The percentage of activated (CD80+) B cells decreased significantly with time ($p=0.0039$) (**Fig. 3.8D**). The percentage of memory (CD27+) B cells changed significantly with time ($p<0.0001$) (**Fig. 3.8E**) but irradiation had no significant effect on the expression of either marker. Collectively, these results imply that while aging led to a drop in the percentage of circulating B cells, as observed in the survivor cohort, radiation did not have a long-term impact on B cell frequency or naïve/memory balance.

While the percentage of circulating B cells fell as the animals aged, the percentage of T cells increased ($p<0.001$) (**Fig. 3.8F**). Although the ratio of CD4+ to CD8+ T cells changed over time ($p<0.0001$) (**Fig. 3.8G**), there was no significant impact of irradiation or interaction between time and irradiation on either the percentage of T cells or the CD4/CD8 ratio. There was no significant difference in CD4+CD8+ frequency between the control and irradiated groups (**Fig. 3.8H**).

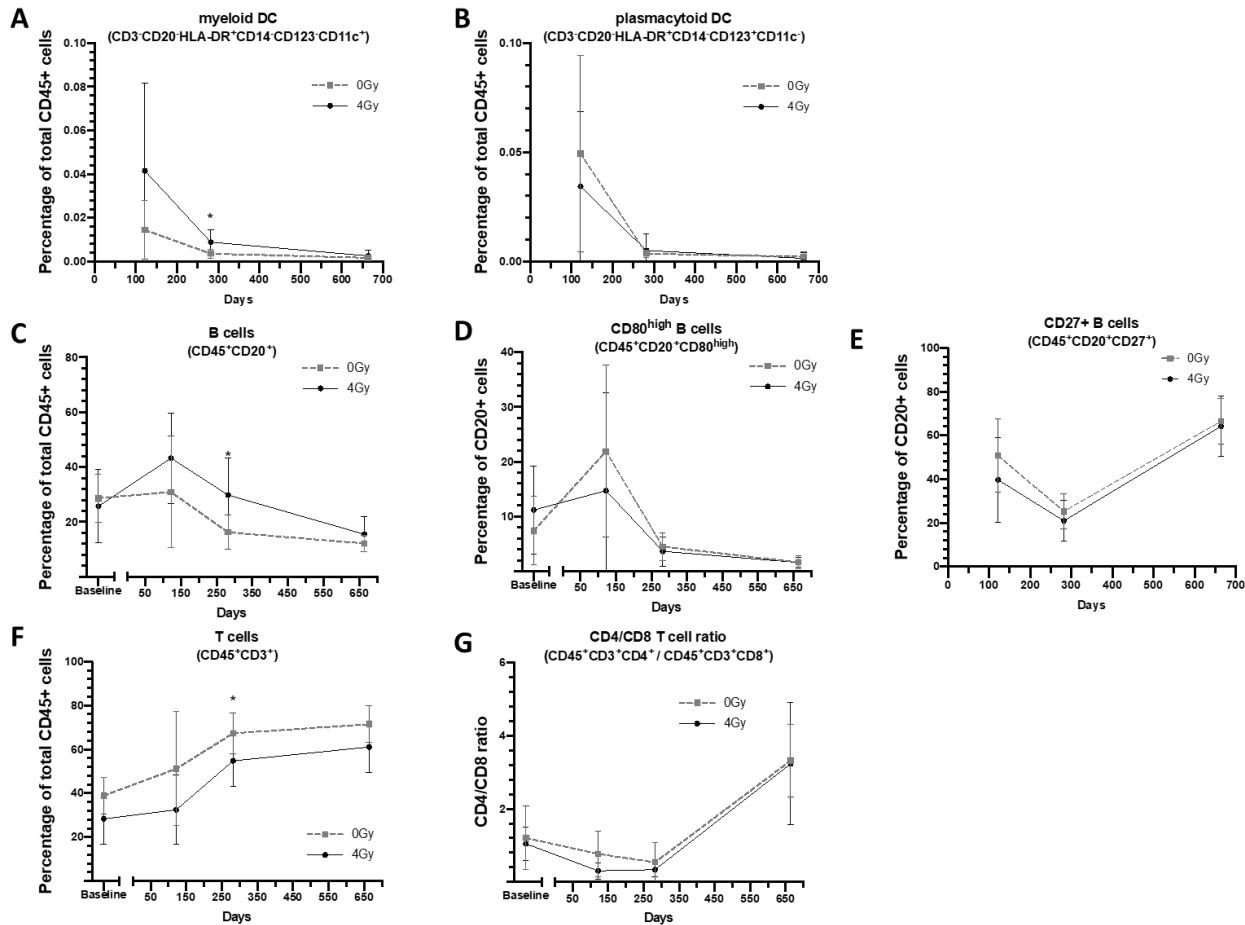


Figure 3.8. Homeostatic Rebalancing of B, T and Dendritic Cells After TBI. PBMC from the prospective study were immunophenotyped at baseline and/or 122, 282, 664 days (4, 9, 21 months) after irradiation. Data are expressed as percentages of the parent population noted on the y-axis. * $p < 0.05$ by Fisher's least significant difference test.

The relative proportion of CD4⁺ and CD8⁺ naïve cells changed with time (CD4⁺: $p = 0.0036$; CD8⁺: $p < 0.0001$) and with radiation exposure (CD4⁺: $p = 0.0099$; CD8⁺: $p = 0.0241$) (**Fig. 3.9A, D**). The percentage of CD4⁺ naïve T cells, but not CD8⁺ naïve T cells, also showed a significant interaction between time and irradiation ($p < 0.0001$). The proportions of naïve T cells in irradiated animals were significantly lower than in age-matched controls at the first post-TBI timepoint tested (CD4⁺: $p = 0.0002$; CD8⁺: $p = 0.0083$). Thus, aging led to a drop in the frequency of naïve CD4⁺ T cells and an increase in the frequency of naïve CD8⁺ T cells. Further, irradiation led to significant decreases in the frequencies of both CD4⁺ and CD8⁺ naïve T cells.

The frequencies of both T_{EM} populations were significantly affected by time (CD4⁺: $p = 0.0102$; CD8⁺: $p = 0.0004$). The frequency of CD8⁺, but not CD4⁺, T_{EM} was also significantly disturbed by irradiation ($p = 0.0002$) (**Fig. 3.9B,E**). These data indicated that aging led to an increase in T_{EM} frequency, with radiation causing a sustained increase in the frequency of CD8⁺ T_{EM}.

CD8⁺, but not CD4⁺, T_{CM} were subject to significant time-dependent effects (CD8⁺: $p = 0.0278$) and were sensitive to irradiation (CD8⁺: $p = 0.001$) (**Fig. 3.9C,F**). In both CD4⁺ and CD8⁺ T_{CM} populations there was significant interaction between time and radiation exposure (CD4⁺: $p = 0.0196$; CD8⁺: $p = 0.0204$). This indicated that aging did not affect the frequency of CD4⁺ T_{CM} over the 21-month study, but that the frequencies of both CD4⁺ and CD8⁺ T_{CM} were affected by radiation exposure, with the impact on CD8⁺ T_{CM} being more prolonged.

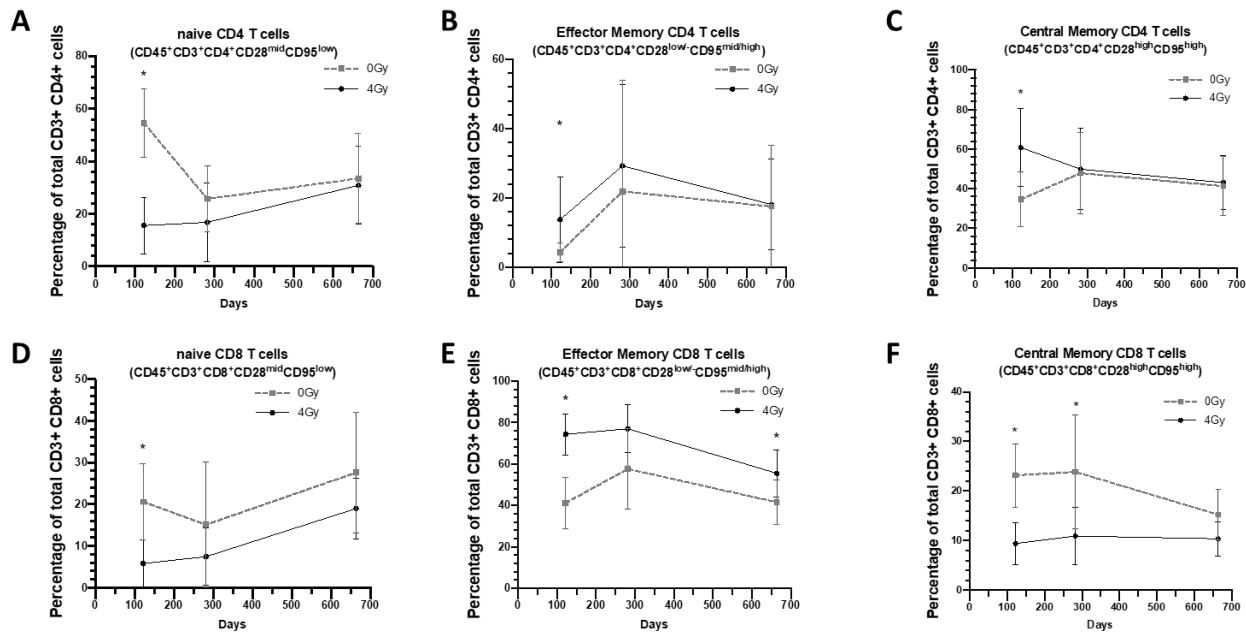


Figure 3.9. Homeostatic Rebalancing of CD4+ but not CD8+ T Cell Populations After TBI. PBMC from the prospective study were immunophenotyped at baseline and/or 122, 282, 664 days post irradiation and gated on the T cell populations indicated. Data are expressed as percentages of the parent population noted on the y-axis. * $p < 0.05$ by Fisher's least significant difference test.

Thymopoiesis and Peripheral Expansion Reconstitute the Peripheral T Cell Compartment After TBI

We quantified sjTREC in CD4⁺ and CD8⁺ T cells isolated from the control and irradiated animals (**Fig. 3.10A,B**). We did not observe a statistically significant change in CD4⁺ or CD8⁺ TREC numbers with respect to either time or irradiation. However, CD4⁺ T cells in one irradiated animal exhibited a transient 70-fold increase in sjTREC at the first timepoint after irradiation, and several of the irradiated animals showed a transient spike in CD8⁺ TREC numbers after irradiation as evidenced by the larger standard deviations of the irradiated samples nine months after irradiation. Interpreting these data is complicated by the animals in the irradiated group having higher and more variable CD4⁺ and CD8⁺ sjTREC counts than the control group pre-irradiation. However, they suggest that if the thymus contributes to CD4⁺ T cell repopulation after irradiation then it primarily does so acutely (earlier than three months after exposure), and that the contribution to CD8⁺ T cell repopulation is transient and variable.

To determine if TBI caused long-term changes to TCR diversity, we sequenced TCR α and β mRNA from PBMC collected at baseline and nine months post irradiation. PBMC from five control and six irradiated animals yielded sufficient RNA for baseline and post-exposure TCR α and/or TCR β sequencing. We first evaluated the top ten α and β clones in each animal at baseline and nine months post TBI. The distribution and identity of the immunodominant clones in each of the non-irradiated animals changed considerably between timepoints, revealing the dynamic nature of the TCR repertoire. We observed similar dynamism in immunodominant clones in the irradiated group (**Fig. 3.10C, D**). To evaluate global TCR diversity beyond the top ten clones, we calculated the TCR α and TCR β Shannon entropy index for each animal at both timepoints. This index incorporates both the number of clones and frequency of each clone, and the higher the index, the greater the diversity of T cell clones in an animal[3]. While entropies in each animal changed over time, there were no consistent trends (**Fig. 3.10E,F**), and mixed-effects modeling did not indicate any effect of time or radiation on the entropies. Thus, radiation did not significantly constrain or enhance overall T cell repertoire diversity. Next, we determined the overlap in clones between baseline and nine months post TBI for each animal. The cumulative frequencies of overlapping TCR α and TCR β clones over time were less in irradiated animals, although these differences did not reach statistical significance (**Fig. 3.10G,H**). There was, therefore, a trend for irradiated animals to have a higher proportion of clones at nine months that were not present at baseline.

Collectively, these data revealed that while dominant TCR clones changed with time, single high-dose irradiation did not lead to a significant long-term loss of TCR diversity or sustained domination of the repertoire through expansion of a small number of clones. New TCR clones only arise during thymopoiesis, and so these findings further support the model that long-term repopulation and homeostasis of circulating T cells following TBI was partially driven by thymic output rather than solely by peripheral expansion.

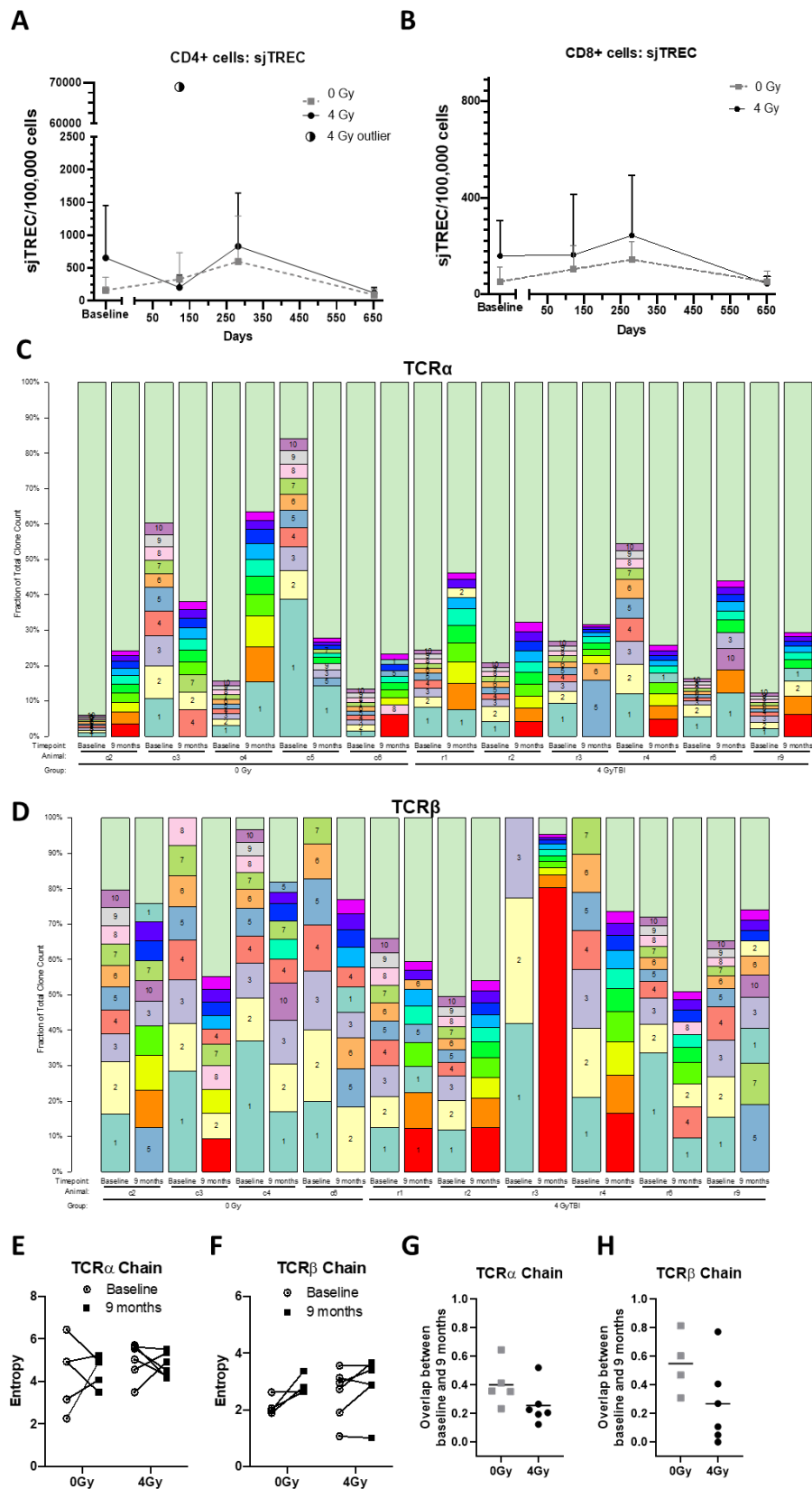


Figure 3.10. Thymopoiesis Contributes to T Cell Repopulation After TBI. (A, B) sjTREC content of CD4+ and CD8+ T cells isolated from PBMC collected at baseline, 122, 282, 664 days post irradiation. (C - H) TCR chains were sequenced from PBMC at baseline and 9 months post irradiation. C, D show cumulative frequency distribution of top ten clones in each animal at each timepoint. Each color represents a distinct clone, and clones found in the same animal at both timepoints are numbered. E, F show entropy for each chain in each animal at baseline and nine months post exposure. Lines link

samples from the same animal. G, H show the mean and per-animal cumulative frequencies of overlapping clones between baseline and nine months post irradiation.

TBI Leads to Long-Term Deficiencies in Responses to Select Antigens

We next evaluated in this cohort the immune response a 23-serotype pneumococcal polysaccharide vaccine (23vP) and a tetravalent 2018/9 seasonal influenza vaccine containing four influenza hemagglutinin (HA) proteins. The pneumococcal vaccine and the first dose of the influenza vaccine were administered approximately 118 weeks after TBI or sham irradiation. A booster influenza vaccine dose was administered four weeks later. Serum was collected from each animal immediately prior to vaccination (baseline) and 1, 4, 5, 8- and 16-weeks post.

HA-specific IgG titers for each animal were normalized to baseline and plotted against time. The area under each curve (AUC) was calculated as shown in **Fig. 3.11A** for a representative animal/HA. The IgG responses for all animals and all HAs is summarized in **Fig. 3.11B**. With one exception, all vaccinated animals had detectable IgG responses to at least three of the vaccine HAs. The majority of control (n=4/6) and irradiated (n=6/10) animals had IgG responses to all four HAs, and there were no significant differences between the control and irradiated groups in the AUC of the IgG responses for any of the HAs. Thus, irradiation did not affect the amount of anti-HA IgG produced in response to vaccine strain HA proteins.

