

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

REPORT DATE: OCTOBER 2020

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				<i>Form Approved</i> <i>OMB No. 0704-0188</i>	
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1. REPORT DATE (DD-MM-YYYY) OCTOBER 2020		2. REPORT TYPE Annual		3. DATES COVERED 30SEP2019-29SEP2020	
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, DACVP Professor Pathology/Comparative Medicine Radiation Oncology				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Wake Forest School of Medicine Medical Center Boulevard Winston-Salem, NC 27157				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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; delayed effects; late effects; heart; immune system; type 2 diabetes mellitus; genomics; transcriptomics; <i>Mus musculus</i> ; <i>Macaca mulatta</i> .					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
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Attachments:

Quad Chart

Manuscript Bacarella et al. 2020

Abstract Michalson et al. 2019

1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

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: Provide a brief list of keywords (limit to 20 words).

Radiation; delayed effects; late effects; heart; immune system; type 2 diabetes mellitus; genomics; transcriptomics; *Mus musculus*; *Macaca mulatta*.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major goals are listed below by project, including relevant dates and percentage completion as indicated.

Core Primate Studies (Cline)

SA1: Provide NHP Investigative Platform

Major Task 1: Regulatory Approval (months 1-6)
 Milestone: Local Institutional Animal Care and Use Committee (IACUC) and DOD Animal Care and Use Review Office (ACURO) approvals in place (100%)

Major Task 2: Access to Long Term Radiation Survivor Cohort (months 1-6)
 Milestone: Scheduling and meetings in progress (90%)
 Sample collections have begun, in support of all projects (planned for months 6-60; 95% 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 in Y2Q1 (100% completed)

SA2: Promote Synergy and Productivity

Major Task 5: Data management/tracking, group interactions (months 1-6)
 Milestones: Data management (100% completed for years 1-4)
 Establish monthly web meetings (100% complete)
 Annual strategic retreat to be scheduled (100% complete for years 1-4)
 Publications (months 6-60; 60% complete in the context of 5 year plan, cardiac, metabolic and genomic papers submitted and accepted; abstracts presented at the annual Radiation Research Society Meeting and other meetings)

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; 80% 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: PRC - Prospective Cohort
Milestones: Create a longitudinal clinical profile of diabetes mellitus (DM) risk with irradiation (70% achieved in context of 5 year plan)
Local IACUC approval (100% achieved)
ACURO Approval (100% Achieved)
Baseline CT imaging (100% achieved)
Characterize changes in skeletal muscle structure & function (50% achieved)
Characterize changes in adipose structure and function (90% achieved)

Major Task 2: RSC - Radiation Survivor Cohort
Milestones: Generate comparable clinical data in monkeys with DM as DEARE to compare with the earlier prospectively irradiated 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 irradiated tissue
Milestones: Compare and contrast early and late changes in redox proteomics following irradiation in mice and monkeys. Verify the role of Akt protein in DM. Identify individual or protein clusters with long-term modifications following irradiation. (95% achieved)

SA3: Track extracellular matrix (ECM) remodeling, including fibrosis, in muscle and adipose depots after irradiation

Major Task 4: ECM characterization and magnetic resonance imaging (MRI).
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 (85% 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 remodeling with treatment (90% 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 will include systolic and diastolic function, ejection fraction, aortic stiffness/pulse wave velocity, wall motion, and electrocardiography. Grid tag mapping will be also used to assess strain and LV volume.

Scheduled Dates: Months 6-48 (6-54 with no-cost extension)

Milestones: Percent completion: 85% in the context of 5 year plan

Detailed cardiac ultrasound (US) evaluations have been 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 plans to move analyses to the TOMTEC. Cardiac MRI image analysis is underway and extensive MR phenotype data has been collected in the PRC. Tissue collections from RSC subjects going to necropsy continue, from PRC subjects were recently completed. We have been carefully evaluating 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 myocardium. We are now 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.

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 will be 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 heart tissues obtained at necropsy.

Scheduled Dates: Months 6-48 (6-60 with no-cost extension)

Milestones: Percent completion: 55%

RSC: blood samples obtained and processed; PRC: baseline and immediate post-radiation blood samples were collected, processed, and archived for future biochemical analyses.

Ongoing blood collections are underway. Two platforms for miRNA analysis have been tested and are being analyzed in preparation for analyses of miRNA in collected samples.

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. The preliminary data suggests that the changes in the heart based on the radiation dose are significantly greater than would have been predicted from prior murine studies. Scheduled Dates: Months 6-48 (6-60 with no-cost extension)

Milestones: Percent completion: 60% 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, planning for ECM and other biomarkers in the radiation survivor cohort; other evaluations are under way). Myocardium from the RSC has been phenotyped for atherosclerosis and is being organized for preparation for RNAseq.

Goals for the past year related to acquiring and analyzing the final post-radiation cardiac MRI and echocardiographic imaging in the PRC, conducting RNAseq and miRNAseq on RNA isolated of myocardium from deceased RSC subjects, additional immunohistochemical analysis of individual cell types and macrophage polarization in myocardium from deceased RSC and Control rhesus monkeys, characterizing blood samples from the PRC and the RSC, continuing plans for analysis of ECM and other biomarkers, ongoing molecular and cellular phenotyping of the monocytes in the PRC and the RSC, submission of manuscript containing comprehensive echocardiographic phenotyping of the RSC describing the effects of radiation on the heart. One manuscript related to the PRC and a second related to the RSC have been published in the past year, another is to be submitted in the near future. Presentations are scheduled for the Radiation Research Society and the American Heart Association.

Project 3 – Immune Recovery (Duke Consortium – 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 (5% complete in context of 5 year plan, first analysis in progress)

Milestones:

- 1.1. Phenotypic analyses in long-term cohort (60 months, 98% complete)
- 1.2. sjTREC in long-term cohort (60 months, 98% complete)
- 1.3. Phenotypic analyses in prospective cohort (60 months, 100% complete)
- 1.4. sjTREC in prospective cohort (60 months, 100% complete)
- 1.5. Determine primary immune response to influenza vaccine (36 months, 100% complete)
- 1.6. Determine primary and recall immune response to influenza vaccines (60 months, 50% complete)

Major Task 2: Murine Studies

Milestones:

- 2.1. Local IACUC Approval (3 months, 100% complete)
- 2.2. Determine the roles of thymopoiesis and peripheral expansion in overall T cell recovery in the classic radiation injury model (18 months, 100% complete)
- 2.3. Determine the roles of thymopoiesis and peripheral expansion in overall T cell recovery in the second radiation injury model (7-24 months, 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 Task 3: HGH Mouse Studies

Milestones:

- 3.1. Determine the effect of HGH on phenotypic T cell reconstitution (25-36 months, 0% complete)
- 3.2. Determine the effect of HGH on functional T cell recovery (37-48 months, 0% complete)
- 3.3. Determine the mechanism by which HGH promote T cell reconstitution (49-60 months, 0% complete)

Major Task 4: IGF-1 Mouse Studies

Milestones:

- 4.1. Determine the effect of IGF-1 on phenotypic T cell reconstitution (25-36 months, 100% complete)
- 4.2. Determine the effect of IGF-1 on functional T cell recovery (37-48 months, 0% complete)
- 4.3. Determine the mechanism by which IGF-1 promote T cell reconstitution (49-60 months, 0% complete)

Project 4 - Genomic Sequencing and Stem Cell Lines (Duke Consortium – Dave)

Aim1: 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

RANseq 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:**

Protocol approvals for all animal studies; establishment of subcontracts and accounts; development of a meeting schedule; review of goals and plans with all investigators; hiring/allocation of effort for personnel; acquisition of NHP subjects for the prospective cohort; pre-irradiation physical examinations, imaging, and blood sampling for all projects; total body irradiation.

Characteristics of our animal populations are briefly described below.

2) **Specific objectives:**

As per SOW. Regulatory approvals, establishment of meeting schedule, hiring, planning, animal acquisition, irradiation.

3) **Results/key outcomes:**

- All animal protocol approvals complete at local and ACURO level
- Subcontracts and accounts established
- Goals and plans reviewed with all investigators
- Monthly web conferences established and continuing
- Hiring/effort allocations made as planned
- Secure server folders established for data exchange
- NHP subjects acquired for PRC, irradiations completed and studies in progress
- Sampling and data acquisition on schedule

4) **Other achievements:**

Data presented at local and national meetings each year.

A brief overview 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.

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

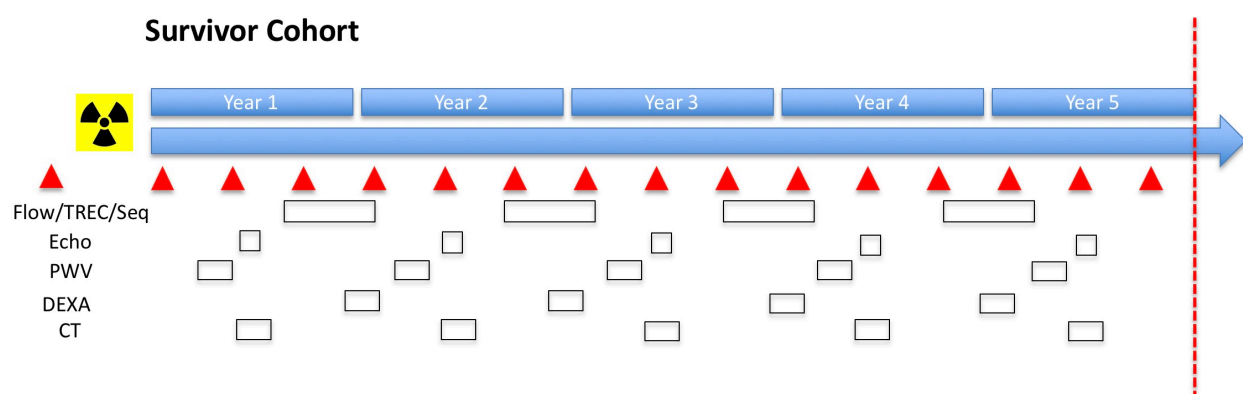


Figure 1: Diagram representing the annual schedule of clinical assessments in the RSC.

The vertical red line indicates the current date. 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.