Sera were then tested for their ability to neutralize the four influenza virus strains represented in the vaccine.

Neutralizing titers for each virus were plotted against time and AUC calculated as shown in **Fig. 3.11C**. The AUC for all four viruses and 16 animals are shown in **Fig. 3.11D**. The responses to the two influenza B vaccine strains were weak in all animals, with no animals generating neutralizing antibodies to B/Colorado/6/2017 and only two generating neutralizing antibodies to B/Phuket/3073/2013. There were no significant differences in influenza A responses between the irradiated and sham-irradiated animals (**Fig. 3.11D**).

The humoral response to 23vP was quantified by measuring serum IgG for each of the 23 pneumococcal polysaccharides using a multiplex bead array. IgG titers for each polysaccharide were normalized, plotted against time and then the above-baseline AUC was calculated. An example plot for one animal and serotype is shown in **Fig. 3.11E** and the AUCs for all serotypes and animals are shown in **Fig. 3.11F**. Some serotypes elicited similar IgG responses in control and TBI animals. However, the irradiated animals showed significantly weaker IgG responses to 10 of the 23 serotypes in the vaccine. The difference in response to serotype 19F was particularly striking, with 100% (n=6/6) of control animals exhibiting a response and only 10% (n=1/10) of the TBI animals responding (p<0.00001). In addition, while all six of the control animals showed some IgG response to a minimum of 17 serotypes, three of the 10 irradiated animals showed responses to 12 or less serotypes. Therefore, despite the total WBC counts (not shown) and frequencies of DC, B and T cell populations all returning to their expected setpoints, irradiated animals mounted significantly weaker humoral responses to select bacterial polysaccharide antigens over two years after radiation exposure.

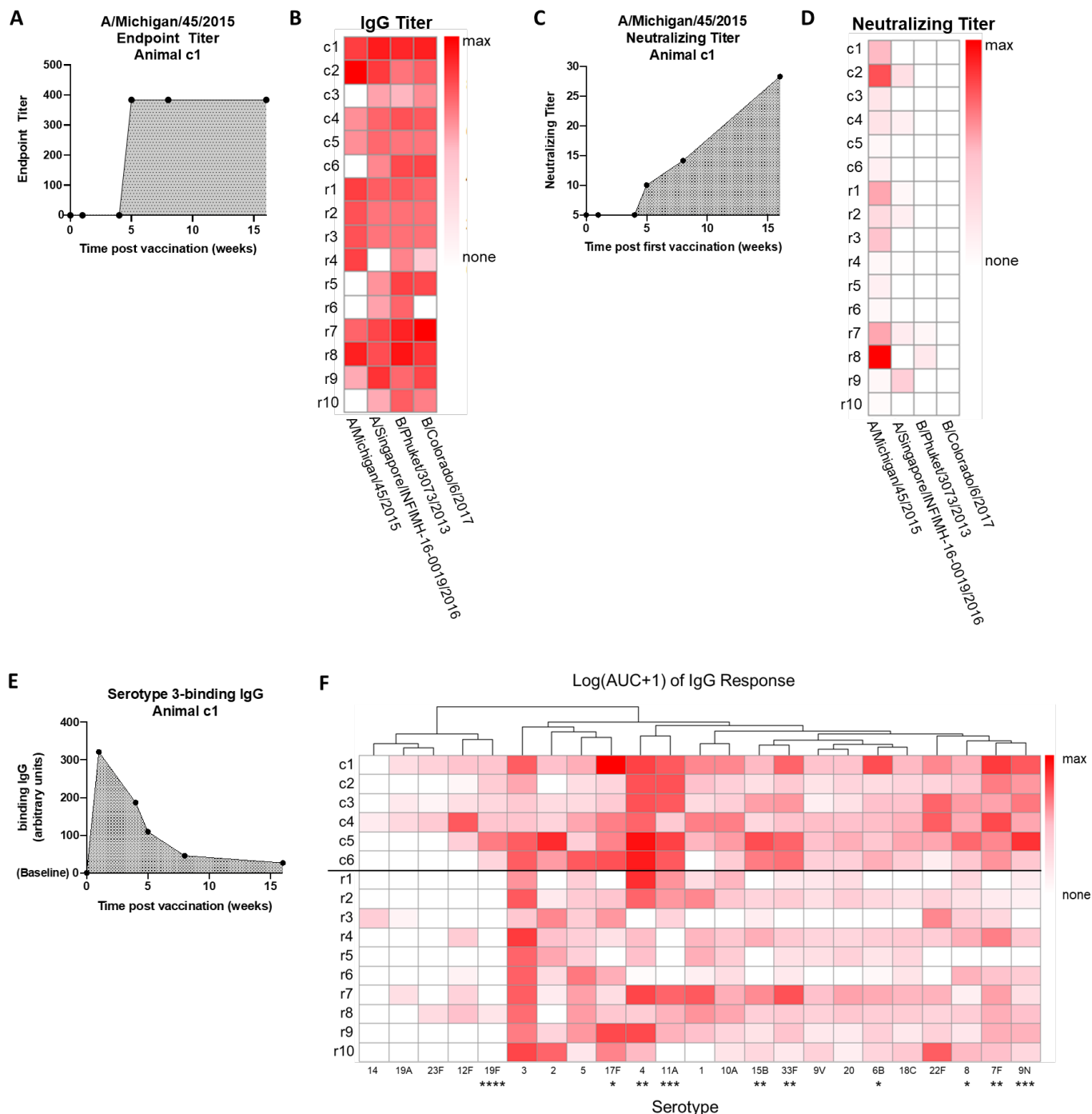


Figure 3.11. Responses to Vaccination Two Years After TBI. Prospective cohort macaques were vaccinated with a 23-valent polysaccharide vaccine and tetraivalent seasonal influenza vaccine 118 weeks after irradiation. Sera were assayed for **(A,B)** hemagglutinin-binding IgG, **(C,D)** neutralizing antibodies against the influenza strains included in the vaccine, and **(E,F)** the 23 pneumococcal polysaccharides. Responses for each animal were plotted as shown in **(A,C,E)** and Area Under the Curves (AUC, shaded) calculated. **(B)** Heatmap of log(AUC+1) for the above-baseline response to each HA over time for all animals and HAs **(D)** Heatmap shows the AUC for the neutralization response over time for all animals and influenza strains. **(E)** Heatmap shows log(AUC+1) for the above-baseline response to each pneumococcal serotype over time. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by t test.

Major Task 2: Murine Reconstitution Studies

As stated in the Statement of Work, the goal of the murine studies under Specific Aim 1 is to define the roles of thymopoiesis and peripheral expansion in overall T cell reconstitution after radiation-induced injury based on radiation dose. As the first step, we characterized how T cells were recovered in peripheral blood and different lymphoid organs after different doses of radiation exposure (2 Gy, 5 Gy, 7 Gy). As shown in the previously submitted annual reports (Year 1-4), we demonstrated that T cell numbers in peripheral blood were able to recover back to normal after irradiation. There was a lasting defect in splenic T cell recovery in the high dose (7 Gy) group. T cells retained the proliferative capacity and repertoire diversity despite long-term thymocyte defects. Mechanistically, both thymopoiesis and peripheral expansion of radioresistant T cells contributed to overall T cell recovery as demonstrated in several different models.

scRNA-seq reveals distinct T cell populations following exposure to TBI

To further understand how T cells recover after radiation injury, we performed single cell RNA sequencing on radioresistant T cells. The results are shown in Year 5 annual report and below. Following exposure to lethal TBI (10.5 Gy) and bone marrow transplantation from CD45.1⁺ donors, we collected splenocytes from recipient-derived (CD45.2⁺) T cells of control (n = 3), 5 days (n = 6), and 21 days (n = 5) groups (**Fig. 3.12A**). The distribution of various lymphocytes prior to purification is shown was assessed by flow cytometry (**Fig. 3.12B**). Early after TBI (day 5), T cell frequency was elevated compared to control group, along with an increase in NKT cell frequency. Later during recovery (day 21), total T cell frequency was reduced, likely contributed by increased proliferation of other cell types. In addition, although $\gamma\delta$ T cell frequency was elevated during recovery, $\alpha\beta$ T cells remained the predominate T cell population.

To characterize how distinct T cell populations respond and recover from TBI, splenic CD4 and CD8 T cells were purified by fluorescence-activated cell sorting (FACS) and pooled for library construction using the 10x Genomics Chromium platform, followed by sequencing using Illumina's NextSeq high output platform. Sequencing depth was approximately 50,000 reads per cell. Quality control was performed using Seurat to filter out doublets and lysed cells. Cells that passed quality control were kept for further analysis, including dimensionality reduction using Uniform Manifold Approximation and Projection (UMAP), differential gene expression analysis, and clustering. We first performed unsupervised clustering analysis, excluding NKT cells and regulatory T cells in attempt to further resolve the populations, yielding six distinct clusters (clusters 0, 1, 2, 3, 4, and 6) (**Fig. 3.12C**). SingleR, an automatic annotation method was used to determine cell identities based on the reference cell types using the murine Immunological Genome Project (ImmGen) database. Clusters 0 and 1 strongly corresponded to CD4 and CD8 naïve T cells (T_N), respectively (Figure 1D). Other top matches for cluster 1 includes CD8 effector and memory T cells (T_{EFF} and T_M), indicating heterogeneity within the cluster. By contrast, the identities of clusters 2, 3, 4, and 6 correlated primarily to T_M or T_{EFF} (**Fig. 3.12D**). Notably, all four clusters matched strongly with thymocytes, indicating elevated proliferative capacity. To better identify each cluster, a second annotation method was applied using representative genes from known T cell subsets, including T_N , T_M , and T_{EFF} (**Fig. 3.12E**). In addition, genes associated with cycling and proliferation were included for annotation. Overall, the phenotype of clusters 0 and 1 indicates the presence of predominately T_N , along with some T_M . In addition, the expression of cycling genes was considerably lower than the remaining clusters. Clusters 2, 3, 4, and 6 primarily exhibited the effector memory T cell (T_{EM}) phenotype of both CD4 and CD8 T cell origins. The four clusters were further divided into 2 categories based on the relative expression of cycling genes: moderately (MP) and highly proliferative (HP) T_{EM1} and T_{EM2} . The above annotation system of the unsupervised clustering analysis suggests that while naïve T cells were shared across control and TBI groups, four distinct proliferative T_{EM} subsets were unique to irradiated animals. Furthermore, the emergence of proliferative T_{EM} subsets indicates that T cells actively undergo homeostatic proliferation following radiation exposure.

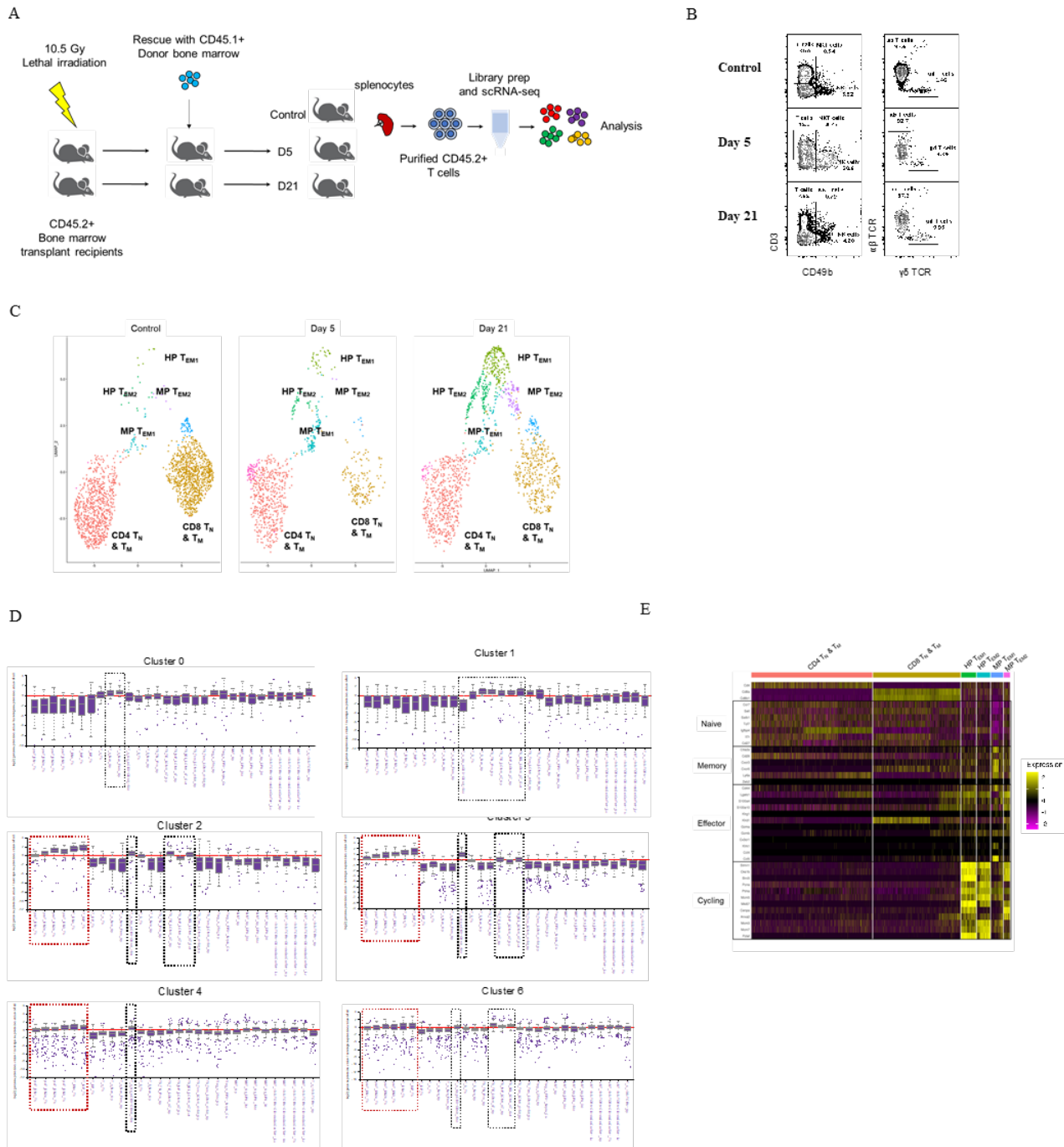


Figure 3.12. Experimental flowchart for single-cell RNA sequencing of splenic T cells from control and irradiated mice. Distribution of distinct lymphocyte cell types prior to T cell purification is shown in the flow cytometry analysis. Splenic T cells were pooled from each group (control, $n = 3$; day 5, $n = 6$; day 21, $n = 5$), isolated by FACS, subjected to library construction and RNA sequencing. Estimated cell numbers used for analysis, sequencing depth, and number of reads were summarized in the table.

Single cell RNA-sequencing revealed unique differentiation paths of radioresistant T cells during recovery

To resolve the relationships of proliferative T_{EM} populations and T_N , unsupervised trajectory analysis for CD4 (Fig. 3.13) and CD8 T cells (data not shown) was performed to determine the differentiation lineage, respectively. CD4 T cells were ordered along a pseudotime axis based on the progression of their transcription profile (Fig. 3.13A). We also examined the placement of control, day 5, versus day 21 cells along the pseudotime trajectory (Fig. 3.13B). Notably, while control and day 5 T cells are located toward the early and middle segments of the path, a great number of day 21 cells were ordered along the latter segments, indicating a progressive change in cellular phenotype over the course of recovery. To determine the identities of cells that are present within each segment, Seurat clusters were annotated along the

trajectory (**Fig. 3.13C**). While T_N were primarily mapped along the root of the trajectory, followed by MP T_{EM} and HP T_{EM} were located in the middle or at the end of the trajectory. Similar to analyses using CD4 T cells, the trajectory of CD8 T cells recapitulated the location of the above population along pseudotime. In addition, further analysis revealed that trajectory construction was based on the graded expression of proliferation markers. Collectively, these findings suggest the potential for radioresistant T_N to serve as the primary source of recovery during homeostatic proliferation.

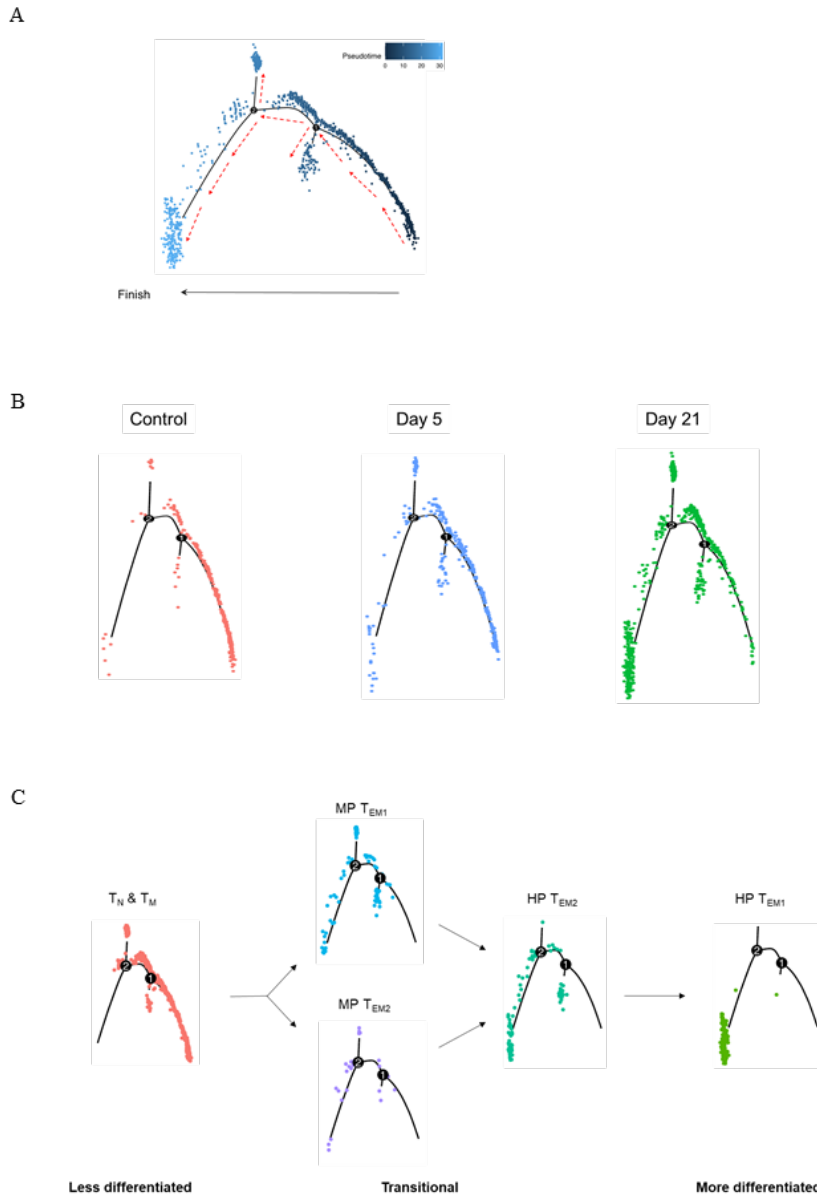


Figure 3.13. T_N & T_M precede other subsets in pseudotime. Pseudotime analysis of CD4 T cells from control and day 5, day 21 irradiated groups. (A) Monocle pseudotime trajectory of T cells from all three groups. Cells are labelled along pseudotime progression. (B) Distribution of CD4 T cells from control, day 5 and day 21 after irradiation on the pseudotime trajectory. (C) Distribution of annotated T cell subsets were identified on the differentiation trajectory, ranging from less differentiated, transition, and more differentiated phenotypes.