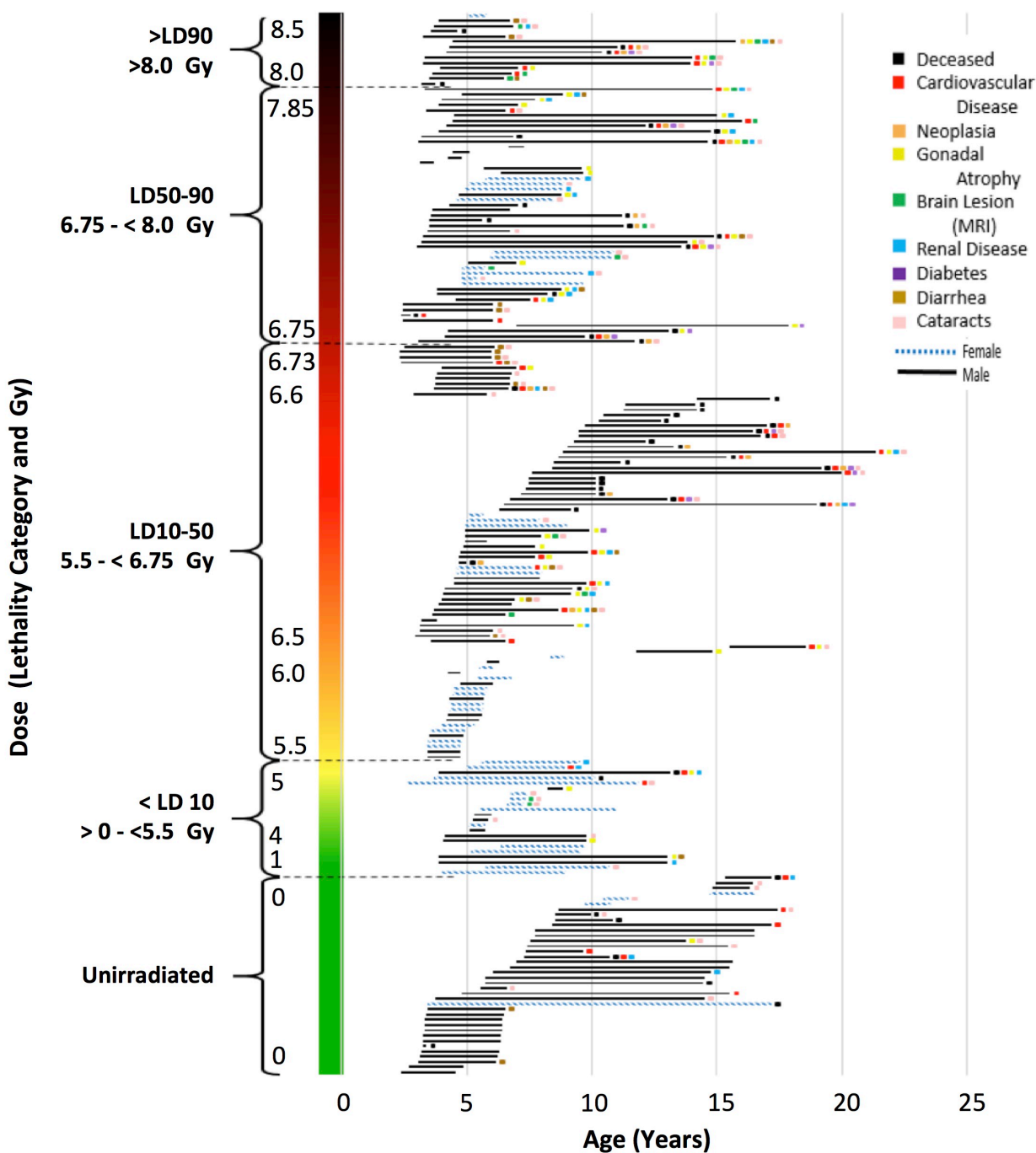


Figure 2: Age and dose distribution of the Radiation Survivor Cohort Each horizontal bar represents a single animal; the left end of the bar is the age at which the animal was irradiated, and the right end of the bar is the animal's current age or age at time of death. Green circles represent the current age of non-irradiated controls. For rhesus monkeys, the typical age a puberty is 3.5 years; typical lifespan is 30 years.

Morbidity

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

Table 1: Threshold-based determinations of morbidity in the long-term Radiation Survivor Cohort (updated 2020).

Organ/Disease	Irradiated	Control	Criteria for Diagnosis
Diabetes	12% (22/184)	2% (1/51)	Hemoglobin A1c (HbA1c) >6.5%; fasted blood glucose > 100 mg/dL x 3 or any non-fasted blood glucose > 200 mg/dL
Kidney	21.2% (39/184)	15.7% (8/51)	BUN > 30 mg/dl or Cr > 1.1 mg/dl Loss of renal volume >50%; Urolithiasis
Lung	15.2% (28/184)	23.5% (12/51)	Pulmonary consolidation on CT scan or Bullous emphysema >25% of lung volume; Hypoxia under sedation (SPO2 < 80% or requiring oxygenation); Respiratory rate >80 breaths per minute
GI	13.6% (25/184)	7.8% (4/51)	Any lesion on endoscopy Chronic diarrhea (severity code >2 for >5 days)
Skin	29.9% (55/184)	39.2% (20/51)	Persistent dermatitis, alopecia or loss of pigmentation
Heart/CV	41.8% (77/184)	35.3% (18/51)	Murmur on auscultation; Valvular insufficiency on echocardiograph Mean arterial pressure >120 Stroke volume <5 mls/stroke & Cardiac output <0.5 L/min at >7 y
Brain (any lesion)	10.9% (20/184)	0% (0/51)	Y/N MRI lesions visible on SWI Neurologic abnormality on clinical exam
Eyes (cataracts)	34.8% (64/184)	17.6% (9/51)	Any lens opacity on annual slit-lamp exam
Behavior	19.6% (36/184)	27.5% (14/51)	Any behavioral abnormality requiring management (customized environmental enrichment plan or medication)
Neoplasia	17.9% (33/184)	2% (1/51)	Any neoplastic disease (imaging or biopsy)
Testicular atrophy	59.7% (86/144)	2.1% (1/47)	Testis volume <10 ml each after 7 years of age; Vol = (p/6)(LxW^2)
Bone	14.1% (26/184)	9.8% (5/51)	Bone mineral content (BMC) < 274g after 7 years of age; Bone mineral density (BMD) < 0.373 g/cm ³ after 7 years of age (Fracture or other gross abnormality on clinical exam or CT scan)
Obese	15.8% (29/184)	43.1% (22/51)	Obesity: Waist > 45 cm or Dexa Body Fat > 30%
Underweight	20.1% (37/184)	15.7% (8/51)	Underweight: Waist < 25 cm or Dexa Body Fat < 12.3% (Excluding animals under 7 yrs old)

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; SP02 = 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.

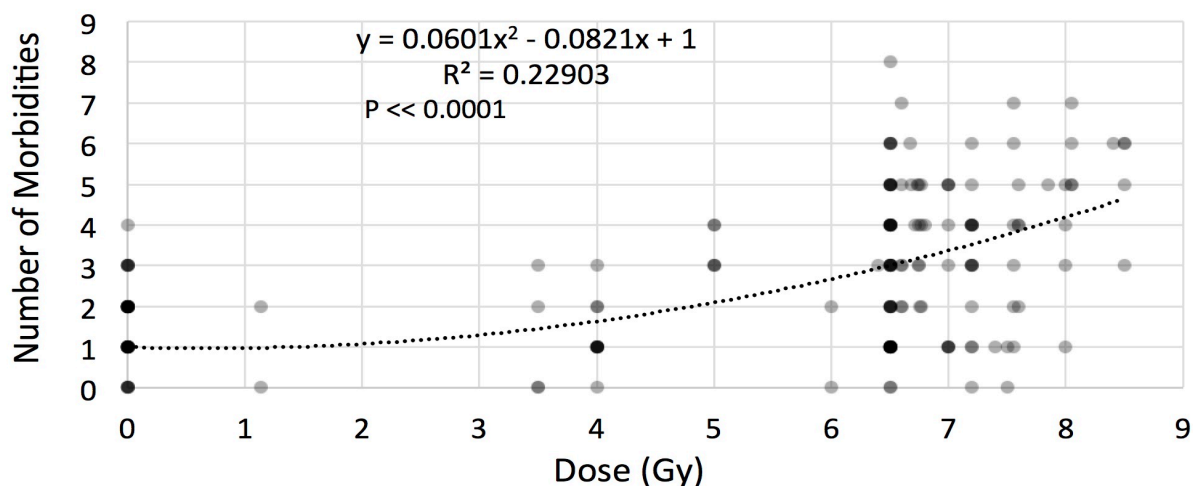


Figure 3. Numbers of disease morbidities plotted against radiation dose for individual animals in the long-term radiation survivor cohort. Darker dots indicate multiple animals at the same plot coordinates. There is a highly significant quadratic relationship between radiation dose and numbers of co-morbid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



Figure 6. 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.

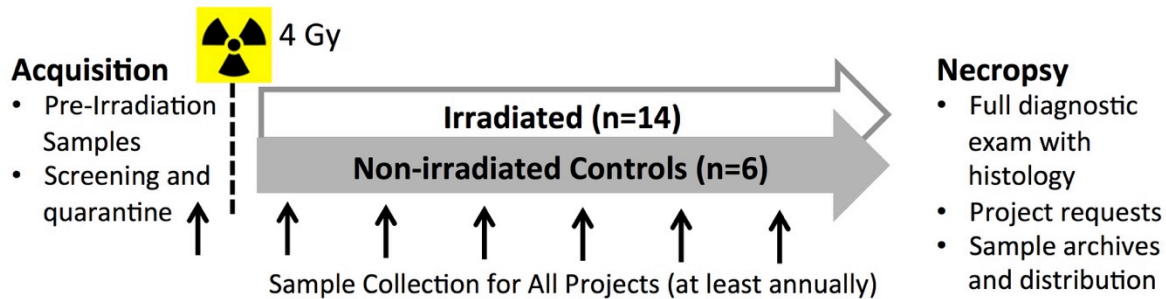


Figure 5: Study design for the Prospective Radiation Cohort (PRC). The in-life portion of this study has been completed.

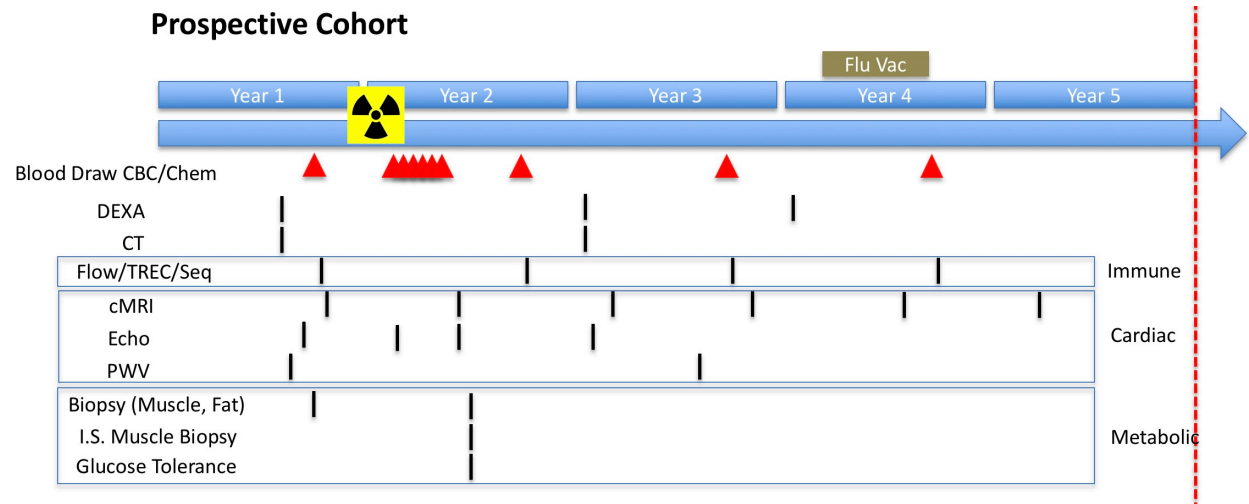


Figure 6: Diagram showing clinical assessments in the Prospective Cohort. The vertical red line indicates the current date. Red triangles indicate blood collection dates. Abbreviations: CBC = complete blood count; Chem = serum chemistry measurements; cMRI = cardiac magnetic resonance imaging; Echo =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

Acute radiation effects have been reported previously (e.g. Michalson et al., 2019). New delayed-effects data are presented in the individual project reports below.

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 (**Figures 1.1 and 1.2**) utilize a unique reverse translational flow whereby diabetes has been reported in survivors of radiation exposures, including our nonhuman primates, and we have designed studies to identify the time course, tissue targets, and interventional strategies that will combat diabetogenesis in at-risk individuals. In accordance with this continuum, this annual 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.