CD4 and CD8 T cells upregulate mitochondrial activity and oxidative phosphorylation following TBI exposure

To investigate T cell responses to irradiation after TBI, we utilized pathway enrichment analysis for biological processes and cellular components to determine pathways upregulated in irradiated T cells compared to control T cells. Analysis was performed for CD4 and CD8 T cells respectively to reveal the presence of shared pathways and potential differences during recovery. The kinetics of cellular responses can be visualized by comparing enriched pathways on day 5 and day 21 (**Fig. 3.14A,B**). Early following TBI, CD4 T cells exhibited upregulation in lymphocyte proliferation and apoptotic pathways (**Fig. 3.14**, left panel). Later during recovery, cellular component analysis revealed upregulation of genes

associated with mitochondrial activity (**Fig. 3.14B**, left panel), indicating that mitochondrial metabolism may play a role in T cell persistence. Interestingly, CD8 T cells shared similar characteristics for increased mitochondrial activity (Figure 3A and 3B, right panel). However, upregulation of mitochondrial-associated pathways occurred as soon as day 5 after irradiation, indicating that CD8 T cells may be more dependent on mitochondrial activity for persistence and recovery. These findings suggest that T cells rely on mitochondrial metabolism such as oxidative phosphorylation to promote survival and proliferation following TBI. GSEA analyses of day 21 T cells further showed that irradiated T cells upregulate mTORC1 signaling pathway (**Fig. 3.14C**), which is linked to overall increase in metabolic activity. Moreover, both oxidative phosphorylation and glycolysis pathways were upregulated in day 21 T cells compared to control T cells. To confirm the metabolic state of day 21 T cells, freshly isolated day 21 or control T cells were subjected to Mito Stress Test to measure mitochondrial respiration (**Fig. 3.14D**). Mito Stress Test, which examines mitochondrial function, revealed higher rate of oxygen consumption rate (OCR) in radioresistantly T cells. Further analysis showed that radioresistant T cells undergo enhanced basal respiration and maximal respiration.

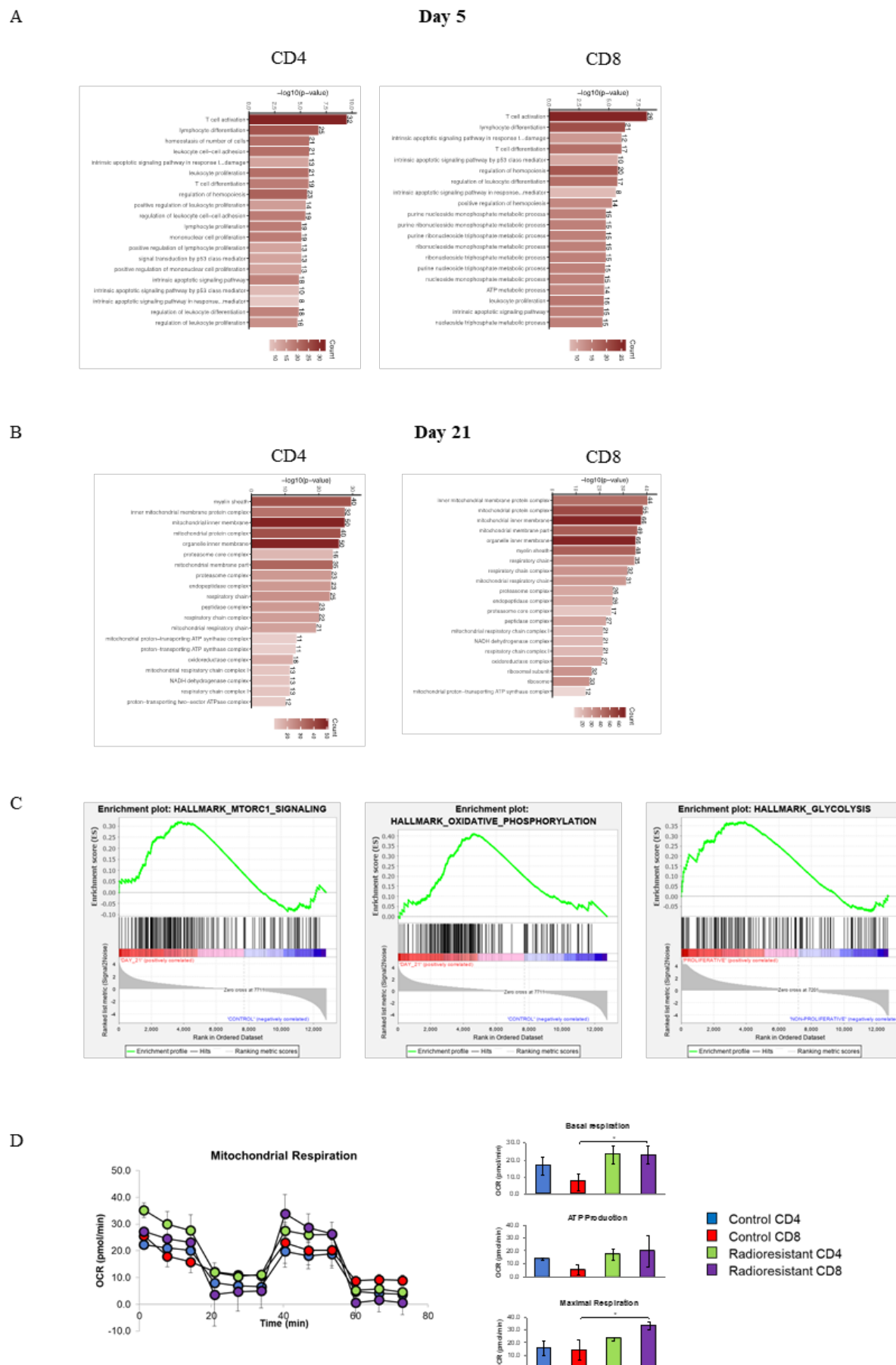


Figure 3.14 CD4 T cells upregulate lymphocyte proliferation and mitochondrial metabolic pathways during recovery after TBI. (A) Naïve T cells (T_N) from 5 days after transplantation were analyzed for enriched pathways compared to

control T cells. Pathways enriched in recipient-derived CD4 and CD8 T_N are displayed in the left and right panels, respectively. (B) Naïve T cells (T_N) from 21 days after transplantation were analyzed for enriched pathways compared to control T cells. Pathways enriched in recipient-derived CD4 and CD8 T_N are displayed in the left and right panels, respectively. (C) GSEA analysis of recipient-derived T cells shows upregulation of mTORC1 signaling pathway, oxidative phosphorylation, and glycolysis. (D) Control splenic T cells (CD45.2⁺) or recipient-derived irradiated T cells (CD45.1⁺) following transplantation were sorted by FACS, followed by Mito Stress Test to determine basal respiration, ATP production, and maximal respiration with an XF24 extracellular flux analyzer. Oxygen consumption (OCR) was measured following injection of oligomycin, FCCP, rotenone and anytimycin A

The above findings indicate that radioresistant T cells increase the utilization of oxidative phosphorylation during the recovery phase. However, the metabolic alteration can also promote oxidative stress, potentially limiting the functional state and reconstitution dynamics. We next examined the expression of various oxidative stress markers, including Hsp90aa1, Prdx1, Prdx2, and Txn1 in control, day 5, and day 21 T cells (**Fig. 3.15A**, left panel) and across previously annotated Seurat clusters (**Fig. 3.15A**, right panel). As T cells gradually increases the utilization of mitochondrial respiration (Figure 3), the expression of oxidative stress markers steadily increases along pseudotime, which is further emphasized in **Fig. 3.15B**, which shows the distribution of cells expressing low, intermediate, and high levels of stress markers across various groups. In day 5 and day 21 T cells, the majority of cells demonstrate higher level of expression compared to control T cells. This was further recapitulated in the expression of different T cell populations (Figure 4A, right panel). As expected, later during the recovery process, clusters 2 through 6, which are annotated as memory T cells, showed greater oxidative stress compared to T_N (clusters 0 and 1). Future experiments will validate the presence of reactive oxygen species. We will also examine the use of small molecule drugs to dampen the oxidative stress and investigate the effect on the persistence, proliferation, functional aspects of irradiated T cells.

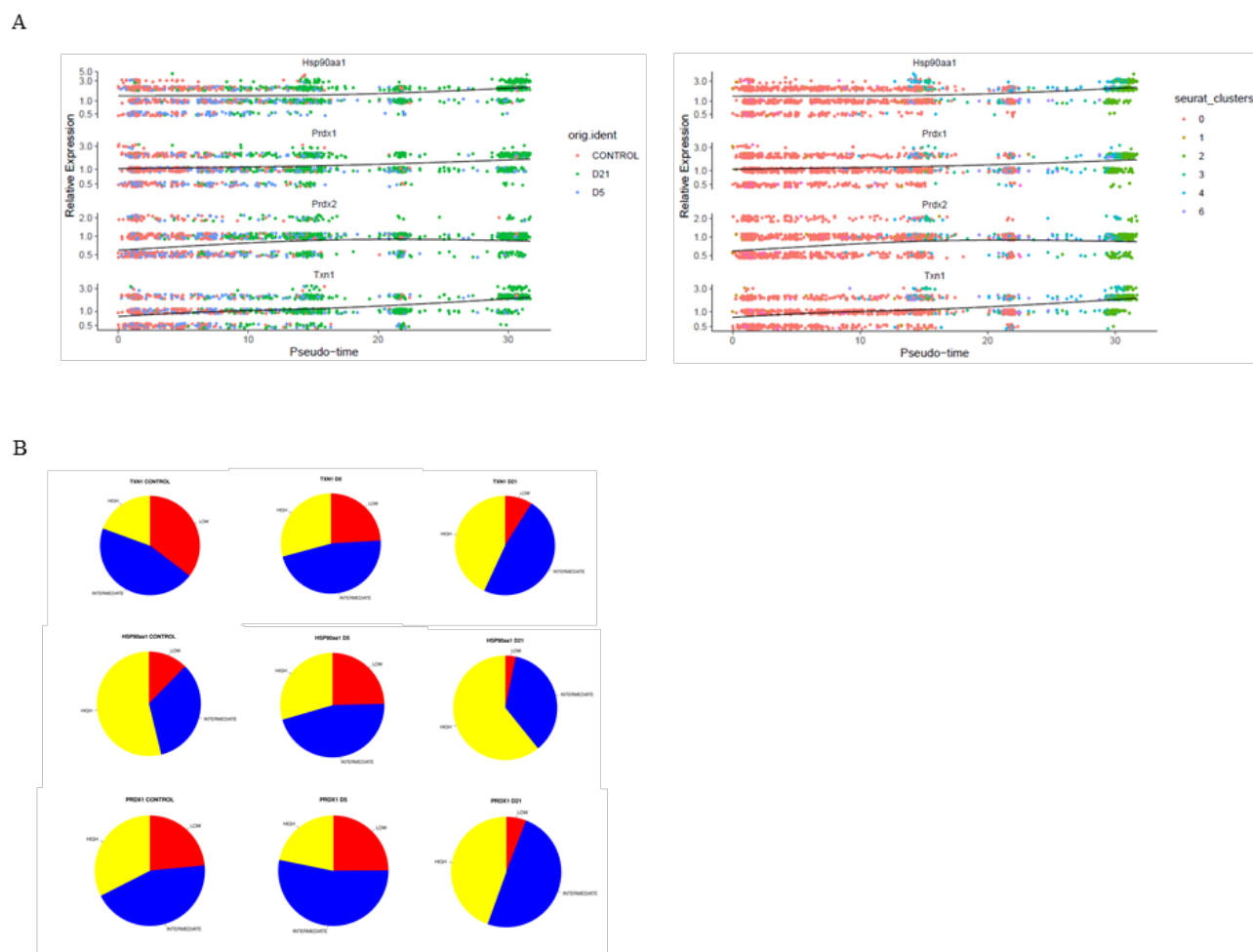


Figure 3.15. T cells upregulate oxidative stress markers during recovery following irradiation. CD4 T cells from control, day 5, and day 21 were analyzed for the expression of oxidative stress markers. (A) Expression of Hsp90aa1, Prdx1,

Prdx2, and *Txn1* in control, day 5, and day 21 T cells (left panel), as well as among various *seurat* clusters annotated in Figure 1 (right panel) along pseudotime. (B) Pie chart representation of oxidative stress marker expression at low, intermediate, and high levels in control, day 5 and day 21 T cells.

Subsets of radioresistant T cells demonstrated increased proliferation capacity in response to distinct types of stimuli *in vitro*

To characterize the phenotype and proliferative capacity of T cell subsets following irradiation, control or recipient-derived radioresistant T cells 21 days after transplantation were sorted based on T_N ($CD44^-CD62L^+$), T_{CM} ($CD44^+CD62L^+$), and T_{EM} ($CD44^+CD62L^-$) phenotypes for subsequent assays. Each subset was then subjected to *in vitro* homeostatic stimulation in the presence of IL-7 and IL-15 to assess the proliferative capacity after labelling with carboxyfluorescein succinimidyl ester (CFSE). Five days following stimulation, both radioresistant CD4 and CD8 T_N and T_{EM} showed enhanced proliferation compared to control T cells. Radioresistant CD4 T_{CM} showed moderately increased proliferation while CD8 T_N showed decreased proliferation compared to control T cells (**Fig. 3.16A**). In addition to homeostatic proliferation, surviving T cells can potentially mediate protection against opportunistic infections following radiation exposure. Therefore, it is critical to assess their capacity to respond to TCR stimulation. To evaluate the proliferative response of radioresistant T cells in the presence of TCR stimuli, sorted T cells were incubated with anti-CD3 and anti-CD28 antibodies. Although radioresistant memory T cells demonstrated comparable proliferation to control T cells, both CD4 and CD8 T_N showed markedly increased ability to proliferate relative to control group (**Fig. 3.16B**). Future studies will aim to assess whether radioresistant T cells are capable of mediating protection from opportunistic infections using *in vivo* models with pathogen challenge.

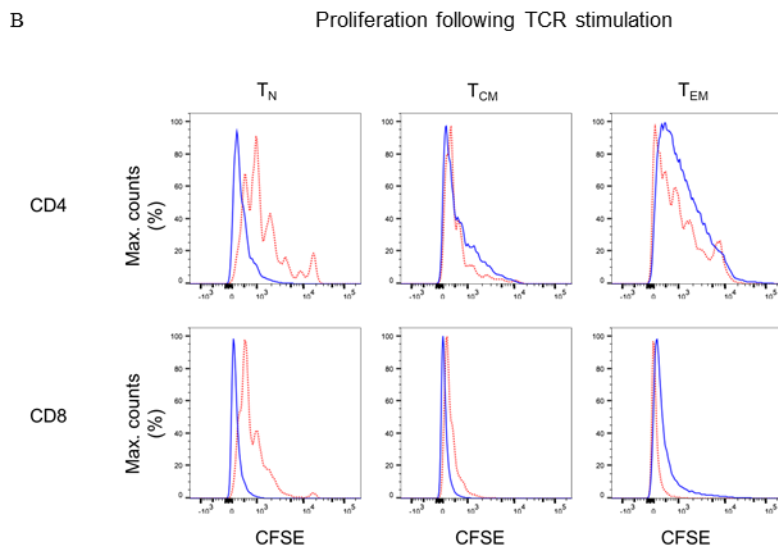
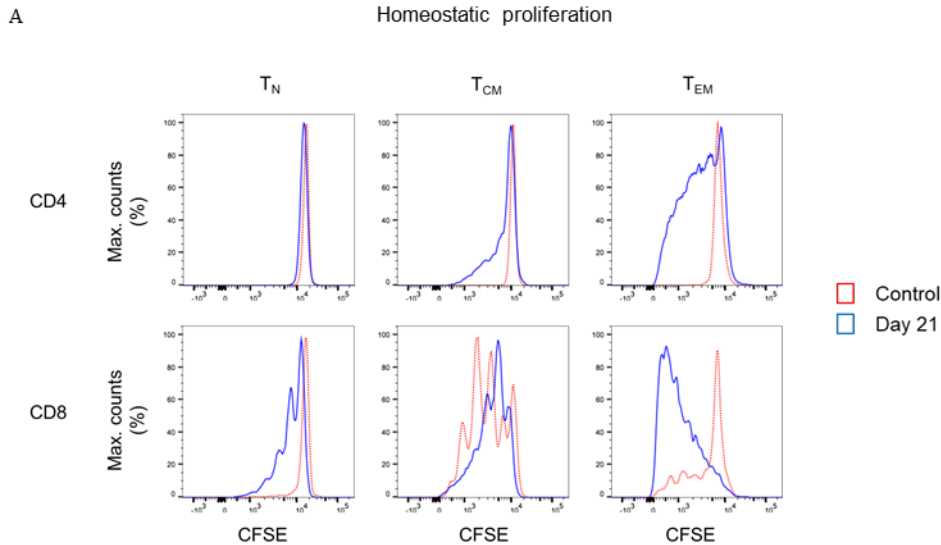


Figure 3.16. Radioresistant T cell subsets demonstrate increased proliferation in response to in vitro stimulation. (A) Radioresistant naïve and effector memory T cells undergo enhanced homeostatic proliferation in vitro. Sorted T cells were labelled with CFSE and stimulated in vitro in the presence of IL-7 and IL-15 for 5 days. (B) Radioresistant naïve T cells demonstrate enhanced proliferation capacity in response to TCR stimulation. Sorted T cells were labelled with CFSE and stimulated in vitro in the presence of anti-CD3 and anti-CD28 antibodies for 5 days.

SA2 Major Tasks 3&4: Mouse Mitigator Studies

The goal of the mitigator studies is to develop medical countermeasures to promote or accelerate overall T cell immunity after radiation injury. In our initial proposal, we proposed to administer HGH and IGF-1 starting at day 28 after irradiation when long-term T cell defect is observed. Due to the unexpected observations that T cell numbers are able to recover back to normal level around 6 weeks post-irradiation, we do not anticipate that the therapeutic window is wide enough for HGH and IGF-1 to work when given at day +28 after irradiation as we initially proposed. To address this issue, we would have to start the treatment early after irradiation. Since we have already demonstrated the positive effects of HGH on T cell recovery when given shortly after irradiation in a published study (Chen et al., PLoS ONE. 2010), we only tested IGF-1 using this alternative regimen. Unfortunately, we could not demonstrate that IGF-1 was able to promote immune reconstitution post-irradiation as we reported in the Year 3 annual report. As an alternative approach detailed in the original proposal, we planned to test other new and more potent agents. One of these agents that we tested was a long-acting IL-7 product, called rhIL-7-hyFc from NeoImmunotech (Rockville, MD). This product has a long half-life in vivo and is currently being testing in several human trials. The results from these studies are presented below.

It is of note that only the data from delayed treatment (day 7) and mechanistic studies were generated under this funding mechanism. The other data were funded by different funding source and are included here as a comparison.

Effects of IL-7-Fc on hematological recovery after radiation injury

C57BL/6 mice, 8-12 weeks old, were irradiated with 5 Gy of TBI. After irradiation, mice received weekly dose of IL-7-Fc subcutaneously at a dose of 10 mg/kg until day +21 (24 hr, day +7, +14, +21). Complete blood counts were determined by automatic hemacytometer before the drug treatment at each time point. As demonstrated in **Fig. 3.17**, 5 Gy of TBI rapidly depleted cells of all linages in peripheral blood. It took at least 4-6 weeks for them to recover back to the baseline levels. Treatment with rhIL-7-hyFc starting at 24 hours led to rapid recovery of total white blood cells and leukocyte subsets counts including neutrophils, lymphocytes, and monocytes. Similaer results were observed when the treatment was started at day +7, when the white blood counts were at the nadir. Unexpectedly, treatmet with IL-7-Fc slowed the recovery of both red blood cells and platelets post-irradiation. These data suggest that IL-7-Fc has positive effects on lymphoid progenitors and myeloid progenitors for netrophils and monocytes but appears to delay reconstitution of red blood cells and platelets.

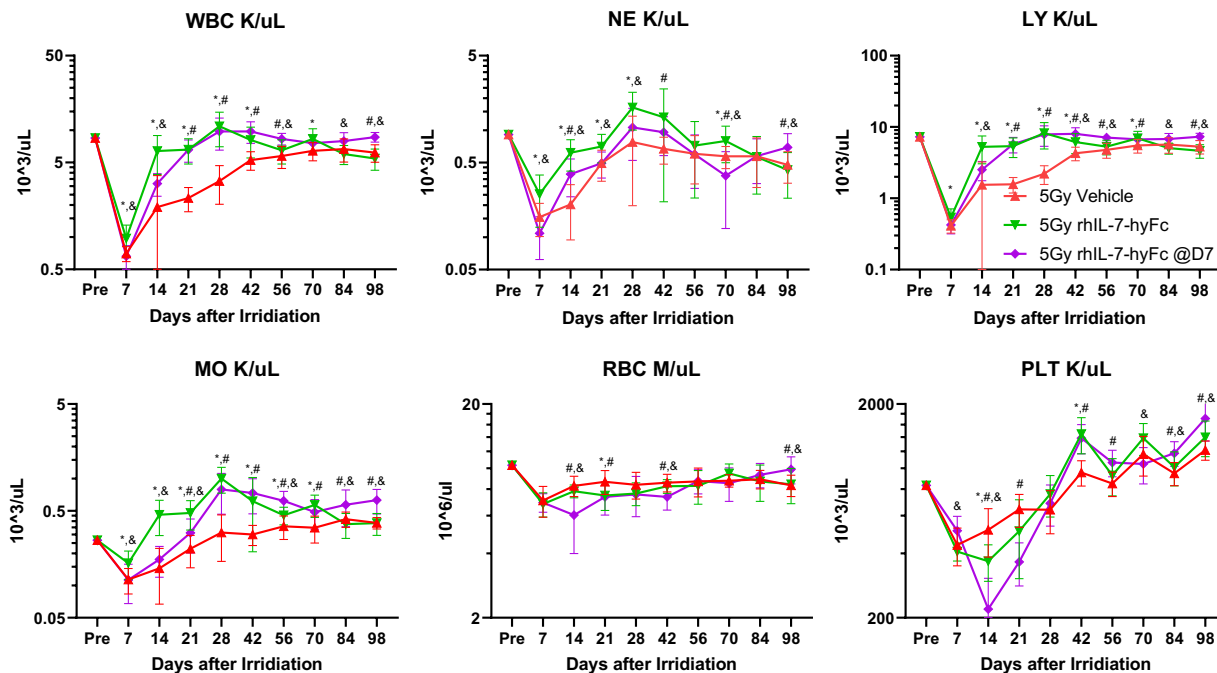


Figure 3.17. Effects of IL-7-Fc on hematological recovery after radiation injury. C57BL/6 mice were first exposed to 5 Gy of TBI. Twenty-four or 7 days later, the mice received weekly s.q. injection of 10mg/kg of IL-7-Fc until day +21. Complete blood counts were obtained weekly before the drug treatment. * $P < 0.05$, rhIL-7-hyFc vs. Vehicle; # $P < 0.05$, rhIL-7-hyFc @D7 vs. Vehicle; & $P < 0.05$, rhIL-7-hyFc vs. rhIL-7-hyFc @D7. WBC, white blood cells; NE, neutrophils; LY, lymphocytes; MO, monocytes; RBC, red blood cells; PLT, platelets.

Effects of IL-7-Fc on lymphocyte subsets after radiation injury

Lymphocyte subsets were determined by flow cytometer in peripheral blood at the same time points as shown in **Fig. 3.17**. As illustrated in **Fig. 3.18**, all lymphocyte subsets dropped 5-200 fold within one week after irradiation. It took them at least 6 weeks to recover back to pre-irradiation level. Treatment with one or two weekly doses of IL-7-Fc either starting at 24 hr or day +7 was able to bring the counts of T and B cells back to normal. In contract to the previous findings with IL-7³³, we did not observe any effect of IL-7-Fc on NK cells. These data suggest that the effects of IL-7-Fc may not be at the common lymphoid progenitor level.

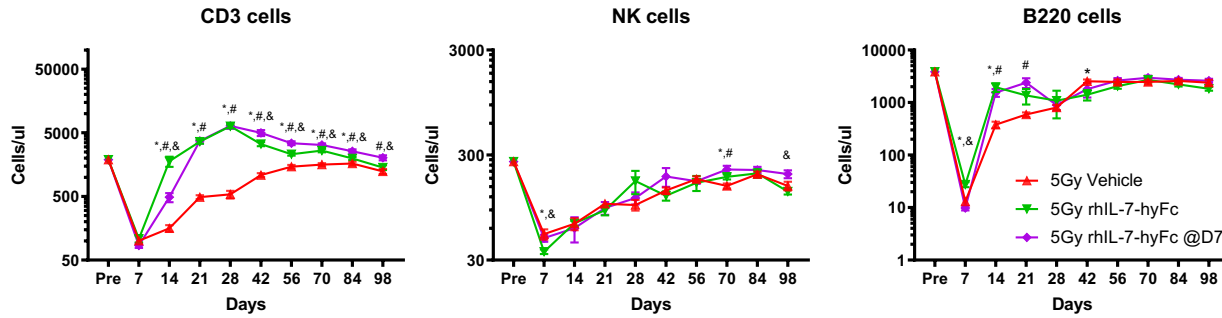


Figure 3.18. Effects of IL-7-Fc on lymphocyte subsets after radiation injury. C57BL/6 mice were first exposed to 5 Gy of TBI. Twenty-four or 7 days later, the mice received weekly s.q. injection of 10mg/kg of IL-7-Fc until day +21. Flow cytometric analysis was performed weekly to determine absolute counts of different lymphocyte subsets in peripheral blood. * $P < 0.05$, rhIL-7-hyFc vs. Vehicle; # $P < 0.05$, rhIL-7-hyFc @D7 vs. Vehicle; & $P < 0.05$, rhIL-7-hyFc vs. rhIL-7-hyFc @D7.

Effects of IL-7-Fc on T cell subsets after radiation injury

To further understand the effects of IL-7-Fc on T cells, we analysed the changes of different T cell subsets after treatment. As shown in **Fig. 3.19**, IL-7-Fc accelerated the recovery of both total CD4 and CD8 cells and their subsets including naïve, central memory, effector memory, and recent thymic emigrants. It is of note that IL-7-Fc appeared to have more rapid effect on CD8 than CD4 T cells.

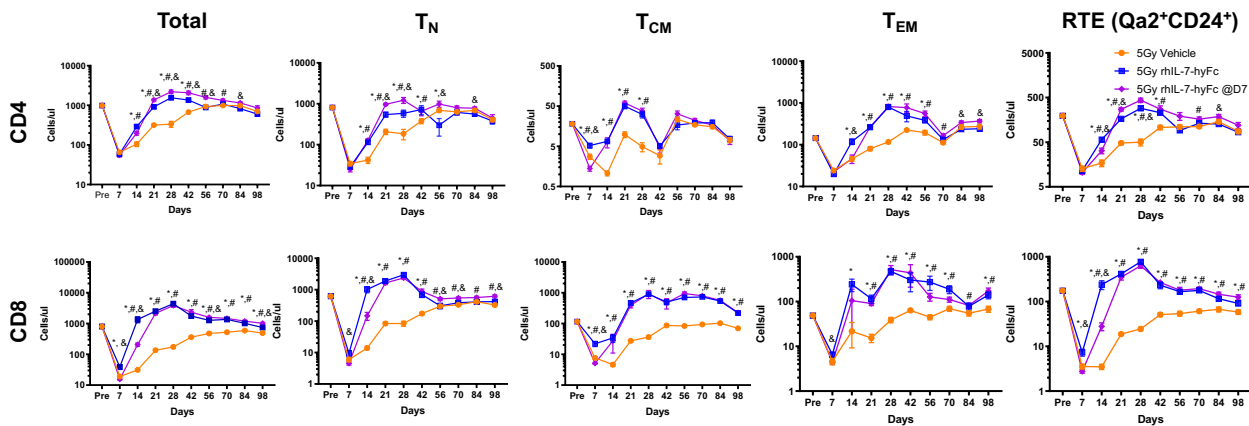
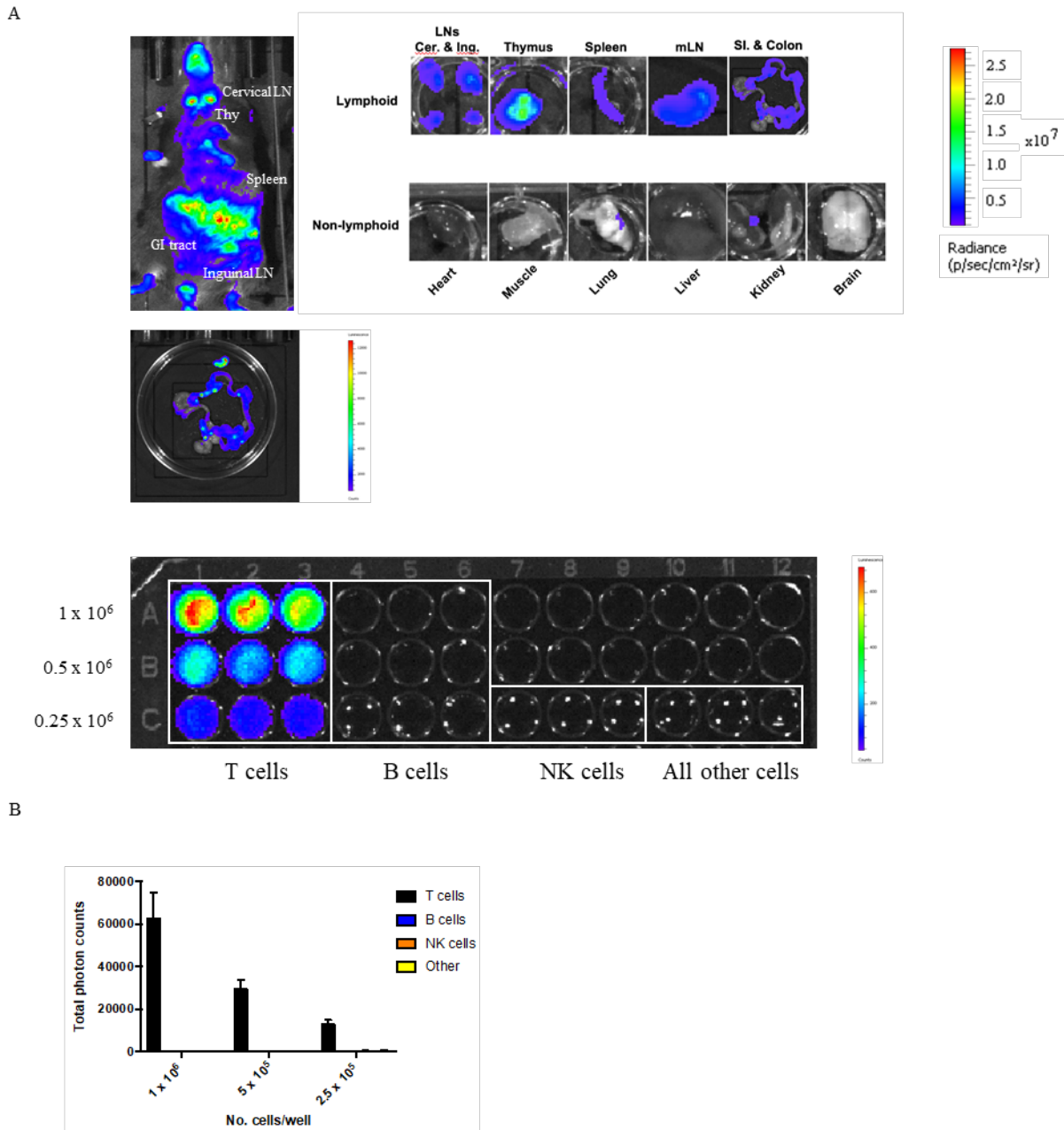


Figure 3.19. Effects of IL-7-Fc on T cell subsets after radiation injury. C57BL/6 mice were exposed to 5 Gy of TBI. Twenty-four or 7 days later, the mice received weekly s.q. injection of 10 mg/kg of IL-7-Fc until day +21. Flow cytometric analysis was performed to determine absolute counts of different T cell subsets in peripheral blood. Different T cell subsets were identified by the following markers: naïve (T_N): CD62L⁺CD44^{low}; central memory (T_{CM}): CD62L⁺CD44^{high}; effector memory (T_{EM}): CD62L⁺CD44⁺; recent thymic emigrant (RTE): Qa2⁺CD24⁺. * $P < 0.05$, rhIL-7-hyFc vs. Vehicle; # $P < 0.05$, rhIL-7-hyFc @D7 vs. Vehicle; & $P < 0.05$, rhIL-7-hyFc vs. rhIL-7-hyFc @D7.

Establishing an imaging system to track T cell recovery in vivo

T cell recovery was studied in peripheral blood by flow cytometry in the above experiments. However, majority of T cells reside in different lymphoid organs instead of blood. Currently, we cannot study T cell reconstitution in other lymphoid organs without sacrificing mice. To address this issue, we established a T cell-specific luciferase reporter strain, which would allow monitoring to T cell recovery in different organs by bioluminescent imaging (BLI). To do this, Luc^{flox/flox} strain was crossed to CD4-Cre strain to generate CD4-Cre x Luc^{flox/flox} (T-Luc) animals. This allows for the constitutive expression of luciferase induced by CD4Cre promoter during thymic development. To assess luciferase expression in vivo, whole body imaging was performed on T-Luc mouse given D-luciferin (**Fig. 3.20A**, top left panel), allowing for the visualization of cervical lymph nodes, thymus, spleen, inguinal lymph nodes, as well as the gastrointestinal (GI) tract. Upon dissection of lymphoid organs and non-lymphoid organs, luciferase expression was primarily detected in lymphoid organs rather than non-lymphoid organs (**Fig. 3.20A**, top right panel). Notably, imaging of T-Luc mice also allowed for the detection of Peyer's Patches (**Fig. 3.20A**, bottom left panel).

Next, to determine whether luciferase expression is restricted to T cells, we sorted splenic T cells ($CD3^+CD4^+$ and $CD3^+CD8^+$), B cells ($B220^+CD3^-$), NK cells ($CD49b^+CD3^-B220^-$), and all other cell types ($CD49b^-CD3^-B220^-$) by FACS. Sorted cells were plated at various concentrations for imaging (**Fig. 3.20B**). While luciferase activity was not detectable in non-T cell populations, luciferase activity in T cells was proportional to the number of cells in each well, further validating that luciferase expression in T-Luc mice is restricted to T cells.