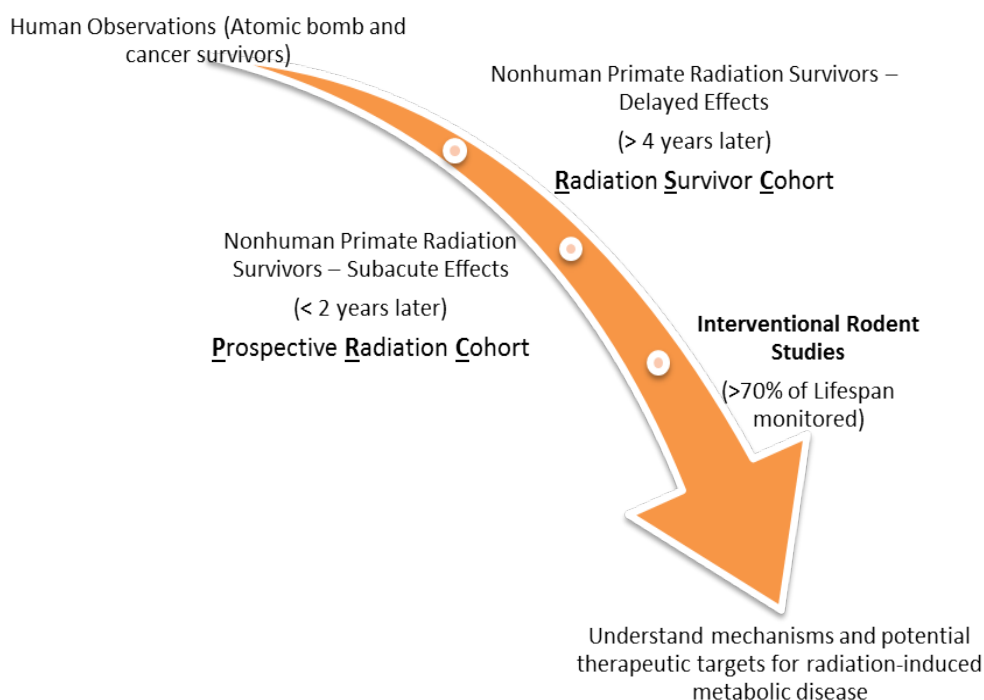


Figure 1.1: Studies reported on for Project 1.

A diagram of the monkey cohorts (RSC and PRC) and the assessment points are shown in **Figure 1.2**. We are halfway through the assessment period.

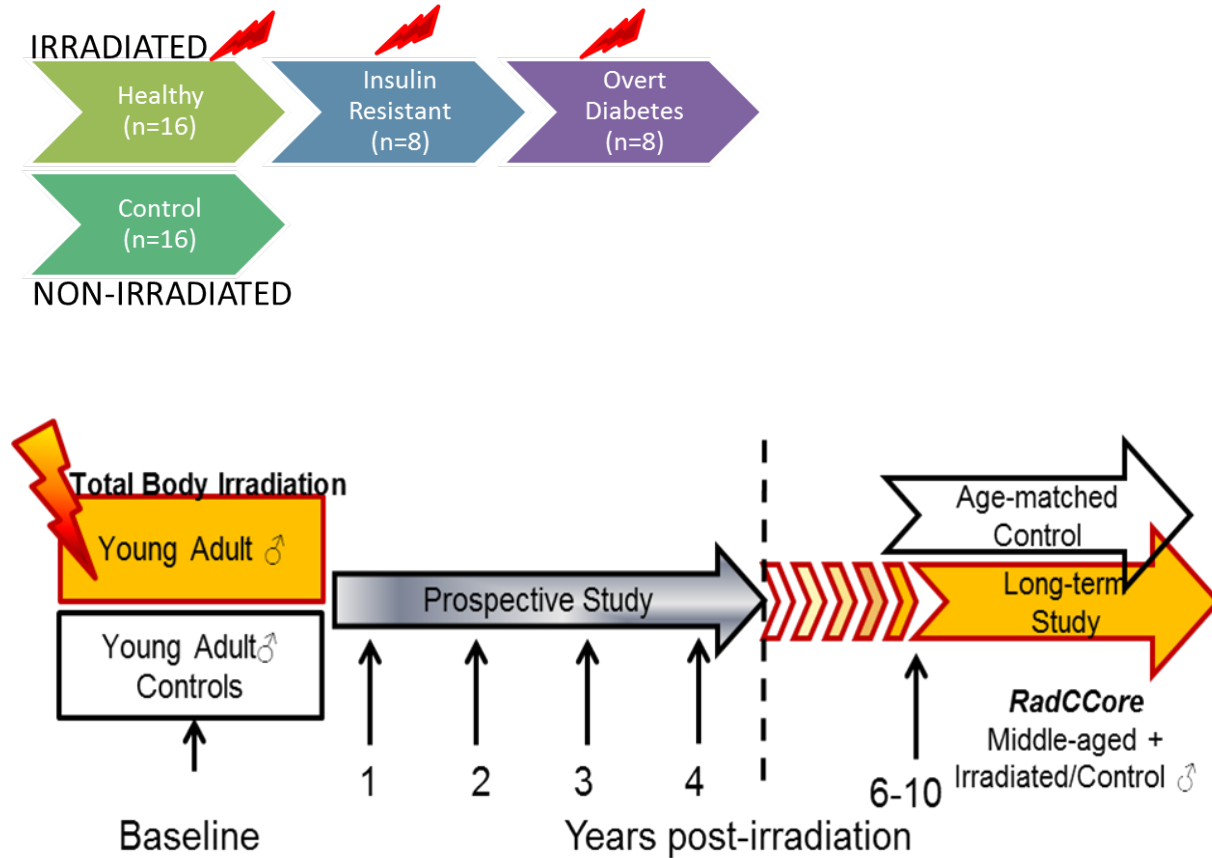


Figure 1.2. RSC monkey groups evaluated within Project 1 (top) and the overlap/continuum achieved with the PRC and RSC

1. Prospective Radiation Cohort

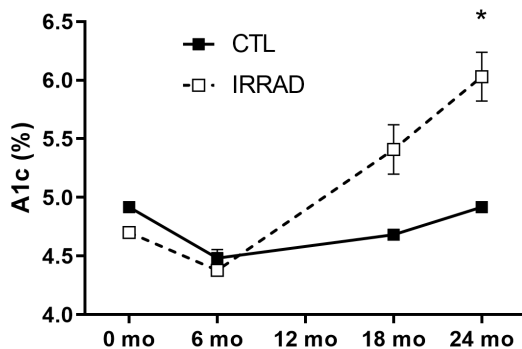


Figure 1.3 Hemoglobin A1c increased over time in irradiated but not control monkeys

Twenty monkeys were stratified into a radiation exposed (RAD) and control (CTL) based on bodyweight and cardiovascular risk factors. In this reporting period, the study terminated with euthanasia of the animals and tissue collections. The key outcome in this year was the publication of our primary study endpoint: diabetes incidence and mechanisms. We evaluated body composition and adipose function and confirmed again that diabetes increases in incidence and prevalence following whole body radiation exposure. Tracking glycemic control over time (**Figure 1.3**) clearly shows a different trajectory in the irradiated animals, with changes not occurring until after a year post-exposure. This sort of chronic timeline is rarely achievable in animal models, and confirms what we suspected from cross-sectional evaluation of radiation survivors.

These reductions in metabolic health and increases in diabetes incidence occurs in the absence of obesity (**Figure 1.4**). Again, this was what was suspected in our cross-sectional studies of radiations survivors, however this prospective study clearly shows that animals are smaller and have less fat mass than non-irradiated control animals. This difference occurs prior to diabetes development and implicates growth and expansion restriction as a mechanism.

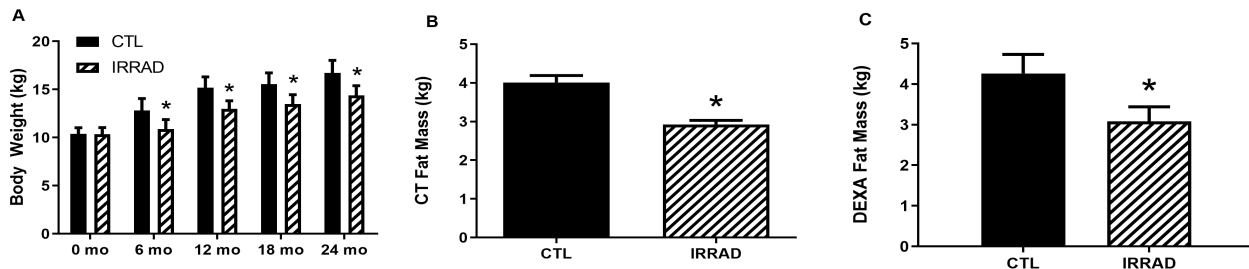


Figure 1.4 Despite the evidence of metabolic disease, irradiated animals are not more obese than controls

Anabolic signaling in adipose is through insulin and insulin like growth factor pathways. We demonstrated that the subcutaneous fat in irradiated monkeys is resistant to insulin signaling which is a hallmark pathway for accelerated aging. Tissue was examined for receptor activation in the presence and absence of insulin and was 30% deficient, which is remarkably similar to the resistance level observed in muscle of radiation survivors. We posit that peripheral tissue insulin resistance is a key mediator of the delayed effects of radiation exposure. In the graph series below (**Figure 1.5**), the insulin resistance (A panel) is coupled with larger fat cell size (B panel) and more macrophages (C panel), which cause inflammation and can further drive more insulin resistance through the secretion of cytokines. Larger fat cells is an unhealthy feature as it indicates lack of new fat cells and existing cells are stressed and hypoxic. The numbers of macrophages negatively correlated with circulating adiponectin, a well-appreciated fat-secreted biomarker of health and insulin sensitivity. These results and others were published in the leading journal for radiation biology (the Red Journal) and is a first of its kind study.

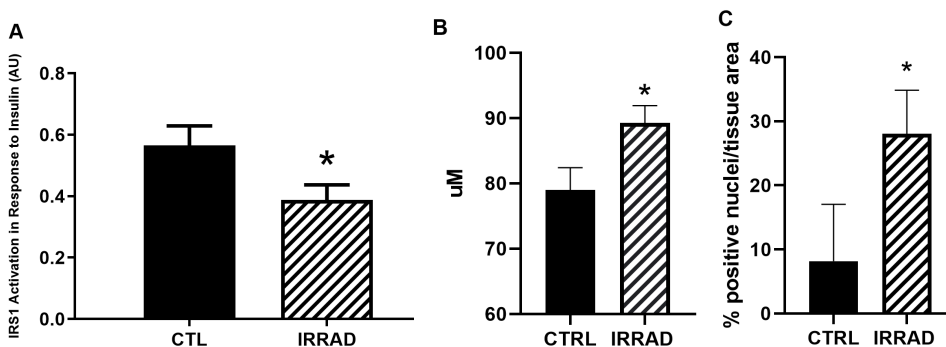


Figure 1.5 Irradiated animals have less insulin signaling (A), larger fat cells (B), and more macrophages in fat (C), relative to controls.

The follow up work being pursued includes looking at adipose precursor cell markers and differentiation potential. We hypothesize that irradiation will decrease precursor markers (CD26 and CD34), and will reduce the ability to differentiate into mature adipocytes. This is known as the “expandability” theory whereby in conditions of nutrient excess, fat has to be able to

hypertrophy and hyperplasia to safely store excess triglycerides. We have isolated preadipocytes within the stromal vascular fraction of fat tissue (this same fraction holds the cells with markers indicated above). In early studies it does look like irradiated fat tissue has a reduced capacity for expansion. Images below (**Figure 1.6**) show cells differentiated at study start and day 11, where fewer cells accumulating triglyceride are present in the irradiated example on the top panel.