IL-7 promotes T cell reconstitution in different lymphoid organs following total body irradiation

In order to investigate T cell reconstitution kinetics in the presence or absence of IL-7, T-Luc mice were subjected to 5 Gy TBI, followed by weekly IL-7 or vehicle treatment through i.v. injections until day 21 (**Fig. 3.21A**). Weekly BLI was used to visualize T cell locations following irradiation. Following irradiation, T cells were visible in cervical lymph nodes, thymus, and GI tract (**Fig. 3.21B**). However, IL-7 induced rapid recovery in both primary and secondary lymphoid organs compared to vehicle group. However, pigmentation in the original C57BL/6 background limits the detection sensitivity when small numbers of T cells are present, particularly during early stages of recovery from radiation. Furthermore, the limited numbers of available T-Luc mice greatly reduce the number of biological replicates. Additionally, the shaving was

performed weekly prior to imaging to improve detection of T cells. To address these issues, we sought to generate chimera mice using white C57BL/6. Following 10.5 Gy TBI, white C57BL/6 recipients were transplanted with 1×10^6 whole bone marrow from T-Luc donors (**Fig. 3.21C**). Following reconstitution, BLI was performed to determine T cell reconstitution from donor cells. 3 months following transplantation, T-Luc-derived T cells populated both primary and secondary lymphoid organs (**Fig. 3.21D**), indicating stabilized chimera generation that simulates the presence of T cells in the physiological condition without TBI. Therefore, the chimeras with stabilized T cell reconstitution can be used to subsequent evaluation of IL-7 in TBI model as shown in **Fig. 3.21A**. Weekly IL-7 or vehicle injections were administered to chimeras until day 21 following 5 Gy TBI. Similar to preliminary findings using T-Luc mice, robust T cell recovery was observed in IL-7 treated animals within the first few weeks following TBI (**Fig. 3.21E**). Notably, the effect on T cell recovery persisted even after the last injection was administered. T cells were readily detected in the thymus, lymph nodes, spleen, and the GI tract in both groups. However, T cell signals were remarkably more saturated in IL-7 treated mice since day 7 through day 63. From day 70, the signal intensity begins to equalize between the two groups, indicating that the effect of IL-7 is more robust during the first few weeks after TBI. These findings demonstrate the clinical implication that IL-7 treatment following radiation exposure induces rapid T cell recovery, which is critical for mediating protection against opportunistic pathogens when T cells are scarce. The functional implications of IL-7 in the context of radiation exposure will be further tested using infection models such as influenza and Cytomegalovirus.

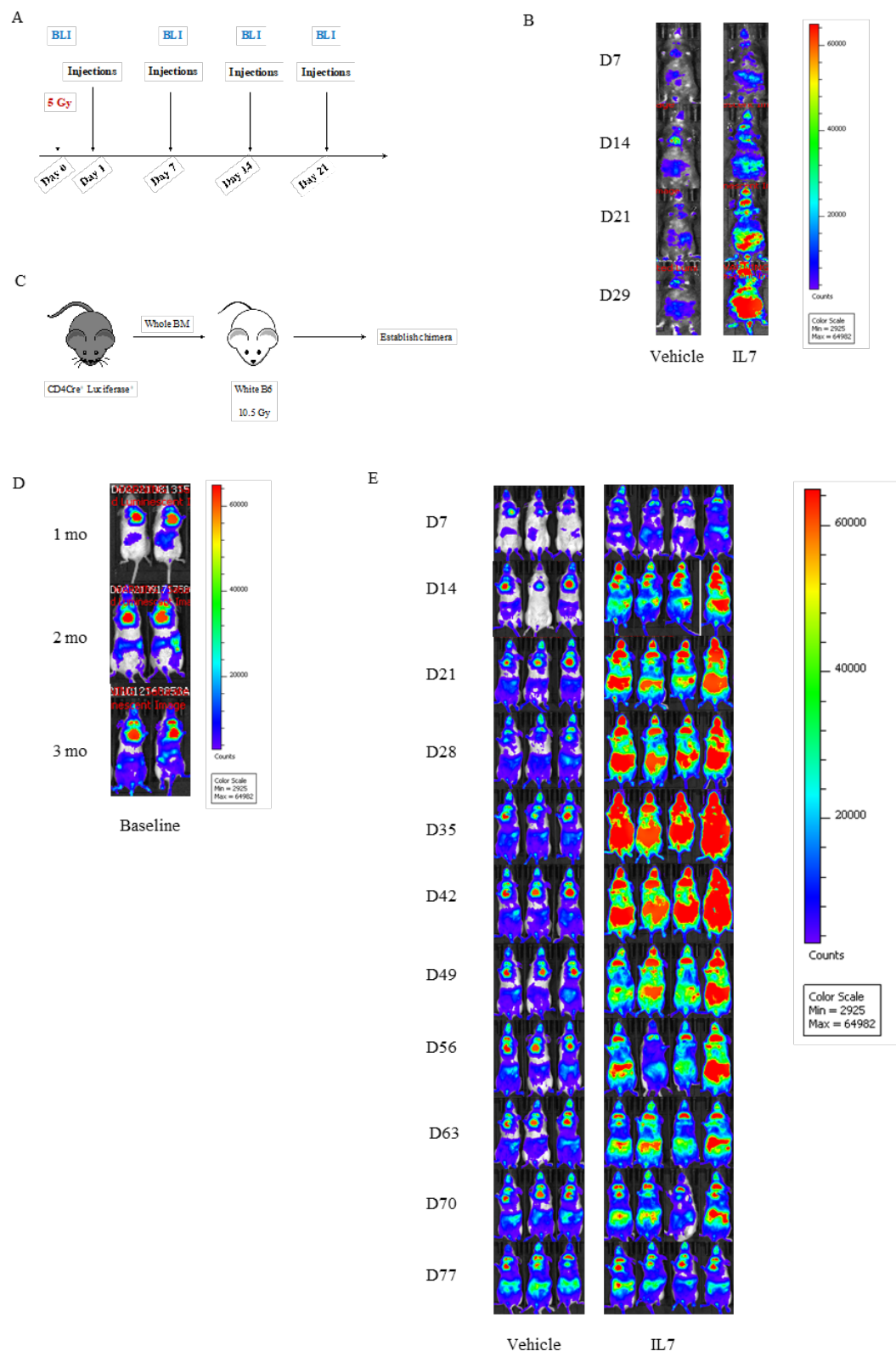


Figure 3.21. IL-7 treatment induces rapid T cell recovery following TBI. (A) Treatment regimen of IL-7 or vehicle through i.v. injections following 5 Gy TBI of T-Luc mice. Mice were shaved prior to imaging. BLI, bioluminescent imaging. (B) BLI of T-Luc mice treated with vehicle of IL-7 following 5 Gy TBI. (C) Generation of white C57BL/6 chimeras using donor bone marrow from T-Luc mice. 1×10^6 whole bone marrow was transferred per white C57BL/6 recipient following 10.5 Gy TBI. BM, bone marrow. (D) BLI of baseline chimera screening for T cell reconstitution at 1 month, 2 months, and 3 months after transplantation. (E) Following chimera generation with T-Luc BM, IL-7 or vehicle was given to chimeras following 5 Gy TBI according to the experimental design in (A), BLI was performed weekly to determine the kinetics of T cell recovery.

IL-7 enhances T cell reconstitution through both thymus-dependent and -independent mechanisms

Chimeras generated using T-Luc bone marrow demonstrated rapid induction of T cell reconstitution following TBI (**Fig. 3.21**). However, it is unclear whether this process is primarily mediated through de novo generation of T cells through radioresistant hematopoietic stem cells during thymic development or through thymus-independent mechanisms such as peripheral expansion of radioresistant mature T cells. To this end, we utilized lethally irradiated white C56BL/7 mice transplanted with either T-Luc-derived lineage negative (Lin⁻) cells from the bone marrow or mature T cells (**Fig. 3.22A**), along with either Lin⁻ cells or T cells from wild type C56BL/7 donors. To simulate the effect of TBI on hematopoietic stem cells and mature T cells, both Lin⁻ cells and T cells were irradiated prior to transplantation so that the contribution of radioresistant Lin⁻ cells and T cells can be detected through BLI. Following transplantation, recipients were treated with either IL-7 or vehicle as shown in **Fig. 3.22A**. Imaging was performed weekly to visualize the T cell recovery in various locations. Following transplantation, IL-7-treated mice began to show T cell signal on day 14 and 21, primarily in the thymus, indicating that radioresistant Lin⁻-derived cells began to contribute to T cell development in the thymus (**Fig. 3.22B**). Over the subsequent weeks, increased signal from the thymus was detected from day 28 through day 56. Moreover, increased T cell signal was detected in the cervical lymph nodes, spleen, and GI tract, suggesting that radioresistant Lin⁻ cells can contribute to the mature T cell pool in the periphery with IL-7 treatment as soon as 21 days after transplantation. In contrast, thymopoiesis from T-Luc-derived Lin⁻ cells was not detected until day 56 in the vehicle group. These results indicate that in the absence of treatments to boost T cell recovery, radioresistant Lin⁻ cells contribute to thymopoiesis albeit in a markedly delayed manner. In contrast, IL-7 treatment considerably accelerates the repopulation of progenitor cells to induce thymopoiesis. These findings also suggest that intervention with IL-7 following radiation exposure may be able to shorten the window of susceptibility to opportunistic infections. In the peripheral expansion model, T cell signals were detected albeit at low intensity 21 days after transplantation (**Fig. 3.22C**). The presence of T cells increased slightly over the course of 56 days. In contrast, T cells can be readily visualized in various secondary lymphoid organs and the GI tract in IL-7-treated group as soon as 14 days after transplantation, at a remarkably higher intensity compared to vehicle-treated group. The results suggest that IL-7 enhances the peripheral expansion of radioresistant T cells. These observations also recapitulated the findings in Figure 7 that the effect of IL-7 persisted even after the end of treatment. Altogether, results from the two distinct transplantation experiments support the role of IL-7 in rapidly enhancing T cell reconstitution after radiation exposure through both thymus-dependent and peripheral expansion mechanisms.

Literature Cited

1. Lynch, H.E. and G.D. Sempowski, *Molecular measurement of T cell receptor excision circles*. Methods Mol Biol, 2013. **979**: p. 147-59.
2. Messaoudi, I., et al., *Nonhuman primate models of human immunology*. Antioxid Redox Signal, 2011. **14**(2): p. 261-73.
3. Stewart, J.J., et al., *A Shannon entropy analysis of immunoglobulin and T cell receptor*. Mol Immunol, 1997. **34**(15): p. 1067-82.

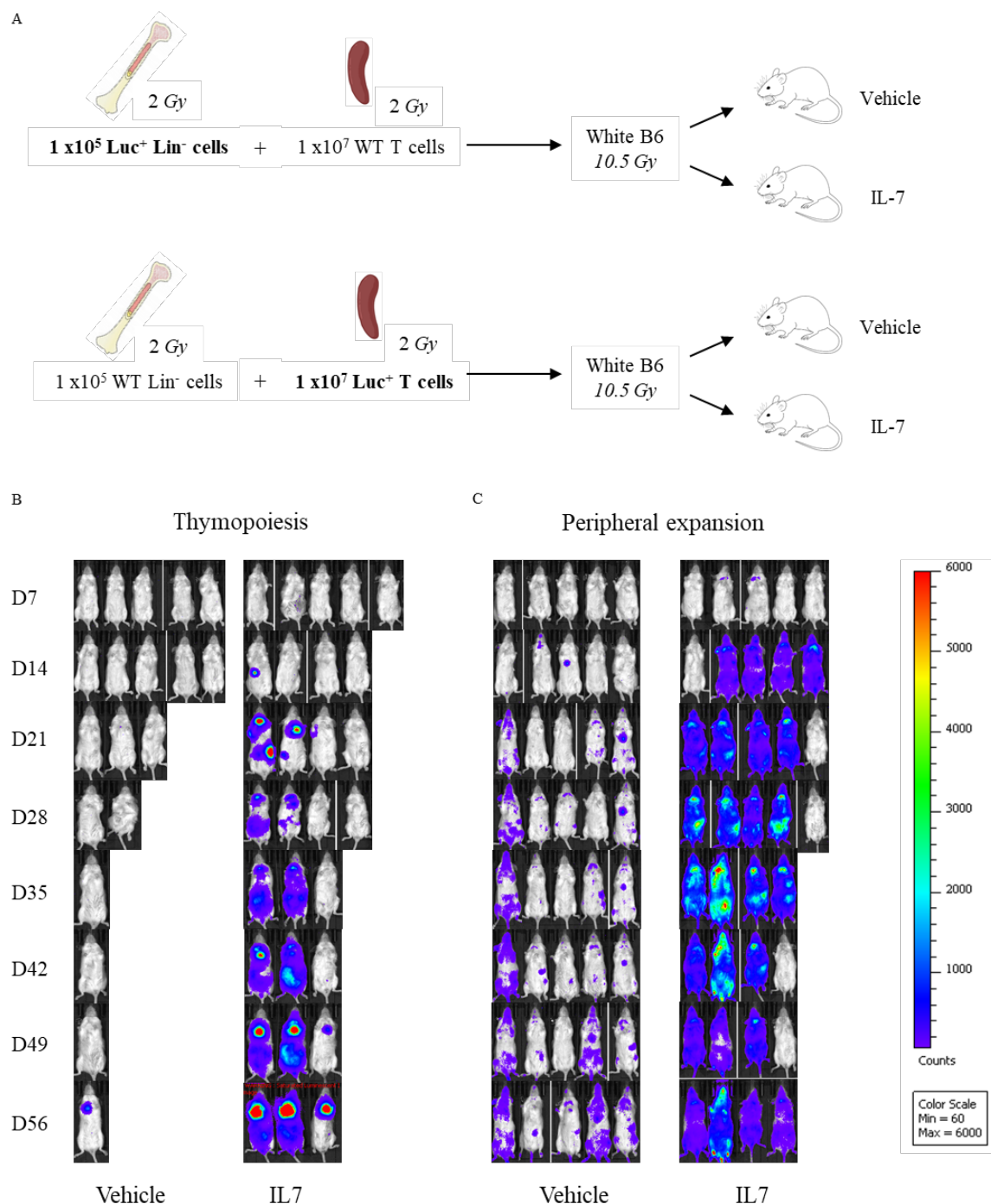


Figure 3.22. IL-7 enhances T cell reconstitution through thymic-dependent and -independent pathways. (A) Schematic of experimental design to determine the mechanisms of IL-7 during T cell recovery. lethally irradiated white C56BL/7 mice transplanted with either T-Luc-derived lineage negative (Lin⁻) cells from the bone marrow or mature T cells, along with either Lin⁻ cells or T cells from wild type C56BL/7 donors. Both Lin⁻ cells and T cells were irradiated at 2 Gy prior to transplantation. Recipients were subsequently treated with vehicle or IL-7 as shown in Fig 7A. (B) BLI of recipients treated with vehicle or IL-7 following transplantation with T-Luc-derived Lin⁻ cells and wild type C56BL/7 T cells. (C) BLI of recipients treated with vehicle or IL-7 following transplantation with T-Luc-derived T cells and wild type C56BL/7-derived Lin⁻ cells.

Project 4 - Genomic Sequencing and Stem Cell Lines (Dr. Dave)

1) Major Activities

- a) Collection and processing of blood samples in the prospective cohort during the subacute post-radiation phase for planned genomic analyses.
- b) Completion of exome sequencing proposed in Aim 1. This include method development for reliable mutation and expression identification from NHPs for which no standard methods currently exist.
- c) Continued discussions with Duke and Wake Forest investigators on cross-project technical collaboration on cell collections, processing, and analysis.
- d) Generation and analysis of RNAseq data from NHPs: We continue to improve the analytical approaches for gapped alignments in NHPs that is important to measure expression from the RNA sequencing reads while skipping the introns in the NHP reference genomes.
- e) Development of deconvolution approaches to resolve different cell types in RNAseq performed on peripheral blood mononuclear cells obtained from NHPs.
- f) Development of in vivo stem cell regeneration model that provides a functional approach to validation of genetic alterations. We performed ex vivo studies of iPS differentiation and are now developing in vivo approaches to overcome the limitations of the ex vivo approaches.
- g) Monthly DOD Conference calls

2) Specific Objectives

There were two main goals of project 4. First, to define the genetic mutations and gene expression signatures induced by radiation. Second, define the molecular basis of immune deficiency and hematologic malignancies induced by radiation.

3) Results and Key Outcomes

SIGNIFICANT RESULTS

Define genetic mutations induced by radiation (Aim #1)

Methods were developed for DNA whole exome sequencing to define the genetic mutations in rhesus macaques. Project 4 investigators also completed the development of methodologies for transcriptome sequencing from rhesus macaque RNA.

Results/Key Outcomes

We have now performed DNA/RNA extraction on a total of 48 cases from 16 individuals before and at two time points after treatment with radiation. Of these, 13 individuals (N=39 cases) yielded sufficient amounts of starting DNA and RNA for sequencing.

All sequencing libraries were carefully quantified using Agilent Bioanalyzer. We also preliminarily sequenced on library on the Illumina Miseq and confirmed even distribution of A/C/G/T bases with very few called as unknown (N). These results indicate that our library construction process has worked well.

Exome sequencing analysis

A critical question in post-radiation exposure and recovery is whether there are clonal effects that guide recovery, i.e. whether radiation leads to the development or selection of clones of hematopoietic stem cells that disproportionately contribute to hematopoiesis. We examined this question by performing whole exome sequencing on the same individuals subjected to RNAseq. We have DNA sequencing from each of the three time points for each individual. Our results suggest that there are no clonal effects post radiation, i.e. there is no dominant clone being selected by radiation. These data will be examined again in the future when more individuals are available to discern whether subclonal or rare allele effects may be associated with post-radiation recovery.

RNA-seq Preprocessing

RNA sequence reads were trimmed (Bolger et al., 2014, PMID: [MC4103590](#)) to remove any remaining Illumina adapter sequences and aligned to the hg19 transcriptome using STAR, a spliced read aligner which allows for sequencing reads to span multiple exons. On average, we were able to align 90% of the sequencing reads for each sample to the reference rhesus macaque genome. Transcript quantification at the gene and transcript level was performed (Li et al., 2011, PMID: [PMC3163565](#)) and expression values were then FPKM normalized, to account for differences in library depth across samples.