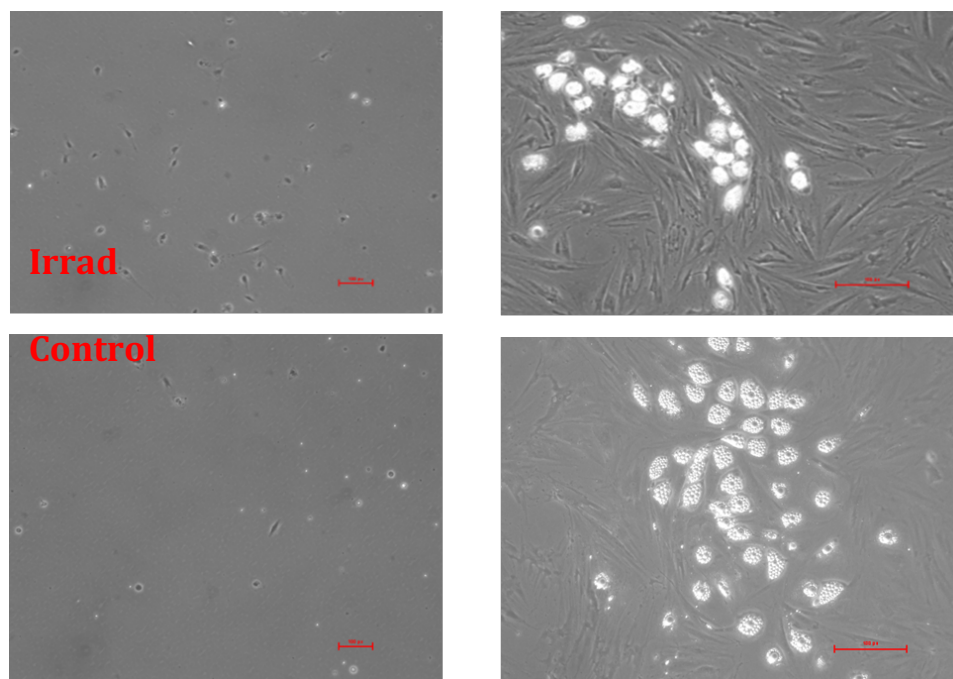


Figure 1.6 Preadipocytes from irradiated animals (top panels) and controls (bottom panels). After 11 days of differentiation (right panels), more adipocytes are storing fat in controls.

We have successfully performed in situ hybridization for precursor markers CD26 and 34 in nonhuman primate fat tissue, which represent mesenchymal and endothelial lineages respectively. Example images are below and we will be quantitating them in the next year.

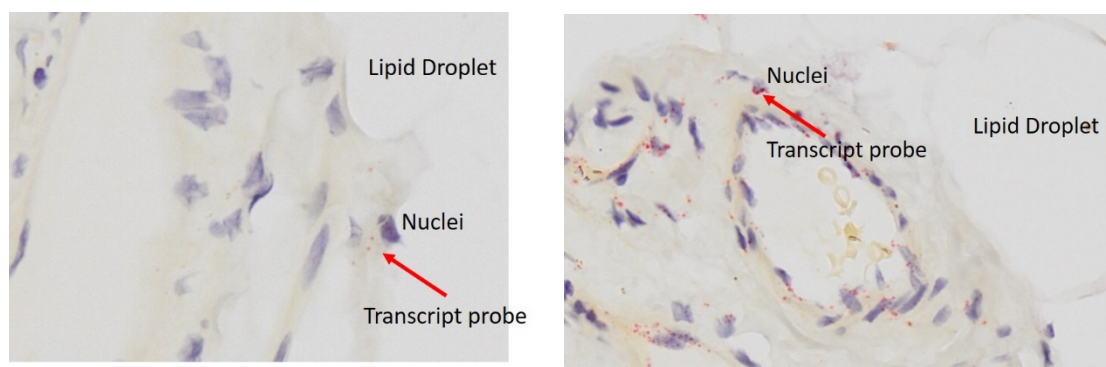


Figure 1.6 In situ hybridization for adipocyte differentiation markers CD26 (left) and CD34 (right) in NHP fat.

Another characteristic of healthy fat is something referred to as “brown fat” – this is highly metabolically active fat that is protective against obesity and diabetes, and actively secretes mediators that affect in a paracrine and endocrine way. We imaged 5 primates from this cohort to look for evidence of brown fat and its relationship with health. In collaboration with Dr. Branca (UNC-Chapel Hill) we used inhaled xenon gas as an agent which is selectively enhanced in brown fat over time and able to be visualized on computed tomography scanning. Our data indicates that abundance relates to seeing dense fat on our images post-xenon, which has been confirmed at necropsy by direct visualization and histology. A table and example are shown below (**Figure 1.7**). These data have supported 2 additional DOD grant applications in collaboration with University of Massachusetts researchers, Dr. Corvera and Czech.

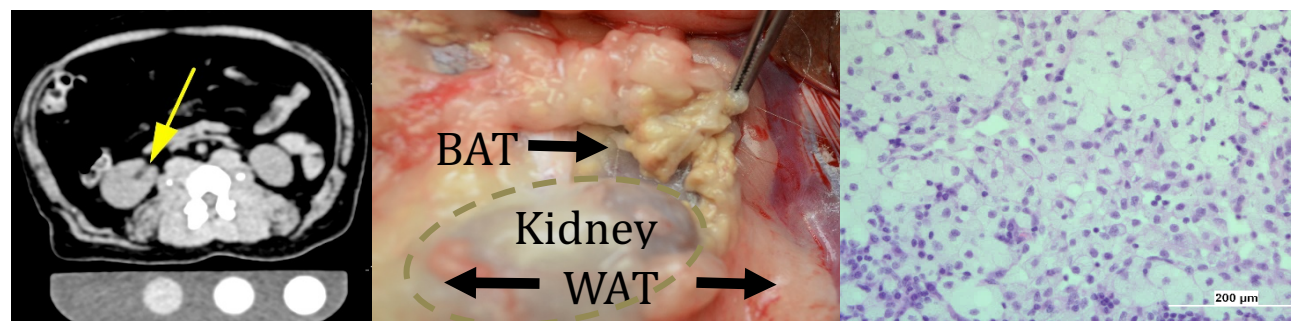


Table 1.1		Xenon tissue enhancement in NHPs		
Health Status	A1c%	Supraclavicular Axilla		Renal Hilus
DM (n=1)	8.5	-	-	-
IR (n=1)	6	+/-	-	-
CTL (n=3)	4.6-4.8	++	++	++

Figure 1.7 *Computed tomography Xenon contrast imaging of brown adipose tissue (BAT), left panel, yellow arrow; brown adipose tissue (BAT) and white adipose tissue (WAT) in the fat around the kidney at necropsy (center panel); histologic appearance of BAT (right panel). Table 1.1 legend: DM = diabetes mellitus; IR = insulin-resistant; CTL = control*

Lastly, we have done evaluation of adipose senescence. Senescent cells are in cell cycle arrest and secreting pro-inflammatory cytokines, affecting tissue around them. Macrophages are known to transiently express senescence markers, whereas the more general rule is that senescence is a permanent state and contributes to tissue dysfunction. Radiation is considered a gold standard for the induction of senescence in vitro. We have assessed senescent cell staining (% of isolated adipocytes) and tissue staining (blinded observer scoring) and found that this long after radiation exposure, senescence associated beta-galactosidase staining is not increased in these animals (**Figure 1.8**). There is far more variability in the irradiated animal's fat tissues, but this could be the result of their metabolic disease and greater macrophage abundance. This data contrasts against dogma and additional markers will be pursued to create a short communication to disseminate.

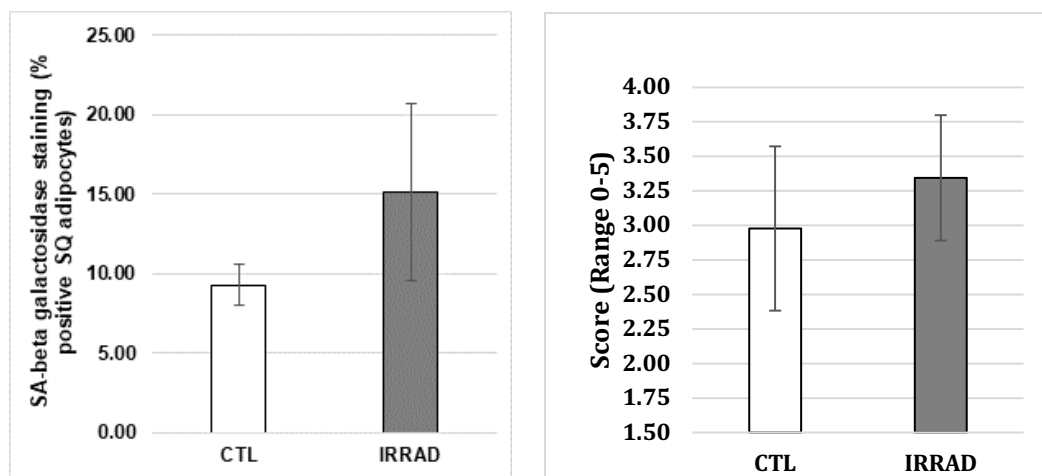


Figure 1.8 *The senescence-associated biomarker senescence associated beta-galactosidase is not increased in adipose tissue of irradiated macaques.*

2. Interventional Rodent Studies

In this study we are evaluating the effects of inhibiting fibrotic signaling (Transforming Growth Factor beta [TGFb receptor inhibition; IPW-5371 by Innovation Pathways). Briefly, the rationale for this intervention is the observation in the RSC that TGFb was increased with radiation (Fanning et al., AJP 2017) and we see an accumulation of collagen subtypes in the muscle tissue. Fibrosis is a well-recognized outcome of radiation exposure and is increasingly important in multiple chronic disease conditions such as heart failure and liver diseases. 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 are also evaluating Tadalafil, a phosphodiesterase-5 inhibitor, marketed as Cialis. This drug has been chosen for emerging evidence that it can enhance perfusion, specifically in skeletal muscle. To date, 3 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 are studied as single agents and in combination after sub-lethal irradiation. The IPW-5371 and Tadalafil are administered to the study mice by compounding them in the Western diet being used to drive insulin resistance and obesity. Mice are fed once daily and drug was introduced 2 weeks after irradiation, mimicking what might be possible in the clinical situation. The drug interventions have had no effect on food consumption and weight gain to date. Due to the Western diet, all groups are considered obese.

In last year's annual report, we described the initial findings of improvements in fatty liver, which is a metabolic epidemic in the U.S and one without any therapeutic options. Our work in the current year has focused on solidifying the anti-fibrotic effects of IPW-5371 (a Transforming Growth Factor beta [TGFb] receptor 1 inhibition; IPW-5371 by Innovation Pathways). We have confirmed that downstream signaling the receptor is reduced in the liver, as measured by immunoblotting for phosphorylated SMAD2/3 (**Figure 1.9**). In the prior year we confirmed this signaling was reduced in lung tissue, indicating the oral administration of the drug has generalized effects. Both IPW-5371 groups were significantly lower. Interestingly, radiation exposure alone does not increase fibrosis signaling at this dose and measured 6 months after.

Liver Fibrosis Signaling

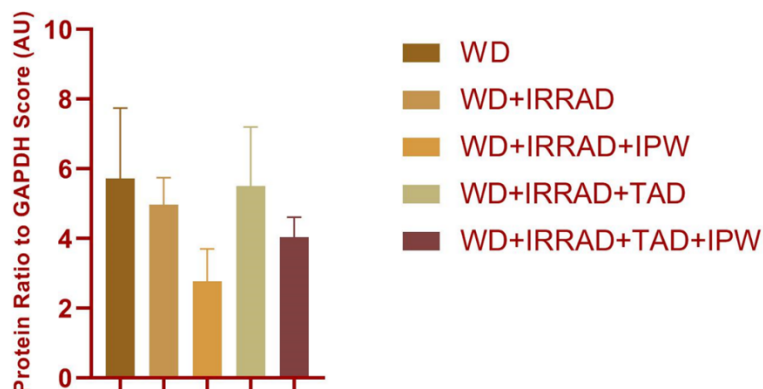


Figure 1.9 Profibrotic signaling (expression of phospho-SMAD2/3), showing lack of a clear radiation effect on fibrosis in the liver, but diminished signaling in the presence of the TGFbeta inhibitor IPW-5371.