Principal Components Analysis (PCA) identified major shifts in gene expression across samples after exposure to radiation. We hypothesized that radiation predominantly affected one or more cell types that resulted in changes in observed gene expression arising from an admixture of cell types. To evaluate the effects of radiation on different cell types, we examined (Newman et al., 2015, PMID: [PMC4739640](#)) the proportions of leukocyte subpopulations in each sample from the normalized expression data. We used validated gene expression profiles to define signatures associated with each lymphocyte type and used 100 permutations to estimate the significance of results. ANOVA tests were then used to determine whether the percentage of each cell type differed across treatment groups.

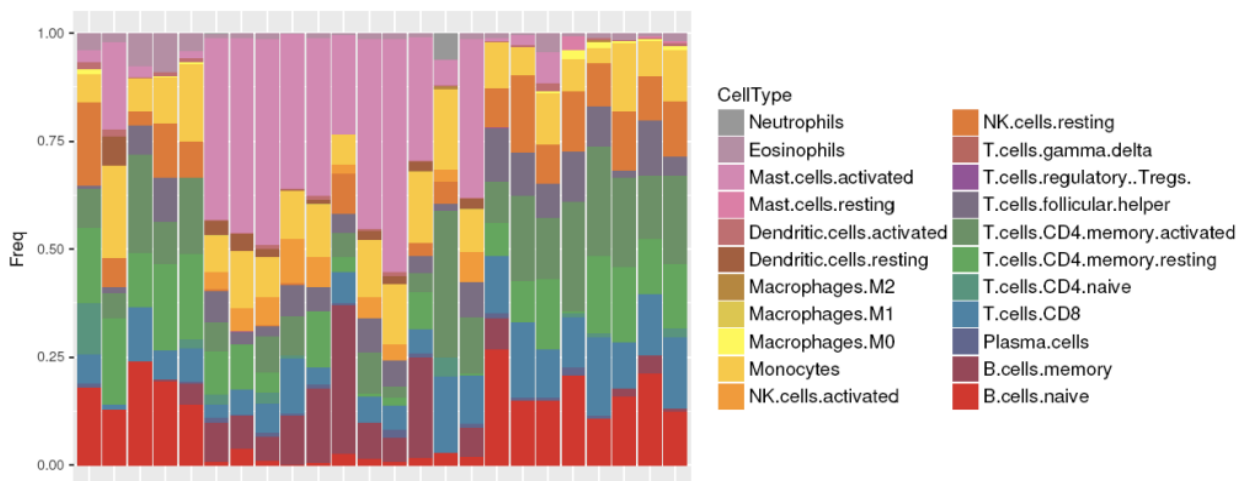


Figure 4.1: Deconvolution analysis of immune populations after exposure to radiation.

Our analysis indicates a large increase ($p < 0.05$) in the proportion of activated mast cells in mid-timepoint samples relative to baseline and late-timepoint samples. We also found that baseline and late-timepoint samples had significantly higher levels of naïve B-cells whereas mid-timepoint samples had significantly higher levels of memory B-cells. There were far fewer significant differences in the proportion of immune cell types between baseline and late-timepoint samples, suggesting the broad effects of radiation on the immune system may be largely reversible in these samples (Figure 4.1).

This analysis is consistent with the observation that memory B cells are best able to withstand the pro-apoptotic effects of radiation. In the memory B cells, we observed two predominant genes that may explain their survival following radiation. The first gene, not surprisingly, is BCL2, which is a known anti-apoptotic factor and is known to be associated with survival of B cells in response to a number of different pro-apoptotic stimuli.

Two other genes emerged from this analysis: CRLF2 and RHOA, which are both transcription factors known to be involved in B cell differentiation and lineage development.

We generated guide RNAs against RHOA and CRLF2 to knockout the gene in B cells using the CRISPR/Cas9 system. In B cell models, RHOA knockout led to rapid cell death, implicating RHOA as a critical survival factor in B cells and a potential role for RHOA in post-radiation survival and recovery in memory B cells. On the other hand, we did not observe any effects of knocking out CRLF2 which remained expressed highly, suggesting possible compensatory mechanisms that overcome the effects of heterozygous knockout.

We are developing follow up studies to identify the roles of RHOA in B cell development which were first identified in this study.

Develop a stem-cell based model to generate immune B cells in vitro (AIM #2)

Given the potential multigenic nature of genetic variation and the emerging role for B cell receptor signaling and other immune functions, we proposed to differentiate stem cells from irradiated macaques (or human stem cells with the genetic modifications present in irradiated macaques) into B cells to better understand their function and the long-term effects of radiation on immune function.

We have previously developed the schema shown in Figure 4.2 below to differentiate B cells from stem cells.

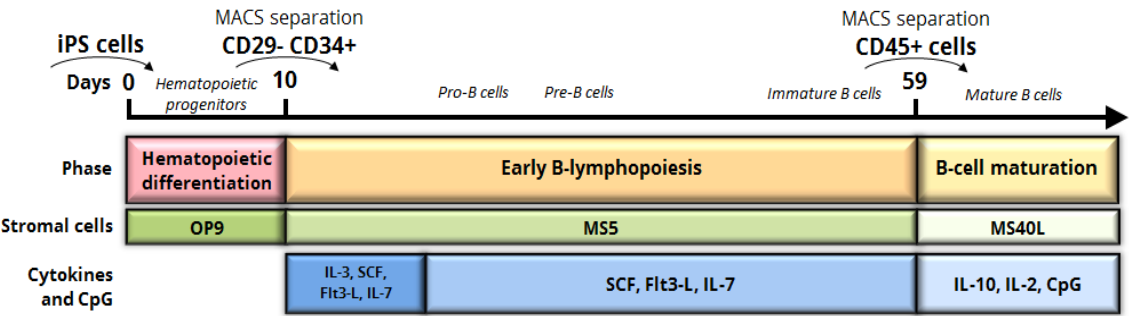


Figure 4.2: Schema for generating B lymphoid cells.

The yields of B cells from three consecutive experiments at 90 days are shown below, and were on-average, less than 1%. Two additional experiments with new reagents yielded no measurable B cells.

Recognizing these critical limitations, we initiated experiments using a humanized mouse model that transplants human stem cells to regenerate intact and functional human immune cells into an immune compromised mouse to generate the data shown in Figure 4.3.

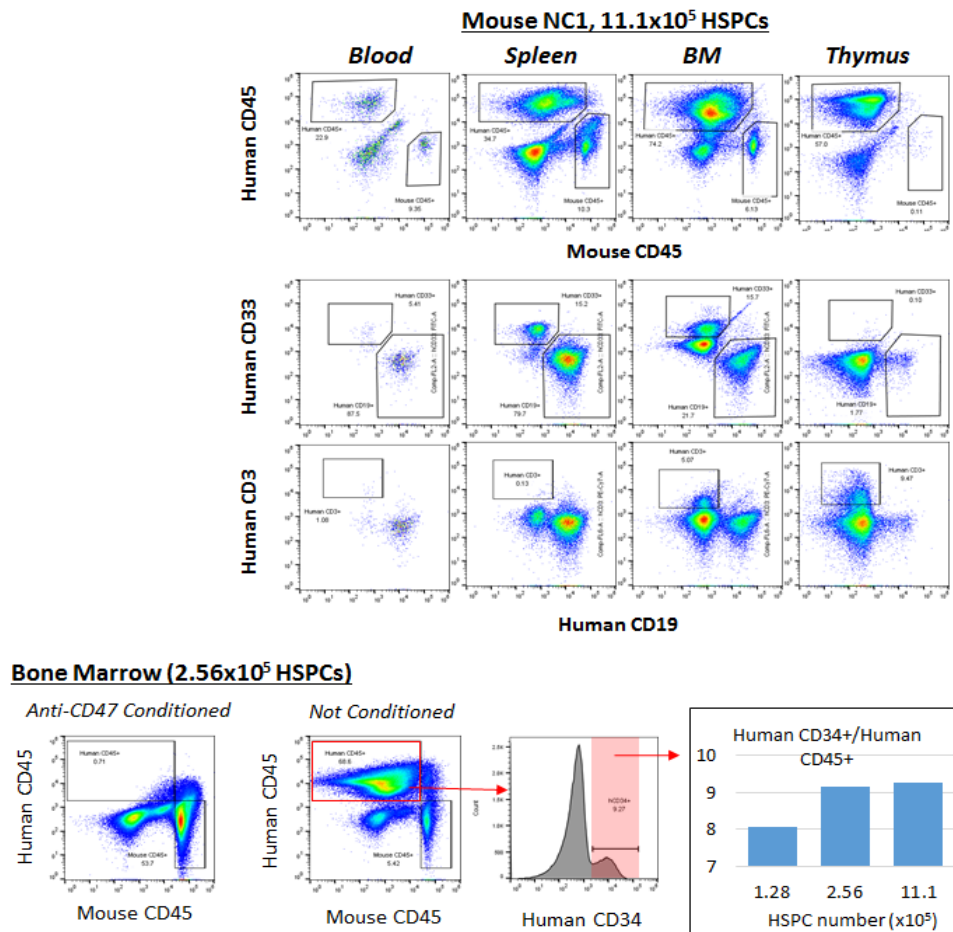


Figure 4.3: Presence of B cells (CD19+), T cells (CD3+), and myeloid cells (CD33+) in blood, spleen, bone marrow, and thymus at 10 weeks post transplantation.

Generation of humanized mouse model:

We used the NOD.B6.SCIDII2ry^{-/-}kit^{W41/W41} (NBSGW) mice as the base model to generate a humanized mouse. NBSGW mice are ideal for this type of project as they contain a polymorphism in the *Sirpa* gene providing for phagocytic tolerance of human cells, PRKDC^{scid} mutation and II2ry^{-/-} mutations which lead to absence of T, B, and NK cells thus preventing xenoreactivity, and a c-kit mutation resulting in impairment of hematopoiesis of the mouse cells, providing a competitive advantage to transplanted cells. We have comprehensively tested this system in experiments transplanting CD34+ hematopoietic stem cells from anonymized human donors.

Figure 4.3 depicts the generation of complete human lineages in mice by implanting as few as 125,000 human stem cells. As shown, we were able to identify substantial numbers of human derived circulating B cells (CD19+), T cells (CD3+) and myeloid cells (CD33+) in blood, spleen, bone marrow and thymus of the mice. Importantly, these humanized mice have an essentially unlimited supply of B and T cells for future experiments.

Interestingly, stem cell dosage did not have a large impact on the recovery of mature human immune cells by the time mice were 10 weeks old. Thus, we can reliably generate humanized mouse models by injecting human stem cells.

Human embryonic stem cell lines can be used as an alternative starting point for stem cells.

After our initial success in generating these humanized mice, we encountered significant difficulties in altering individual genes. In addition, we transduced these cells with GFP to be able to track the cells containing the CRISPR constructs using their fluorescence. We found that these cells containing the correct CRISPR constructs failed to engraft in the recipient mice, leading to us to test several iterations of the viral vector used to transduce the human stem cells with the appropriate CRISPR constructs. We found that, although the CRISPR elements worked as designed in our prior

tests, the process of transplantation somehow resulted in alterations in chromatin to a closed conformation leading to silencing of expression.

We finally tested a CRISPR construct that was coupled to a previously described (Zhang et al, Mol Ther, 2010) ubiquitous chromatin opening element. This resulted in persistence of expression and maintenance of GFP expression (Figure 4.4).

Peripheral Blood (19 weeks)

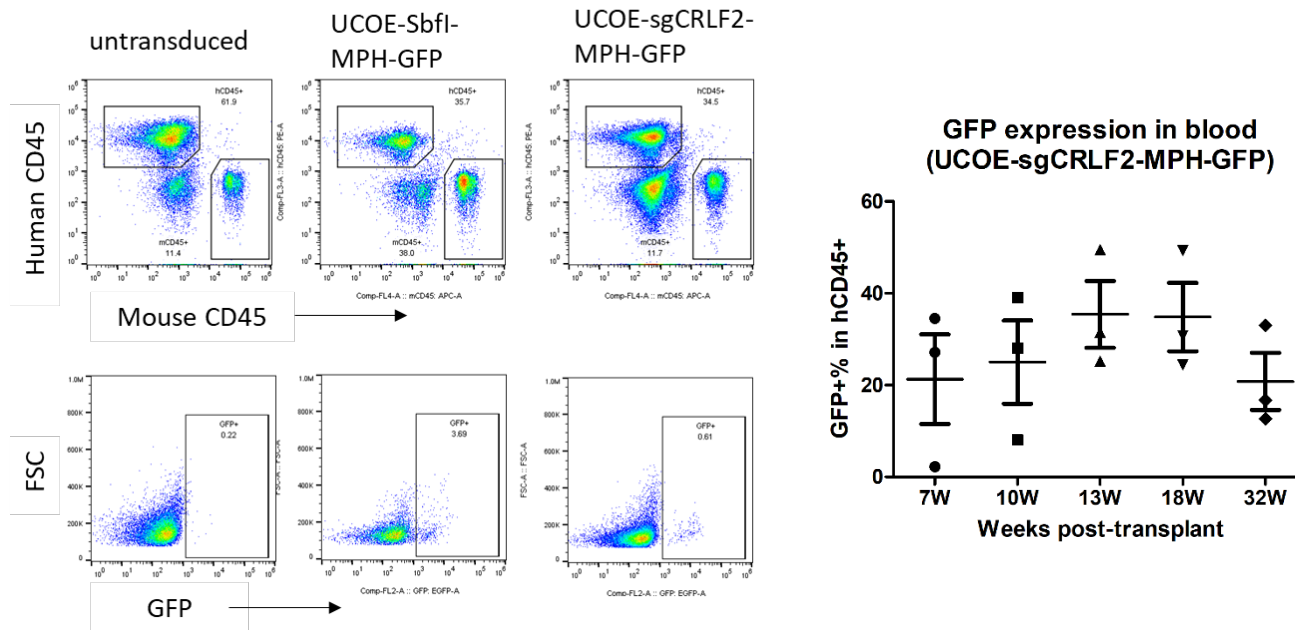


Figure 4.4: Engraftment of B cells occurs successfully in both untransduced and transduced cells, but only the UCOE containing elements have significant numbers of GFP⁺ cells. Right panel shows long term follow up of GFP expression is maintained in the presences of these UCOE elements.

Interestingly, we also found vastly improved engraftment with these modified stem cells with engraftment exceeding an average of 50% at 21 days, which is well within expected time frames.

We thus have an important new model for defining the genetic effects identified from the DEARE models.

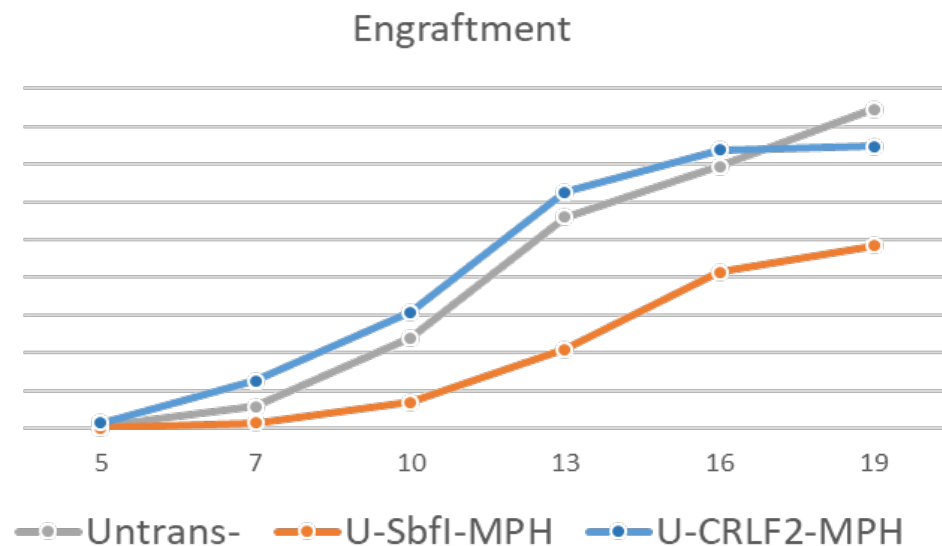


Figure 4.5: Engraftment of B cells occurs successfully in both untransduced and transduced cells and human cells are detectable under all three conditions by 10 days.

Conclusion:

This proposal enabled critical method development and data generation from effects observed in DEARE macaques. We have demonstrated the feasibility of our in vivo approach for modeling complex genetic interactions.

These results will be taken forward to develop viable models for understanding the roles of RHOA in recovery from radiation and other critical developmental functions in the B cell and T cell lineages.

The genomic data will be utilized throughout the other projects to inform future development and interpretation of experimental studies.

4) Other Achievements

Nothing to report.

What opportunities for training and professional development has the project provided?

Overall (PI Cline)

1. All investigators participated in monthly teleconferences and our annual retreats.
2. Core Investigators (Drs. Cline, Dugan, and Register) and Fellows (Drs. Andrews, Debo, Michalson and Thakur) attended annual Radiation Research Society meetings, which provided education and professional development.
3. Fellows and Graduate Students were involved in most projects.

Project 1 Diabetes (PI Kavanagh).

1. Trainees: Three trainees worked on the project (Alistaire Ruggiero, Dr. Nicole Bacarella, and Dr. Ravi Vemuri). Dr. Bacarella is a laboratory animal medicine resident (veterinarian) who finished her residency this year, and her publication (PMID: 31805366) has resulted in her being eligible for her board examinations for specialty certification in Laboratory Animal Medicine.
Alistaire Ruggiero is a graduate student who shared first authorship on this publication regarding structural and functional changes related to adipose tissue in irradiated animals. This has formed the basis of her PhD project whereby she will be looking at adipose macrophages as determinants of health in obese and non-obese primates.
Ravi Vemuri is a post-doctoral fellow that is interested in the microbiome and is working on the dataset previously reported on, which documents that blood pressure is related to intestinal barrier function and microbiome shifts over time.
2. Professional Development: Dr. Kavanagh attended a DOD-focused research retreat (2nd of June, 2020) to share research progress and discuss collaborative opportunities.