The improvements in fatty liver with reduced TGFb signaling did lead to an improvement in insulin sensitivity as measured by insulin tolerance testing, and no changes in insulin sensitivity were seen in response to irradiation or Tadalafil treatment. This is consistent with the dramatic findings relating to liver steatosis observed. The change observed with treatment restored insulin sensitivity to that of a non-irradiated control mouse. Irradiation induced insulin resistance, consistent with what we have observed in all our nonhuman primate studies (**Figure 1.10**).

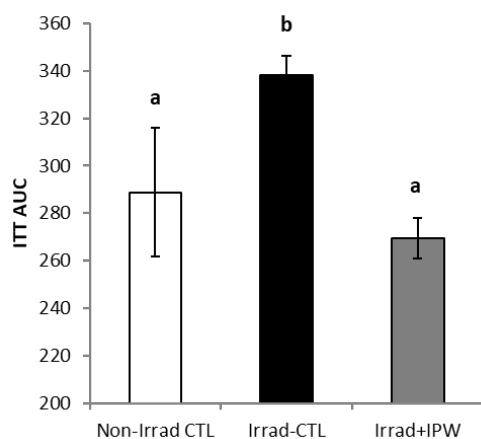


Figure 1.10 Insulin tolerance test results in mice indicating that irradiation increased glucose in the face of insulin treatment (insulin resistance), and that IPW-5371 reduced the radiation effect.

This year we have further evaluated thymic cell profiles (**Figure 1.11**), confirming that despite no differences in thymic weights – IPW-5371 improved cellularity – however the recovery was dominated by CD3 and CD4 single positive and double positive cells, and no effect on CD8 positive or double negative cells. Our next step is to evaluate the histopathology of the tissue to understand the stromal differences that support cell recoveries. Similar observations have been made in genetically modified rodents with altered TGFβ signaling and acute radiation exposure, however our “natural” model and protracted time course is a new aspect.

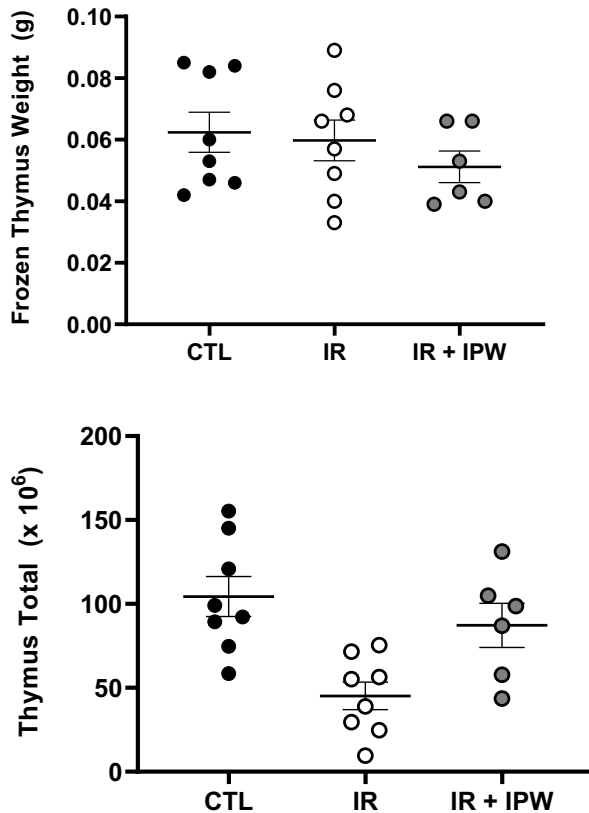


Figure 1.11 Mice. Top - thymic weight is not altered by irradiation or IPW treatment. Bottom - Thymic cellularity was reduced by irradiation, and that effect was mitigated by IPW-5371 treatment.

We have also continued to investigate the pharmacological interaction observed between Tadalafil and IPW-5371, and have found consistent cardiovascular lesions in males that were exposed. Last year we reported the significant death rates from combined exposures, and this year we confirm the presence of inflammatory and necrotic lesions. Our new data this year expanded histological lesion scoring in males at the 6 month time point. We used 2 blinded observers to pathological scoring of heart sections based on presence or absence of inflammation, erosion, degeneration and hemorrhage. Each lesion was given a score of 1=present, 0=absent and a sum total created (range 0-4). A total of 75 mice were evaluated in 6 groups (group sizes range 3 -20). The scores are shown below and indicate that the highest scores were observed in drug treated mice.

Table 1.2 - Pathological scoring of mouse heart sections.

GROUP	MEANS	N	STD.ERR.
NONIRRAD	0.54	13	0.18
IRRAD	0.25	4	0.25
IRRAD + IPW	1	3	0.58
IRRAD + TAD	1.15	20	0.18
IRRAD + TAD + IPW	1.13	15	0.27
NONIRRAD + TAD + IPW	1.25	20	0.23

Due to the uneven group sizes and multiple factors, we did factorial ANOVA to tease out the drug effects. Comparing groups exposed to no experimental drugs versus any experimental drugs, scores were 2.5 fold higher ($p=0.005$). To evaluate the effect of IPW-5371, we compared scores from mice with no exposure (including Tadalafil as a single agent) to those with exposure either alone or in combination, and scores were 2.4 fold higher ($p=0.002$). Irradiation had no effect on heart scores ($p=0.39$) and there was no interaction between radiation and drug exposure ($p=0.75$ for any drug and $p=0.92$ for IPW-5371). The lesions induced by drug exposure appear to be primarily driven by IPW-5371 exposure and was characterized by hemorrhage most commonly, followed by inflammation, and then degeneration.

Table 1.3 - Pathological scoring of mouse heart sections.

	HEMORRHAGE (% PRESENT)	EROSION (% PRESENT)	INFLAMMATION (%PRESENT)
NO IPW	4%	0%	22%
IPW EXPOSED	25%	0%	42%
CHI SQU P-VALUE (YATES CORRECTED)	$p<0.001$	N/A	$p=0.004$

The cause of death appears to be great vessel rupture and death with hemorrhage observed in body cavities. These outflow tracts are subject to high oscillations in pressure and require enough structural integrity to move ejected blood down to the abdomen and brain. Sudden death by vascular accident is an important side effect as it is difficult to predict or quantitate risk. The mechanisms behind why a sex difference in risk exists (males affected) are unknown. The current year will be used to verify the histological grading and write up results.

4) Other Achievements:

Nothing to Report

Project 2 – Radiation/Heart Disease (Register)

1) Major Activities

- a) Collection and processing of blood samples in the prospective cohort during the post-radiation phase for planned serum and plasma biomarker analyses.
- b) Monitoring and surveillance of animals in the prospective cohort
- c) Collaborations with Duke investigators on cross-project cell collections, processing, and data analysis, manuscript development, and future grant applications
- d) Analysis of gene expression in iliac arteries obtained from RSC cohort subjects which had gone to necropsy. Key target genes being investigated include collagen, macrophage and T cell markers, as well as selected cytokines and chemokines.
- e) 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.
- f) Cardiac tissue analyses: Processing of myocardial and coronary artery tissue blocks (RSC, & 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. Manuscript submitted to AHA journal.
- g) Cardiac Ultrasound Analysis Software: Ongoing discussions with the vendors of our cardiac Image Analysis software system (TOMTEC). We upgraded the computer and software for customized output to accommodate the smaller NHP heart sizes, and recently renewed the TomTec software.
- h) Cardiac Ultrasound Data collected from the PRC studies have been collected and evaluated from the 1st 4 years. Ultrasound images from the past 4 years in the RSC are 100% completed and a new manuscript has been placed on a preprint server and submitted for publication.
- j) Cardiac MRI studies on the 16 PRC monkeys were completed for the final timepoint. Analyses of the images from have been partially completed. We are working to have specialized image analyses completed in the coming months. We also acquired new software (SEGMENT) from Medviso AB Imaging for measuring strain in our cardiac MRI scans, this work is currently underway.
- k) Monthly DOD Conference calls
- l) Work has been initiated on a related 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) has been initiated.
- m) Final whole body CT scans were completed on the PRC

2) Specific objectives

Collections of samples, US, and cMRI images from the prospective cohort as described above. Planning for next cardiac imaging and biomarker assessments. Completions of necropsies in RSC subjects undergoing euthanasia and the PRC at the termination of the premortem period. Analysis of myocardium and data from RSC. Acquisition of premortem and postmortem data in the PRC.

3) Results/key outcomes:

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

Table 1. Demographics of Previously Irradiated Short Term (ST), Long Term (LT), & Deceased Male Rhesus Macaque Survivors (IRR) and Controls (CTL) [Mean (SEM)]

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

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 2.1**). 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 2.2**). 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 ($p=0.01$) (Table 4). 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.

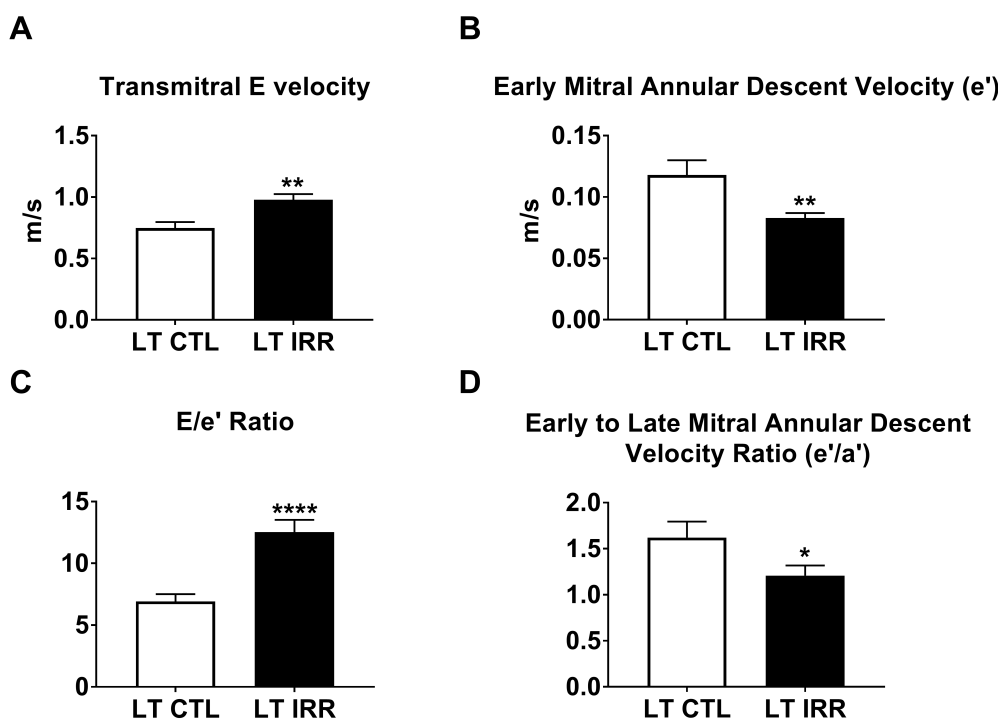


Figure 2.1 Echocardiographic Diastolic Function in Long Term Irradiated (LT IRR) and Long Term Controls (LT CTL): LT IRR animals had significantly increased transmitral early filling peak velocity (E) (Panel A), decreased early mitral annular descent velocity (e') (Panel B), increased E/e' ratio (Panel 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. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

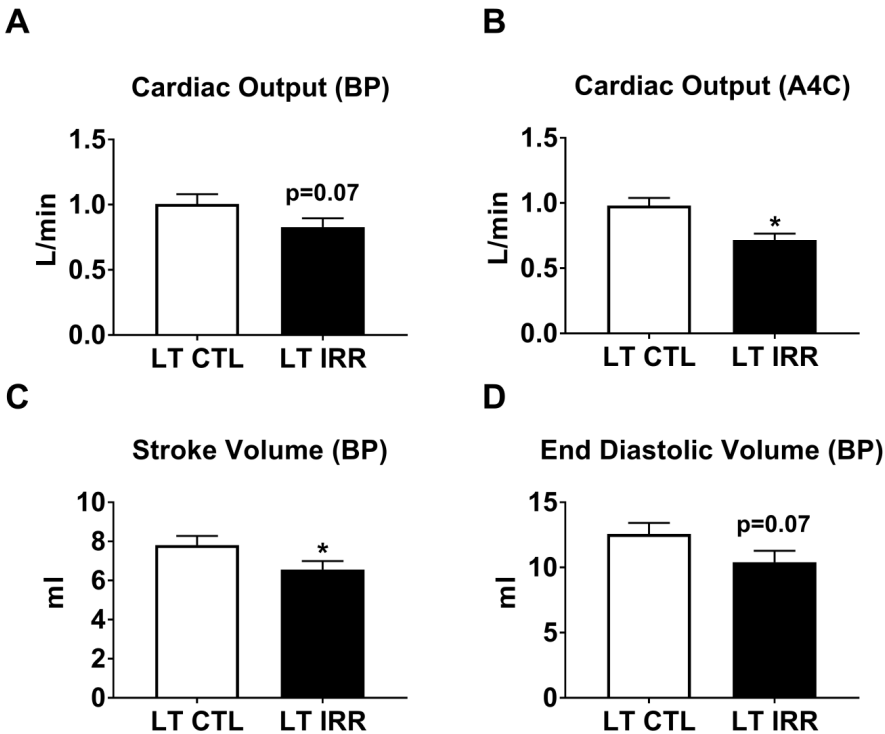


Figure 2.2 Echocardiographic Cardiac Volume Measures in Long Term Irradiated (LT IRR) and Long Term Controls (LT CTL): LT IRR animals tended to have decreased cardiac output calculated by method of disks measurements using the perpendicularly oriented apical 2 chamber (A2C) and apical 4 chamber (A4C) 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. * $p < 0.05$

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 2.3**), 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.

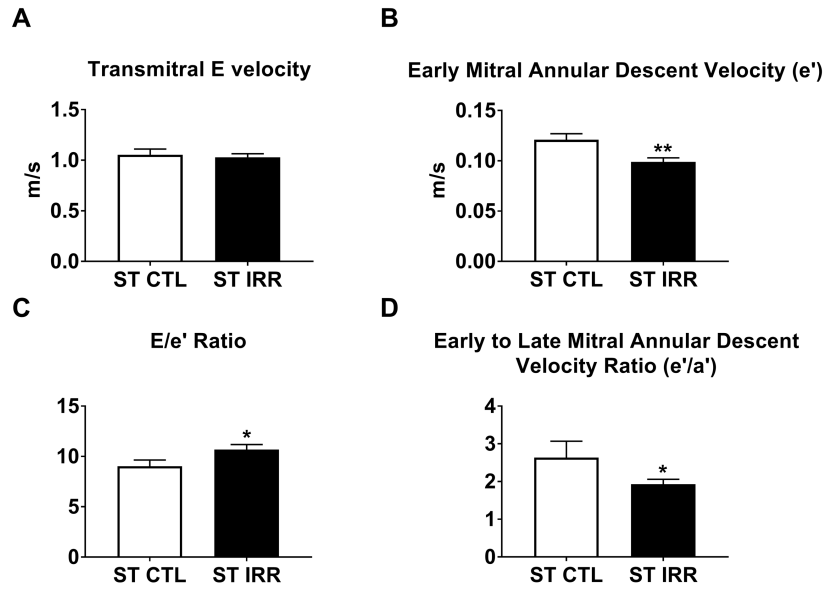


Figure 2.3 Echocardiographic Diastolic Function in Short Term Irradiated (ST IRR) and Short Term Controls (ST CTL): ST IRR animals had no differences in transmitral early filling peak velocity (E) (Panel A), but decreased early mitral annular descent velocity (e') (Panel B), increased E/e' ratio (Panel C) and decreased early to late mitral annular descent velocity ratio (e'/a') (Panel D) compared to ST CTL animals. Data are mean ± SEM. * $p < 0.05$, ** $p < 0.01$

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 2.4A**) Capillary density was reduced in the IRR group in the LV ($p < 0.05$) and IVS ($p < 0.01$), but not the RV. (**Figure 2.4B**). Examples are shown in **Fig 3.4CD**.

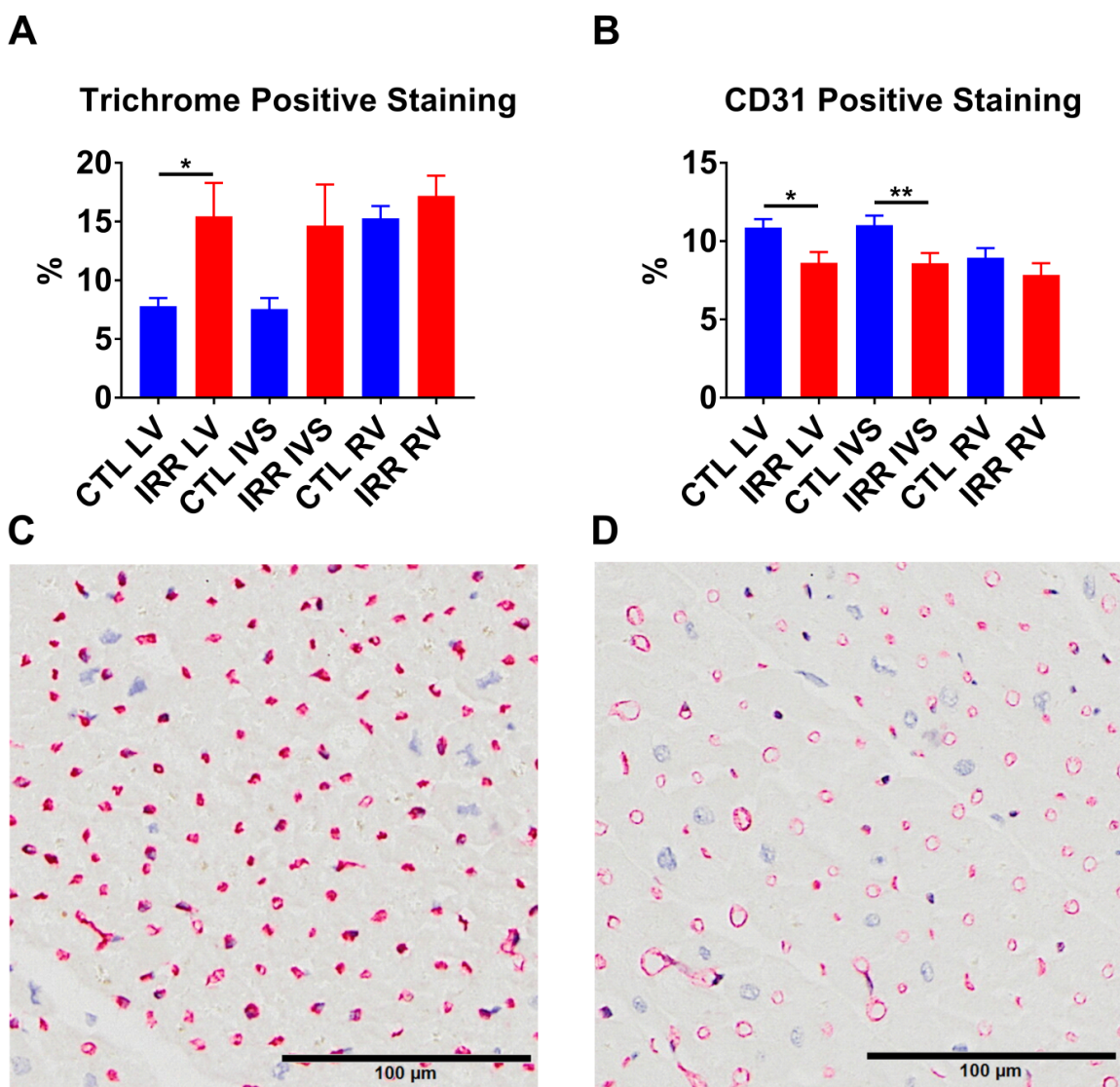


Figure 2.4 Fibrosis and Capillary Density Immunostaining in Deceased Irradiated (IRR) and Control (CTL) Animals: IRR animals had significantly increased percent positive trichrome staining of the left ventricle (LV) but not the interventricular septum (IVS) or right ventricle (RV) compared to CTL. IRR 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. * $p < 0.05$, ** $p < 0.01$

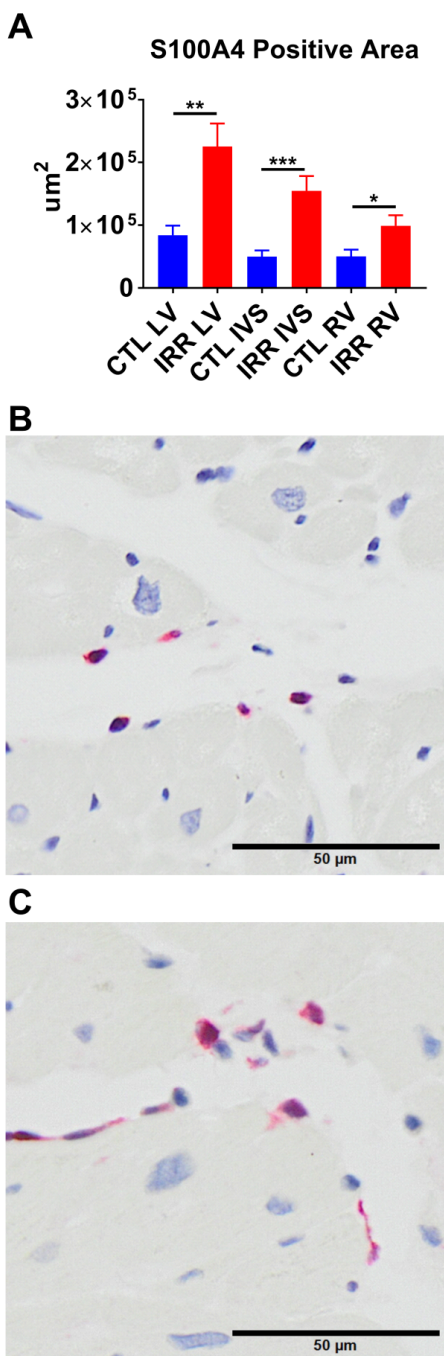


Figure 2.5 Fibroblast Immunostaining in Deceased Irradiated (IRR) and Control (CTL) Animals: IRR animals had greater S100A4 staining in all evaluated sections of myocardium compared to CTL animals (Panel A). Panels B and C are representative mid-myocardial S100A4 stained sections from a CTL and IRR animal respectively illustrating typical positive staining increases in IRR animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ 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 2.5**).

Macrophage content assessed by CD163 and CD68 was increased across all sections of the heart (**Figure 2.6AB**). 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 2.6CD**).