A large group effort ensued in 2019 to leverage this existing body of research on the delayed effects of radiation into an-NIAID program project (P01). A grant was written, with preparatory meetings conducted at Duke (4th September, 2019) and an in-person presentation and NIH (18th September, 2019). The review of the P01 was thorough and ultimately not funded, however Dr. Kavanagh's project was listed as a strength (second strongest project of the 5 submitted) and a respectable score of 39 was given. This grant writing and collaborative experience was very helpful, the review very insightful and aids in decision making about the next steps in this research.

The brown adipose preliminary data generated in this cohort of nonhuman primates has resulted in the submission of 2 DOD applications this year. We have applied for a new DOD peer reviewed medical research program award called "Advancement of CRISPR-Based Adipose Therapies for Type 2 Diabetes to Non-Human Primates" in which Dr. Kavanagh is the principal investigator of Project 3 and the program principal investigator is Dr. Michael Czech of the University of Massachusetts (W81XWH-20-PRMRP-FPA). We have also applied for a DOD expansion award called "CRISPR-Based Gene Editing to Induce Thermogenic Adipose Tissue in Type 2 Diabetes" and this expansion specifically leverages the radiation survivor monkeys as a population relevant to military interests.

Project 2 RIHD (PI Register)

1. Image Analysis Software, Cardiac Ultrasound, Cardiac MRI, and RNAseq analysis
2. Tissues: VisioPharm, programmable, development of semi-automated programs to assess myocardial, arterial, and other histologic and immunohistochemical phenotypes including fibrosis and atherosclerosis. Some of this work has resulted in training experience for undergraduate students (Ben Williams, University of South Carolina Sophomore), Matthew O'Brien, Wake Forest University Junior-Senior, Abby Anderson, Wake Forest University Junior).
3. Cardiac Ultrasound: CV ultrasound applications and measurements has been optimized through interactions with Cardiology faculty and vendors, establishing new SOPs for prospective cohort.

4. Cardiac MRI: Register has ongoing interactive discussion sessions with Clinical Cardiology staff and faculty
5. Gene expression analyses: Ongoing practical experience in the utilization of Bioconductor and R to conduct RNAseq and other genomics analyses and in the use of Ingenuity Pathways software.
6. New studies employing single cell and single nuclei gene expression profiling.

Project 3 (Immune Recovery) PI Sempowski and Chen

1. Dr. Chen attended 2016 Federation of Clinical Immunology Societies meeting held in June in Boston. This is the meeting in translational immunology delivering the latest breakthroughs across immune-mediated diseases.
2. Several visiting scholars including Drs. Ying Huang, Xinhua Chen, Xiaoli Nie, and Xiaoyan Yang have worked on this project. They have gained significant research skills including experimental design, flow cytometry, animal experiments, and BLI.
3. Dr. Olabunmi Agboola was a Pediatric Allergy/Immunology fellow mentored by Dr. Chen. She was involved in flow cytometric experiments and data analyses.
4. Yujing Zou is a PhD student mentored by Dr. Chen. She was involved in many aspects of this study including flow cytometry, in vivo experiments, single cell RNA sequencing, BLI.

Project 4 (Genomics) PI Dave

Nothing to Report

How were the results disseminated to communities of interest?

Overall Program (Cline)

Study design, public health and strategic relevance, and opportunities for collaboration were presented at several venues:

- The Radiation Research Society Annual Meeting (each year)
- The NIH/NIAID Centers for Medical Countermeasures Against Radiation (each year)
- Reports during monthly teleconferences with the CMCR Steering Committee (2020-2022)
- The NIH Radiation/Nuclear Program Immune Dysfunction Workshop, (virtual) Sept 9-10, 2020
- The annual Radiation Countermeasures Center of Research Excellence (RadCCORE) meeting at Duke University, Durham, NC
- MD Anderson Cancer Center, Houston, TX, March 9-10, 2020 (Invited Speaker)
- Comparative Medicine Research Strategy Meetings, Wake Forest School of Medicine
- Department of Radiation Oncology, Wake Forest School of Medicine
- Wake Forest Animal Resources Program Continuing Education Seminar

Project 1 - Diabetes (Kavanagh)

- Comparative Medicine Research Strategy Meetings

Project 2 - Radiation-Induced Heart Disease (Register)

- Comparative Medicine Research Strategy Meetings
- Presentations at the annual Radiation Research meeting, at the annual RADCCORE meeting at Duke University, in Departmental and other seminars at Wake Forest, along with special presentations and discussion with visiting Cardiologists from other institutions.

Project 3 - (Immune Recovery) (Sempowski/Chen)

- Nothing to report

Project 4 - Genomics (Dave)

- Nothing to report

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Overall (PI Cline):

The findings produced by this program have increased awareness of the multi-systemic nature of late radiation effects, in the domains of radiation biology, radiation mitigation, and medical management of affected individuals post-exposure.

Project 1: Diabetes (Kavanagh)

Our data has confirmed the relationship between whole body irradiation and metabolic disease. This has impact on oncological practitioners regarding a patient's future risks for disease and thus post-treatment follow up. Our data has also corroborated the importance of transforming growth factor beta in both health and disease. Although this is a complicated signaling pathway to modify with pharmacological agents, the potency of has been demonstrated and effect sizes can be estimated for future experiments. Drug agents exist currently and are in development which could be used to treat people who develop post-radiation metabolic disease. We have also identified stem cell loss as a potential mediator of the delayed effects of radiation exposure. There are avenues to pursue in this domain to treat delayed effects. We may be able to reawaken or reprogram stem cells, or perform autologous transplantation of one's own expanded stem cell population.

We have also characterized new models of accelerated disease development, and these can be used to probe questions relating to aging and resilience.

Project 2: Cardiovascular Disease (Register)

The project has produced new data on the effects of radiation on myocardial fibrosis and cardiac function, on myocardial fibroblast populations, on myocardial macrophage polarization, on myocardial capillarization, and on monocyte polarization, implicating monocyte and macrophage driven pathways in promoting pro-fibrotic pathways and producing evidence of restrictive cardiomyopathy and diastolic dysfunction. Papers and investigators (DeBo, Michalson) involved in these studies have been selected for awards based upon the content and quality of the research presented. Figures have been highlighted on the cover of Radiation Research (DeBo et al 2016) see below and appendix.

Project 3: Immune Injury (Sempowski/Chen)

The NHP studies completed for this study indicate that total body irradiation leads to subtle but important defects in immune cell populations and immune responses for at least two years after exposure. The findings imply that irradiated individuals may be less able to respond to some vaccines, and by extension some pathogens, even after their white blood cell counts and other clinical measurements of immune health return to baseline.

The results from the murine studies revealed for the first time that, even though total body irradiation has long-lasting effect on thymopoiesis, its effects on T cell numbers, individual T cell function, and T cell repertoires are minimum. Single cell RNA sequence studies revealed for the first time the signaling pathways used by T cells to recover from radiation injury. Confirmation of these signaling pathways by subsequent studies will fill the knowledge gap in our understanding of T cell recovery post-irradiation. The knowledge will lead to novel therapies for delayed immune recovery after radiation exposure.

Project 4: Genomics (Dave)

The creation of a data bank of the underlying genomic and transcriptomic signatures of radiation injury is key to understanding mechanisms of DEARE.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

Projects 1 and 2

Nothing to report; all changes have been previously reported and approved in writing.

Project 3

- In addition to flow cytometry, we also used BLI to study T cell recovery. This imaging technique allows tracking T cell recovery in different lymphoid organs over time and generate more complete picture of T cell recovery compared with flow cytometric assay.
- Because radiation does not induce long-lasting phenotypic T cell defect, we started IGF-1 treatment 1 hour instead of 28 days after irradiation hoping that this will increase the chance to detect a positive signal.
- Since HGH and IGF-1 did not work as initially expected, we tested a long-acting IL-7 product, as an alternative approach detailed in the original proposal. This change was discussed with and approved by our program officer Dr. Zachary Zabarsky before the experiment was initiated.

Project 4

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Core Primate Studies (Cline)

Previously reported delays in animal acquisition in years 1-2, and COVID-19 related delays in year 5 slowed the completion of work with the Prospective Cohort (PRC) and some analysis of data, particularly for Project 2 for which both in vivo and post mortem assessments are necessary. These delays were the impetus for the year 6 of no-cost extension.

Projects 1 and 2

Nothing to report; all changes have been previously approved and reported in writing.

Project 3

- In 2017, BD Biosciences recalled the majority of the fluor-conjugated human/NHP antibodies used in our immunophenotyping of the long-term survivor cohort from 2016-2017. The recall was due to unpredictable loss of fluorescent signal arising from incorrect warehousing. We re-analyzed all the flow data collected using the recalled lots to accommodate shifts in fluorescent signature and eliminated data in which we were not confident.
- Even though we have completed majority of work related to the testing of novel medical countermeasures, we have not completed the functional assay yet. Since the funding has been ended, we will complete these experiments using different funds.

Project 4

Nothing to report; all changes have been previously approved and reported in writing.

Changes that had a significant impact on expenditures

Nothing to report; all changes have been previously reported in writing.

Significant changes in use or care of human subjects

Not applicable. No human subjects research.

Significant changes in use or care of vertebrate animals

Nothing to report; all changes submitted and approved in writing and approval by institutional animal care and use committees and ACURO have been documented in previous reports. There is no ongoing DOD-supported animal work at this time.

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS

Journal Publications

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 186(1): 55-64. Federal support acknowledged. **Note: Dr. Debo was awarded the 2017 Radiation Research Editors' Award for this paper, and a key figure from this manuscript was selected for the journal cover.**

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 Biology* 2017 313(3): R290-297. Federal support acknowledged.

Sherrill C and Kavanagh K. Fight Club: Using Housing Conditions to Curb Male Cage Aggression. 2019 *Laboratory Animal Science Professional*; March: 38 – 40. Federal support acknowledged.

Bacarella N, Ruggiero A, Davis AT, Uberseder B, Davis MA, Bracy DB, Wasserman DH, Cline JM, Sherrill C, Kavanagh K. Whole Body Irradiation Induces Diabetes and Adipose Insulin Resistance in Non-human Primates. *Int J Rad Onc Biol Physics* 106 (4):878-886 2019 Federal support acknowledged.

Vemuri R, Ruggiero A, Whitfield JB, Dugan GO, Cline JM, Block MR, Guo H, Kavanagh K. Hypertension drives microbial translocation and shifts in the fecal microbiome of non-human primates. *American Journal of Physiology, Heart and Circulation* at <https://www.biorxiv.org/content/10.1101/2021.07.30.454379v1> (in review). Federal support acknowledged.

Ruggiero AD, Davis MA, Davis A, DeStephanis D, Williams AG, Vemuri R, Fanning KM, Sherrill C, Cline JM, Caudell DL, Kavanagh K. Delayed Effects of Radiation in Adipose Tissue Reflect Progenitor Damage and Not Cellular Senescence. *Int J of Radiation Oncology Biology Physics* (in review). Federal support acknowledged.

Michalson KT, Macintyre AN, Sempowski GD, Bourland JD, Howard TD, Hawkins GA, Dugan GO, Cline JM and 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;192:121-134. Acknowledgement of federal support (yes). **Note: Dr. Michalson was awarded the 2020 Radiation Research Editors' Award for this paper.**

Hale LP, Rajam G, Carlone GM, Jiang C, Owzar K, Dugan G, Caudell D, Chao N, Cline JM, Register TC and Sempowski GD. Late effects of total body irradiation on hematopoietic recovery and immune function in rhesus macaques. *PloS one*. 2019;14:e0210663. Acknowledgement of federal support (yes).

Caudell DL, Michalson KT, Andrews RN, Snow WW, Bourland JD, DeBo RJ, Cline JM, Sempowski GD and Register TC. Transcriptional Profiling of Non-Human Primate Lymphoid Organ Responses to Total-Body Irradiation. *Radiation research*. 2019;192:40-52. Acknowledgement of federal support (yes).

Thakur P, DeBo R, Dugan GO, Bourland JD, Michalson KT, Olson JD, Register TC, Kock ND and Cline JM. Clinicopathologic and Transcriptomic Analysis of Radiation-Induced Lung Injury in Nonhuman Primates. *International journal of radiation oncology, biology, physics*. 2021;111:249-259. Acknowledgement of federal support (yes).

Macintyre AN, French MJ, Sanders BR, Riebe KJ, Shterev ID, Wiehe K, Hora B, Evangelous T, Dugan G, Bourland JD, Cline JM, Sempowski GD. Long-Term Recovery of the Adaptive Immune System in Rhesus Macaques After Total Body Irradiation. *Adv Radiat Oncol*. 2021 Apr 19;6(5):100677. doi: 10.1016/j.adro.2021.100677. PMID: 34646962; PMCID: PMC8498734. Acknowledgement of federal support (yes).

Kristofer T. Michalson, DVM, PhD, Gregory O. Dugan, DVM, David L. Caudell, DVM, PhD, J. Mark Cline, DVM, PhD, Dalane Kitzman, MD, Thomas C. Register, PhD. Cardiac Structural and Functional Characteristics in Long Term Rhesus Macaque Survivors of Ionizing Radiation Exposure. (biorxiv; doi: 10.1101/2020.02.18.95319). (Submitted; under revision) Acknowledgement of federal support (yes).

Books or other non-periodical, one-time publications

Abstracts Published in Meeting Proceedings

Kavanagh K, Pfisterer BR, Presley TD, William IM, Wasserman D, Cline JM. The long term effects of ionizing radiation on muscle extracellular matrix and perfusion in monkeys. 62nd Annual Meeting of the Radiation Research Society, Waikoloa HI, 2016 Acknowledgement of federal support (yes).

Fanning KM, Pfisterer B., Davis AT., Presley T., Wasserman DS., Cline JM, Kavanagh K. Skeletal muscle perfusion protects against diabetes in nonhuman primate model of accelerated aging. The Gerontological Society of America Annual Scientific Meeting, New Orleans LA, 2016 Acknowledgement of federal support (yes).

Cline JM, Dugan G, Andrews R, Kavanagh K, Bourland D, Hanbury D, Peiffer A, Register T, Debo R, Caudell D, Chao N. Multisystemic late effects of radiation exposure in nonhuman primates. Radiation Research Society, Waikoloa/Kona, HI, October 15-19, 2016 Acknowledgement of federal support (yes).

Fanning K, Sherrill C, Davis AT, Kavanagh K. Healthspan measures in a western diet conditioned, irradiated rodent model of accelerated aging. 21st IAGG World Congress of Gerontology and Geriatrics, San Francisco, CA 2017 Acknowledgement of federal support (yes).

Sherrill C and Kavanagh K. Comparison of Housing Conditions to Ameliorate Aggression in Male Mice. 69th American Association of Laboratory Animal Sciences National Meeting, Baltimore MD, 2018 Acknowledgement of federal support (yes).

Kavanagh K, Sherrill C, Davis AT, Hart B. Transforming Growth Factor Beta 1 Receptor Antagonist Therapy Improves Non-Alcoholic Hepatic Steatosis In A Novel Diet And Irradiated Mouse Model. Digestive Diseases Week, San Diego 2019 Acknowledgement of federal support (yes).

Stephens J, Blas U, Kock N, Cline JM, Sherrill C, Hart B, Kavanagh K. Pathologic Cardiovascular Tissue Alterations Associated With Experimental Anti-Fibrotic Therapies. American College of Veterinary Pathology Annual Meeting, 2021 Acknowledgement of federal support (yes).

Thomas C. Register; Ryne J. DeBo; Kris Michalson, DVM; Cynthia J. Lees, DVM, PhD; Greg Dugan, DVM; Greg Sempowski, PhD; Kylie Kavanagh, DVM; David Caudell, DVM, PhD; and J. Mark Cline, DVM, PhD. Serum IL-10 and IL-15 Levels are Increased with Prior Exposure to Gamma Irradiation and are Associated with Radiation Sensitive Cardiac Phenotypes". Radiation Research Society Meeting October 2016

William W. Snow, DVM, Thomas C. Register, PhD, Gregory D. Sempowski, PhD, Timothy D. Howard, PhD, H. Daniel Bourland, PhD, Kouros Owzar, PhD, Chen Jiang, PhD, J. Mark Cline, DVM, PhD, David L. Caudell DVM, PhD. Transcriptional Analysis of Lymphoid Tissue Responses of Ovariectomized Female Cynomolgus Macaques Exposed to Total Body Radiation ASH 2016

Kristofer T. Michalson, Gregory Dugan, Ryne J. DeBo, Dalane Kitzman, J. Mark Cline, Thomas C. Register. Effects of Total Body Irradiation on Echocardiographic Phenotypes in Male Rhesus Macaques. Radiation Research Society Meeting October 2017

Kristofer T. Michalson, Andrew N. McIntyre, Ryne J. DeBo, Gregory D. Sempowski, J. Mark Cline, Thomas C. Register. Evaluation of Monocyte Polarization in Long Term Non-Human Primate Survivors of Acute Ionizing Radiation Exposure Radiation Research Society Meeting October 2017

Xinhua Chen, Ying Huang, Yiqun Jiao, Xiaoli Nie, and Benny J. Chen. T cell reconstitution following ionizing irradiation. Abstract was presented in The 63rd Radiation Research Society Annual Meeting 2017*

David L. Caudell, Kristofer T. Michalson, Rachel N. Andrews, William W. Snow, J. Daniel Bourland, Ryne J. DeBo, J. Mark Cline, Gregory D. Sempowski, Thomas C. Register. Transcriptional Profiling of Nonhuman Primate Lymphoid Organ Responses to Total Body Irradiation.