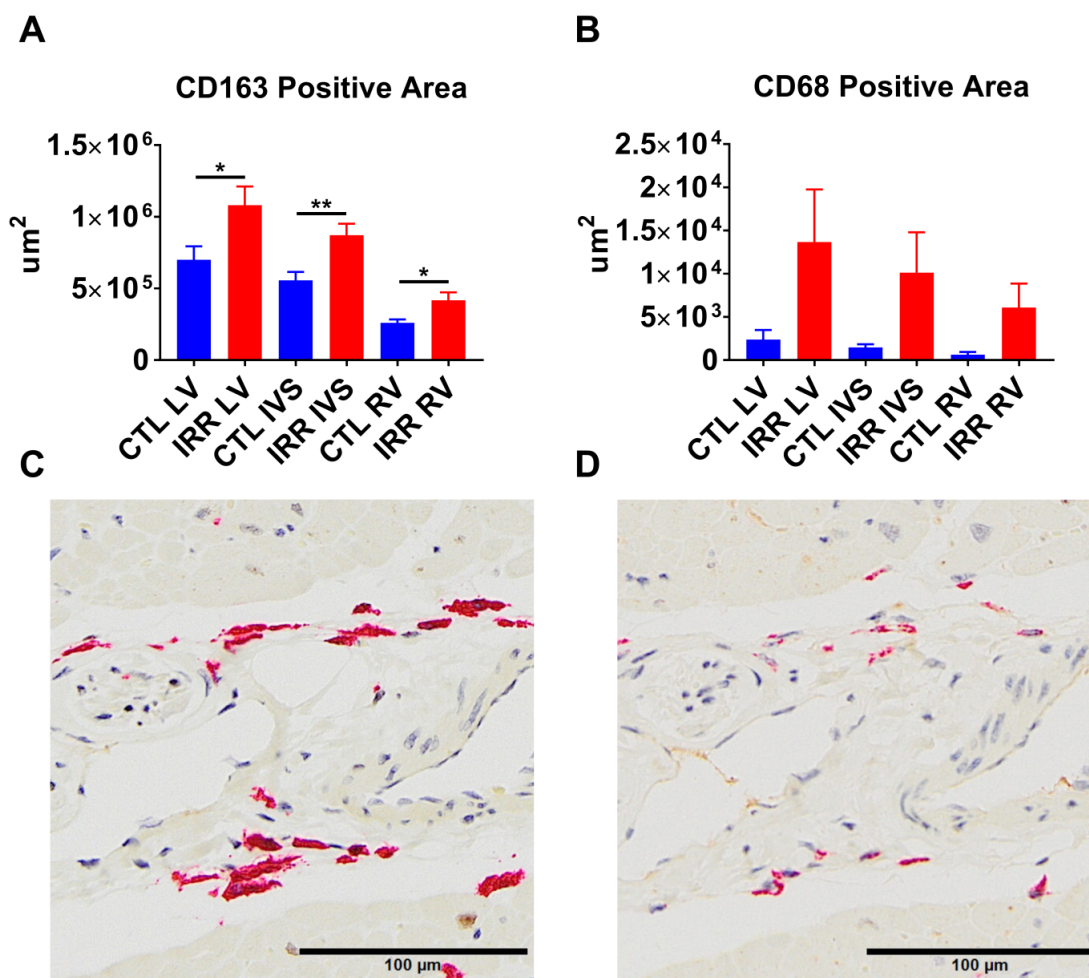


Figure 2.6 Macrophage Immunostaining in Deceased Irradiated (IRR) and Control (CTL) Animals: IRR animals had increased normalized CD163 positive staining area in the left ventricle (LV), interventricular septum (IVS), and right ventricle (RV) compared to controls (CTL)(Panel A). In contrast no differences 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⁺. * $p < 0.05$, ** $p < 0.01$

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 $c\text{-Maf}^+/\text{CD163}^+$ macrophages in the LV ($p < 0.05$) and IVS ($p < 0.01$), but not RV, and increased $p\text{STAT1}^+/\text{CD163}^+$ macrophages in the LV ($p < 0.01$), IVS ($p < 0.01$), and RV ($p < 0.05$). (Figure 8) Finally, we compared the $c\text{-Maf}^+/\text{CD163}^+$ (M2) to $p\text{STAT1}^+/\text{CD163}^+$ (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 2.7)

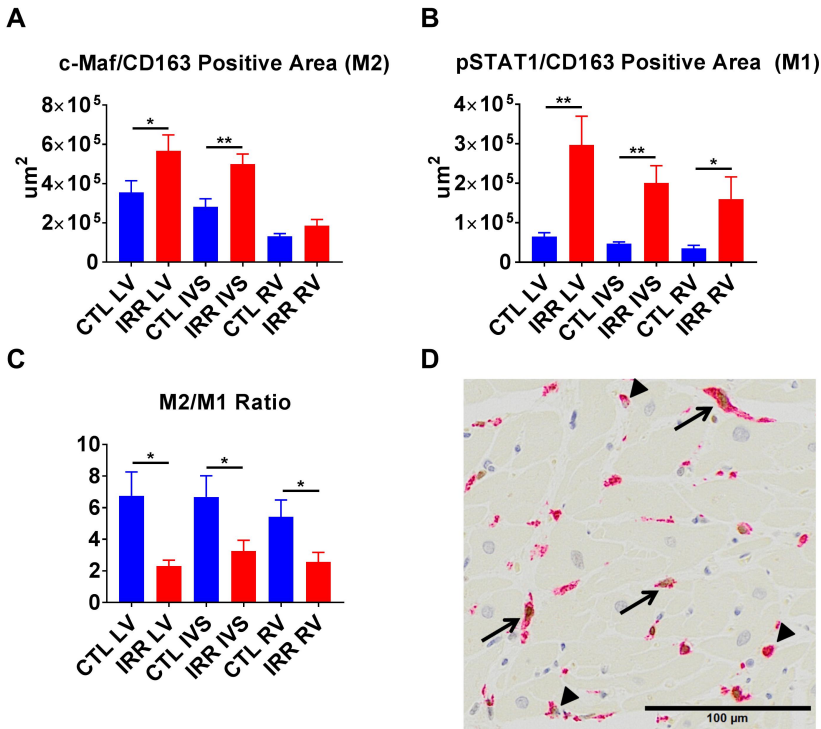


Figure 2.7 Macrophage Polarization in Irradiated (IRR) and Control (CTL) Myocardium: IRR animals had increased $c\text{-Maf}^+/\text{CD163}^+$ staining (M2 polarization) in the left ventricle (LV), interventricular septum (IVS), but not right ventricle (RV) compared to controls (CTL) (Panel A). Increased $p\text{STAT1}^+/\text{CD163}^+$ 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 $c\text{-Maf}^+/\text{CD163}^+$ 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 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.

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.

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. Data analysis is underway.

Preliminary Analysis of RNAseq dataset: RNAseq data was evaluated using MultiQC v0.7 (Phil Ewels, available on GitHub). Alignment was conducted with STAR using the rhesus genome (Macaca mulatta v 8.0.1). Cutadapt was used to find and remove primer adapter sequences, primers, poly-A tails and other unwanted sequence high-throughput sequencing reads. Analyses were carried out using R, Bioconductor, and custom codes. Quality control for high throughput sequence data was assessed using FastQC (Simon Andrews at the Babraham Institute in Cambridge). The Adjusted P, is the genomic adjusted raw p value, FDR_Genomic_Adjusted_P is Benjamini & Hockberg. The genomic adjustment is evidenced by the pp plots, which takes the raw p value and removes inflation typically found in data analyzed with DESeq2.

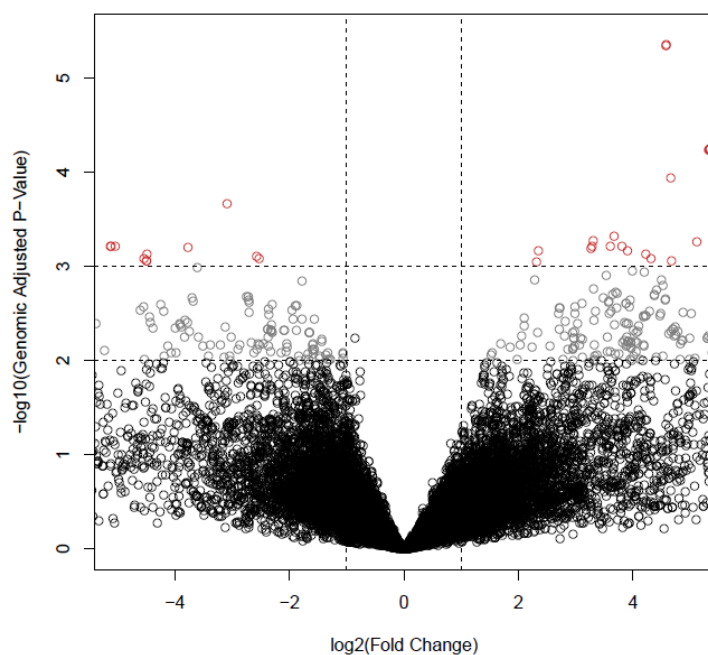
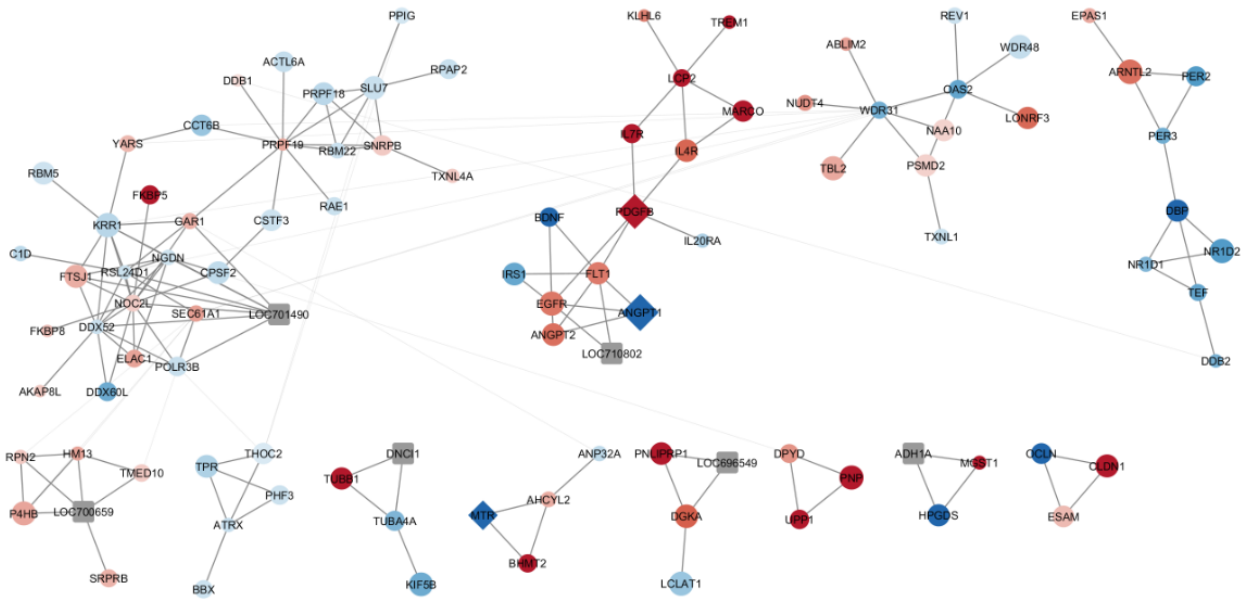
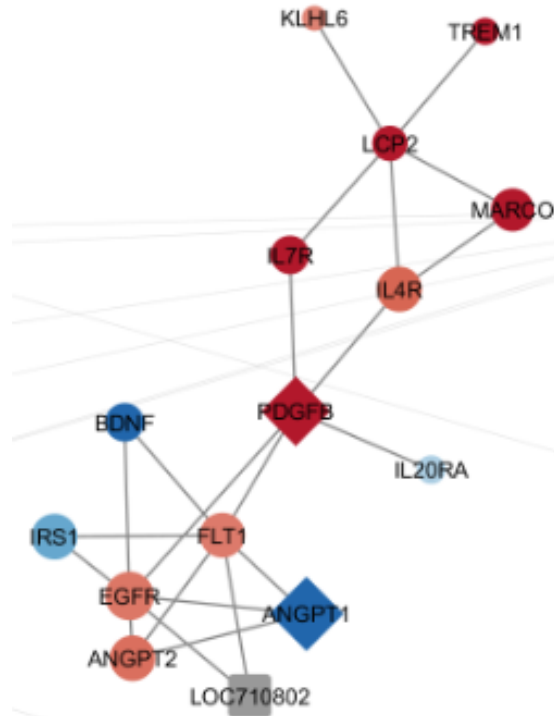
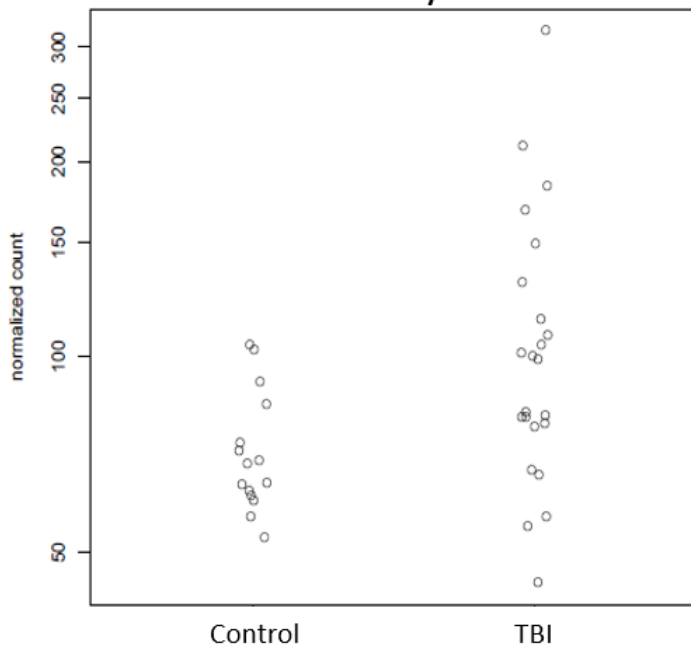