Kristofer T. Michalson, Andrew N. McIntyre, Gregory D. Sempowski, Gregory O. Dugan, J. Mark Cline, Thomas C. Register. Monocyte Polarization in Acute and Delayed Responses to Total Body Irradiation in Male Rhesus Monkeys. Radiation Research Society Meeting October 2018

Yujing Zou[^] Xinhua Chen[^], Yiqun Jiao, Ying Huang, Xiaoli Nie, Xiaoyan Yang, Suijing Wu, Andrew N. Macintyre, Nelson J. Chao, Gregory D. Sempowski, and Benny J. Chen. Long-term T cell reconstitution in peripheral blood and lymphoid organs following total body irradiation (TBI). Abstract was presented in The 65th Radiation Research Society Annual Meeting 2019

Dugan GO, Olson JD, Rahbar E, O'Donnel LM, O'Donnell RP, Bourland JD, Cline JM Caudell DL. Assessment of platelet function in rhesus macaques that received total body irradiation and developed diabetes. Radiation Research Society, 2020

Thomas C. Register, PhD; John P. Post, BS; Kristofer T. Michalson, DVM, PhD; Greg Dugan, DVM; Stephen J. Walker, PhD; Kip Zimmerman, BS; Carl D. Langefeld, PhD; JM Cline DVM, PhD. Radiation Induced Alterations in Myocardial Immunology Related mRNA Profiles in Male Rhesus Macaques. October 2020

Sakamoto CK, Olson JD, Dugan G, Bourland JD, Caudell D, Sempowski GD, Hale LP, Cline JM. Whole thorax irradiation induces long-term thymus injury in male rhesus monkeys. Radiation Research Society, 2020

Thomas C. Register, Kristofer T. Michalson, Timothy D. Howard, Carl D. Langefeld, Kip D. Zimmerman, J. Mark Cline. Total Body Irradiation Induces Significant Long Term Alterations in Myocardial DNA Methylation Profiles Accompanied by Increased DNA Methylation Age. Invited talk Radiation Research Society Meeting October 2021

Yujing Zou, Yiqun Jiao, Xiaoyan Yang, Yuxian Huang, Jianhong Ou, Simon Gregory, Vaibhav Jain, Nelson J. Chao, and Benny J. Chen. Characterizing the transcriptional and functional profile of radioresistant T cells. Abstract was accepted for presentation in The 67th Radiation Research Society Annual Meeting 2021.

Cline JM, Olson JD, Dugan GO. Age at irradiation, age, dose, mitigator use, and patterns of delayed multi-organ morbidity in irradiated rhesus macaques. Radiation Research Society, 2021 (platform presentation/virtual)

PhD Dissertations:

Ryne DeBo, MS, PhD. Non-Human Primate Models of Ionizing Radiation Exposure and Evaluation of the Effects of a Potential Radiation Mitigator. Molecular Medicine and Translational Sciences Graduate Program, Wake Forest School of Medicine, May 2017. Acknowledgement of federal support (yes).

Kristofer T. Michalson, DVM, PhD. Functional, Structural, Cellular, and Molecular Cardiac Responses in Nonhuman Primate Models of Menopause and Ionizing Radiation Exposure. Integrative Physiology and Pharmacology Graduate Program, Wake Forest School of Medicine, May 2018. Acknowledgement of federal support (yes).

Other Publications, conference papers, and presentations

Ryne J DeBo "Molecular, Cellular and Cardiovascular Effects of Total Body Irradiation and the Investigation of a Potential Radiation Mitigator in Non-Human Primate Models" Molecular Medicine and Translational Sciences Seminar Series, Wake Forest School of Medicine, December 2016

Kavanagh K. Intestinal Aging. Bandera TX. NIA Nathan Shock and Barshop Institute for Longevity and Aging Studies Annual Conference. October 2016

Kavanagh K. What is Comparative Medicine? Perth, Western Australia. Murdoch University College of Veterinary Medicine March 2017

Ryne J. DeBo 2017 Radiation Research Editors' Award Lecture for the publication: 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." 2017 Radiation Research Society Scholars in Training Workshop, Cancun, Mexico, October 2017

Thomas C. Register. "Radiation Induced Heart Disease" Radiation Countermeasures Center of Research Excellence, J. B. Duke Conference Center, Duke University, July 2017

Kristofer T. Michalson and Thomas C Register. "Monocyte Programming and Fibrotic Responses to Total Body Irradiation", Third Annual Comparative Medicine Research Symposium, Wake Forest Bowman Gray Center for Medical Education, July 2017

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" Third Annual Comparative Medicine Research Symposium, Wake Forest Bowman Gray Center for Medical Education, July 2017

Ryne J DeBo, "Effects of a Potential Radiation Mitigator on Nonhuman Primate Heart and Lung" Comparative Medicine Research Strategy Meeting, Wake Forest School of Medicine, February 2017

Kristofer T. Michalson "Monocyte Programming and Fibrotic Responses to Total Body Irradiation in Nonhuman Primates" Comparative Medicine Research Strategy Meeting, Wake Forest School of Medicine, September 2017

Team: Department of Defense PRMRP Meeting, Westfields Marriott Washington Dulles Chantilly VA, November 2017.

Team: NIH NIAID Meeting with Program Officers regarding follow up P01 (Regenerative Immunology in Radiation Injury [unfunded]), NIH Bethesda MD September 2018

Kris Michalson, DVM: "Understanding Health Effects of Irradiation: Radiation-induced Heart Disease". 2018 Deans Research Symposia Series featuring the Department of Pathology & Section of Comparative Medicine, Bridging the Gap Using Novel Preclinical Models, Pathology and Forensic Science. May 31, 2018

Thomas Register, PhD: "Late Effects of Radiation Exposure: Nonhuman Primate Models". 1st Annual Wake Forest Radiation Sciences Forum. May 2, 2018

Kristofer T. Michalson: 2018 "Monocyte Polarization is Altered Post-Irradiation in Male Rhesus Macaques", 4th Annual Thomas B. Clarkson Comparative Medicine Research Symposium, Wake Forest School of Medicine, July 26th, 2018.

Kavanagh K, Sherrill C, Davis AT, Hart B. Transforming Growth Factor Beta 1 Receptor Antagonist Therapy Improves Non-Alcoholic Hepatic Steatosis In A Novel Diet And Irradiated Mouse Model. Abstract presentation Digestive Diseases Week 2019, San Diego CA May 2019

Kavanagh K. Host - Microbial Interactions in Health and Disease. 2020 Annual Meeting of the Society for Leukocyte Biology, Invited Speaker, Portland OR June 2020

Kavanagh K. Metabolic Regulation of Inflammation and its Resolution. June 2021

Thomas C. Register. "Total Body Irradiation Induces Significant Long Term Alterations in Myocardial DNA Methylation Profiles Accompanied by Increased DNA Methylation Age". Radiation Research Society Meeting October 2021

Websites or other internet sites

Nothing to report

Technologies or techniques

Developed new dual staining immunohistochemical methods and image analysis approaches to evaluate macrophage subpopulations in nonhuman primate tissues. These methods were adopted and are now routinely used by other investigators within the program.

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Data or Databases:

Collected and preserved the following 'omic datasets:

- RNAseq of monocyte subpopulations at baseline and 5-month follow-up in PRC
- RNAseq and miRNAseq of RSC and PRC myocardium
- Nanostring datasets of Immune function mRNA transcripts and miRNAs from RSC myocardium
- Nanostring dataset of miRNA from RSC myocardium
- Ongoing analyses will also be preserved for future analyses

Physical Collections:

- Myocardium and other tissues were carefully collected and preserved for ongoing and future analyses
- Circulating monocytes preserved for future analyses

Audio products:

Thomas C. Register, recording of invited talk for virtual meeting, "Total Body Irradiation Induces Significant Long Term Alterations in Myocardial DNA Methylation Profiles Accompanied by Increased DNA Methylation Age" Radiation Research Society 2021

Software:

Software developed as part of this work related to specific macros and customized analyses on the Tomtec platform for echocardiography image analysis and phenotyping and on the Visiopharm platform for quantitation of various histologic and immunohistologic stains of myocardium and other tissues.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Provide the following information for: (1) Project Directors (PDs)/ PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort).

Core Primate Studies (Cline)

Name: J. Mark Cline
Project Role: Principal Investigator
Researcher Identifier: N/A
Nearest person month worked: 2.4
Contribution to Project: No change

Name: David Caudell
Project Role: Co-Investigator
Researcher Identifier: N/A
Nearest person month worked: 0.6
Contribution to Project: Reduced as project was ending

Name: Janet Tooze
Project Role: Co-Investigator
Researcher Identifier: N/A
Nearest person month worked: 0.6
Contribution to Project: No change

Name: Jean Gardin
Project Role: Project Coordinator/Laboratory Manager
Researcher Identifier: N/A
Nearest person month worked: 0.6
Contribution to Project: No change

Project 1 – Diabetes (Kavanagh)

Name: Kylie Kavanagh
Project Role: Project Director
Researcher Identifier: N/A
Nearest person month worked: 4.8
Contribution to Project: No change

Name: Cristina Furdui
Project Role: Project Collaborator
Researcher Identifier: N/A
Nearest person month worked: 0.0
Contribution to Project: Effort on project complete

Name: Masha Block
Project Role: Research Technician III
Researcher Identifier: N/A
Nearest person month worked: 2.4
Contribution to Project: Adjusted as project was ending

Name: Darla DeStephanis
Project Role: Research Assistant
Researcher Identifier: N/A
Nearest person month worked: 5.4
Contribution to Project: No Change

Name: Jingyun Lee
Project Role: Research Associate
Researcher Identifier: N/A
Nearest person month worked: 0.0
Contribution to Project: Effort on project complete

Name: Alistaire Ruggiero
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 0000-0002-1942-2750
Nearest person month worked: 5
Contribution to Project: Data generation in the laboratory regarding senescence
Funding Support: NIAID T32 Scholarship (AI006401 PI Alexander-Miller)

Name: Ravi Vemuri
Project Role: Post-doctoral research fellow
Researcher Identifier (e.g. ORCID ID): 0000-0002-3238-426X
Nearest person month worked: 5
Contribution to Project: Microbiome and senescence analyses
Funding Support: Institutional support and sU01AI50578 (PI Cline)

Project 2 – Radiation/Heart Disease (Register)

Name: Thomas Register
Project Role: Project 2 Director
Researcher Identifier: 0000-0002-4078-0166
Nearest person month worked: 1.8
Contribution to Project: No change

Name: Sujethra Vasu
Project Role: Co-Investigator
Researcher Identifier: N/A
Nearest person month worked: 0.0
Contribution to Project: Dr. Vasu is a cardiologist and worked with Drs. Hamilton and Register in the conduct and analysis of cardiac MRI studies.

Name: Craig Hamilton
Project Role: Co-Investigator
Researcher Identifier: N/A
Nearest person month worked: 0.6
Contribution to Project: Dr. Hamilton worked with Drs. Vasu and Register in the conduct and analysis of cardiac MRI studies.

Name: Julie Johnston
Project Role: Laboratory Technician III

Researcher Identifier:	N/A
Nearest person month worked:	10.80
Contribution to Project:	Adjusted as project was ending
Name:	Maryanne Post
Project Role:	Laboratory Technician IV
Researcher Identifier:	N/A
Nearest person month worked:	1.2
Contribution to Project:	No change

Project 4: Genomic Sequencing and Stem Cell Lines (Dave)

Name:	Sandeep Dave
Project Role:	Project 4 Director
Researcher Identifier:	N/A
Nearest person month worked:	0.0
Contribution to Project:	No effort; project 4 work ended in 2020

Name:	Anupama Reddy
Project Role:	Bioinformatics specialist
Researcher Identifier:	N/A
Nearest person month worked:	0.0
Contribution to Project:	No effort; project 4 work ended in 2020

Name:	Cassandra Love
Project Role:	Research Technician
Researcher Identifier:	N/A
Nearest person month worked:	0.0
Contribution to Project:	No effort; project 4 work ended in 2020

Name:	Guojie Li
Project Role:	Bioinformatics and Biostatistics Specialist
Researcher Identifier:	N/A
Nearest person month worked:	0.0
Contribution to Project:	No effort; project 4 work ended in 2020

Name:	Qiu Qin
Project Role:	Bioinformatician
Researcher Identifier:	N/A
Nearest person month worked:	0.0
Contribution to Project:	No effort; project 4 work ended in 2020

Name:	Alexander Waldrop
Project Role:	Research Technician
Researcher Identifier:	N/A
Nearest person month worked:	0.0
Contribution to Project:	No effort; project 4 work ended in 2020

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

J. Mark Cline (PI)

Changes in *Current Support* as reported since the last reporting period (annual report):

Project Title: Development of a Wake Forest Multi-Species NHP Biorepository to Support Interdisciplinary Aging Studies

Change: New Award

Project Title: Role of the Fecal Microbiome in the Radiation Resilience of Nonhuman Primates

Change: New Award

Project Title: The Wake Forest Nonhuman Primate Radiation Survivor Cohort-Supplement #1

Change: New Award

Project Title: The Wake Forest Nonhuman Primate Radiation Survivor Cohort-Supplement #2

Change: New Award

Project Title: The Wake Forest Nonhuman Primate Radiation Survivor Cohort-Supplement #3

Change: New Award

Project Title: NHP Covid-19 Immunogenicity Studies

Change: New Award

David Caudell (Co-Investigator)

Changes in *Current Support* as reported since the last reporting period (annual report):

Project Title: Reducing Colorectal Cancer Mortality with CF10

Change: New Award

Kylie Kavanagh (Project 1 PI)

Changes in *Current Support* as reported since the last reporting period (annual report):

Project Title: Advancement of CRISPR-based Adipose Therapies for Type 2 Diabetes to Non-human Primates

Change: New Award

Project Title: Role of the Fecal Microbiome in the Radiation Resilience of Nonhuman Primates

Change: New Award

Thomas Register (Project 1 PI)

Changes in *Current Support* as reported since the last reporting period (annual report):

Project Title: Development of an Innovative Vervet (*Chlorocebus aethiops sabaeus*) Model of Early Alzheimer's-like Neuropathology and Symptomatology

Change: New Award

Janet Tooze (Co-Investigator)

Nothing to Report

Greg Sempowski (Project 3 PI)

Nothing to Report

Benny Chen (Co-Investigator)

Nothing to Report

Sandeep Dave (Project 4 PI)

Nothing to Report

What other organizations were involved as partners?

Project 1: Diabetes

Organization Name: Vanderbilt University

Location of Organization: Nashville, TN

Partner's contribution to the project:

- Collaboration (e.g., partner's staff work with project staff on the project)

Dr. David Wasserman and his team were collaborators on our project and had a subcontract awarded to evaluate extracellular matrix proteins in irradiated primate tissues. They have expertise in this area and imaging/quantification capabilities in place.

8. SPECIAL REPORTING REQUIREMENTS

See attached Quad Chart

9. APPENDICES

None

Long Term Follow-up of the Late Effects of Acute Radiation Exposure in Primates

Proposal Log Number PR141508



PI: J Mark Cline, DVM, PhD

Org: Wake Forest Health Sciences **Award Amount:** \$9,999,998

Study/Product Aim(s)

To study delayed effects of acute radiation exposure (DEARE) with a focus on our newly-described radiation-induced pathologies, through phenotypic and mechanistic studies, including:

- 1.Type 2 diabetes mellitus
- 2.Radiation-induced heart disease (RIHD)
- 3.Chronic immune impairment with restriction of the antigenic response repertoire; and
- 4.Radiation-specific genomic signatures underlying 1-3.

Approach

We will use unique nonhuman primate (NHP) resources, including a pre-existing long-term (10+ year) radiation-exposed survivor cohort and development of a prospective cohort allowing baseline pre-exposure assessments and 4 years of follow-up. Complementary immune, metabolic, and interventional studies to be done in mice. Priority will be given to pathways and strategies with high potential for translation to human exposures and outcomes.

Projects	Project 1: Diabetes Body composition, Redox Injury	Project 2: Radiation Induced Heart Disease Cardiac function and biomarkers	Project 3: Immune Recovery Thymopoiesis and immune function	Project 4: Genomic signatures Genomic basis for observed injury
Platforms				
NHP Survivor cohort Long-term outcomes	✓	✓	✓	✓
NHP Prospective Cohort Baseline-controlled	✓	✓	✓	✓
Mouse Studies and Treatments	✓		✓	
NHP Study Archives Including Treatments		✓		

Figure 1. Schematic overview of the matrix relationship between populations of irradiated animals studied and investigative approaches used.

Accomplishments: NHP and mouse irradiation studies under way. Data collection and analysis in progress. Publications and abstracts presented at national meetings. Spin-off grant funding acquired

Timeline and Cost

Activities CY	15	16	17	18	19	20	21
Oversight/Core Primate Studies							
Proj 1 Diabetes							
Proj 2 Radiation/Heart Disease							
Proj 3 Immune Recovery							
Proj 4 Genomic sequencing and stem cell lines							
Estimated Budget (\$K) Based on budget period 09/30/15 – 09/29/20. Budget period in () after dollar amounts.		1	2	3	4	5	6
	\$2,150,541 (1)				\$1,979,721 (4)		
	\$1,975,657 (2)				\$1,863,796 (5)		
	\$2,030,283 (3)				\$826,722 (6)		

Goals/Milestones

CY15 Goal – Program/Project “Kickoff”

- ✓ Regulatory approvals, establishment of admin structure, scheduling/planning
- ✓ Animal acquisition and NHP quarantine & sampling/analysis, both cohorts

CY16 Goals – Prospective NHP studies begin, preliminary results presented nationally

- ✓ Exome sequencing and IPSCs from NHP studies (in progress)
- ✓ Irradiation of NHP prospective cohort and post-irradiation sampling/analysis for cardiac, redox, tissue composition, RNA profiling, and biomarker studies; presentation of early results (in progress)
- ✓ Mouse radiation studies: classic model

CY17 Goal – Continued NHP and mouse studies

- ✓ Mouse radiation studies: secondary model development
- ✓ Continued analysis of NHP composition/biomarkers as above based on preliminary results; presentation of data

CY18 Goal – Continued NHP and mouse studies

- ✓ Mouse intervention study of HGH and IGF-1 (phenotypic)*
- ✓ Continued analysis of NHP composition/biomarkers as above; publication
- ✓ Preliminary cell lineage data from NHPs presented nationally

CY19 Goal – Continued NHP and mouse studies

- ✓ Mouse intervention study of HGH and IGF-1 (functional)*
- ✓ Primate studies as above continue; publication

CY20 Goal – Analysis and publication of results

- ✓ Mouse intervention study of HGH and IGF-1 (mechanistic)*
- ✓ Necropsy of prospective cohort, analysis of terminal outcomes; publication

Comments/ Challenges/ Issues/Concerns

• In 1-year no-cost extension for completion of work

•*mouse studies as amended with SO approval