Figure 2.8 Volcano Plot of RNAseq Data from Male Rhesus RSC Subjects: Plot shows adjusted p values for different up and down regulated genes. At $p=0.001$, there were in 22 upregulated genes in TBI vs 16 in Control myocardium.

Figure 2.9: MCODE Pathways identified from DEGs illustrated in Figure 2.8.**Differential Expression of PDGFB
Control vs TBI Myocardium****Figure 2.10: Differential expression of PDGFB in Control vs TBI Subjects.** (left panel) PDGFB related pathway illustrated with DEGs noted by red (upregulated) or blue (downregulated genes). PDGF gene itself. (right panel)

Preliminary analyses have identified PDGFB and PDGFB related pathways as a potential key mediator of radiation effects in the heart (Figure 2.10). PDGFB overexpression in mouse models leads to moderate cardiac fibrosis (Gallini et al. PMID: 27816607). Interestingly, stem cell antigen expressing (Sca-1) positive cells have been shown to overexpress PDGFB in addition to being able to home to injured heart (Samal et al. PMID: 22749858). Analyses underway.

nCounter XT Gene Expression Assay: Purified RNA samples were diluted with RNase free water to a final concentration of 10 ng/ μ L. 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).

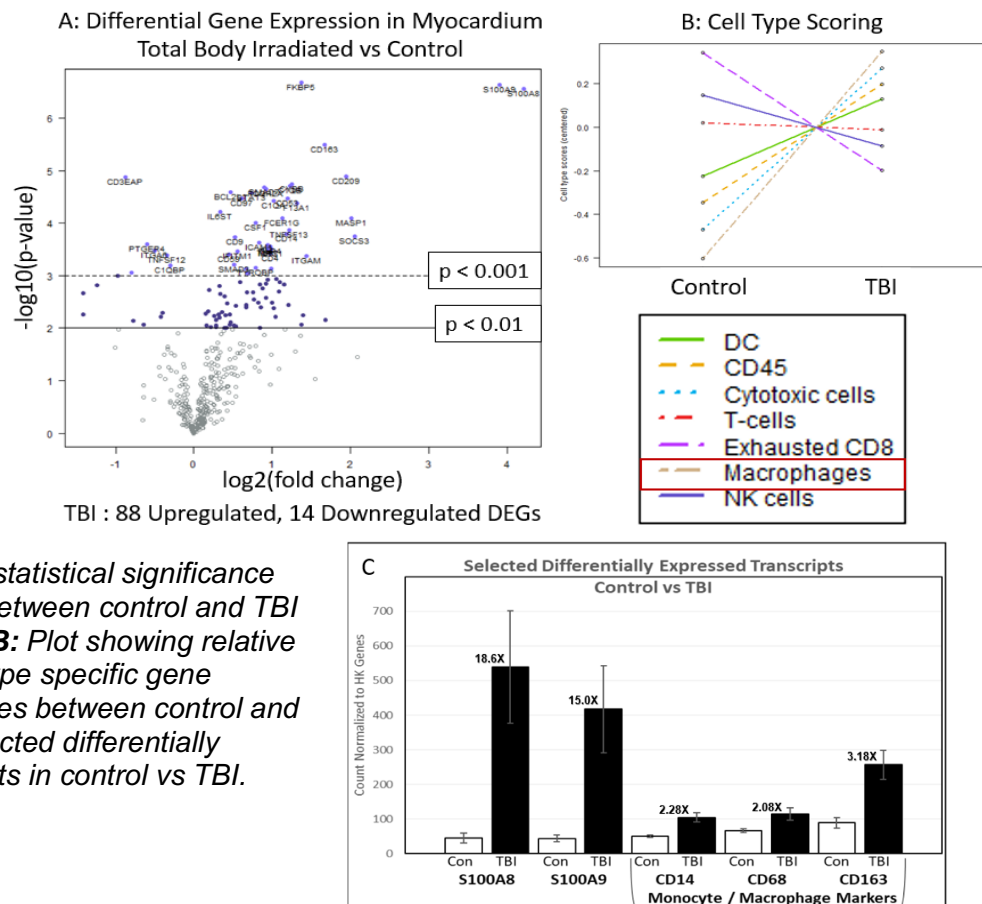
Preliminary Results: At $p=0.05$, 125 transcripts were higher and 23 genes were lower in TBI myocardium relative to control (**Figure 2.11A**). The top ten upregulated transcripts were: S100A8, S100A9, C6, SOCS3, MASP1, CD209, FOS, CD163, ITGAM, and CCL2. Numerous transcripts for macrophage markers were 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 2.11A, C**). The top ten downregulated transcripts were: CD38, KIT, CCL24, THY1, DPP4, DEFB1, CD3EAP, MYL2, MAP2K6, and OAS2. **Figure 2.11B** 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 2.11C**.

Figure 2.11:
Immunology Related Targeted Myocardial Gene Expression

Analyses: A:

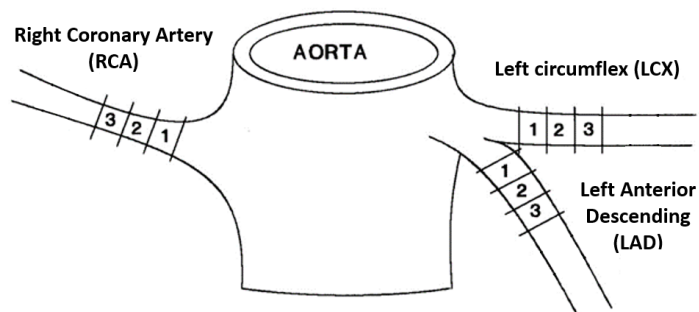
Volcano plot of the statistical significance vs. fold difference between control and TBI myocardial genes. **B:** Plot showing relative differences in cell type specific gene expression signatures between control and TBI groups. **C:** Selected differentially expressed transcripts in control vs TBI.



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 2.12**) 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 2.13**. 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.

Figure 2.12: Illustration of the sources of the coronary arteries evaluated in the RSC.

Sources of Coronary Artery Blocks



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

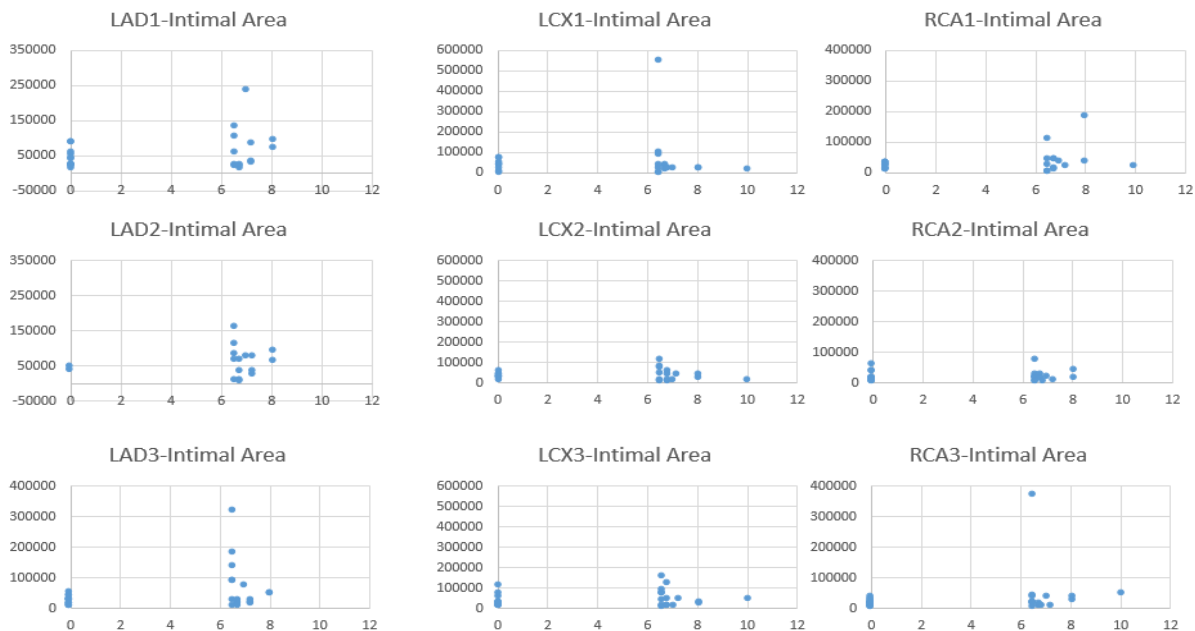


Figure 2.13: Raw data of atherosclerosis (intimal area in μm^2) from the 9 blocks of coronary arteries illustrated in Figure 2.12

4) Other Achievements:

Awards:

Dr. Kristofer T Michalson, DVM, PhD (former graduate student in Comparative Medicine/Pathology), was selected as the recipient of the **2020 Radiation Research Editors' Award** for our manuscript "*Monocyte Polarization is Altered Post-Irradiation in Male Rhesus Macaques: Implications for Delayed Effects of Acute Radiation Exposure*" which appeared in the August 2019 publication of RADIATION RESEARCH 192, 121-134. Co-authors were Andrew MacIntyre, Greg Sempowski, Dan Bourland, Tim Howard, Greg Hawkins, Greg Dugan, J. Mark Cline and Thomas C. Register. The manuscript describes TBI induced alterations in the proportions of 'classical' and 'intermediate' monocyte subfractions, and changes in the transcriptional profiles of the intermediate monocyte fraction, which provide supportive evidence that these cells in particular may have pro-fibrotic effects. The work is important in that it provides potential new avenues for intervening on radiation induced fibrosis in the heart and other tissues. Former fellow graduate student Ryne DeBo received the 2017 Radiation Research Editors' award.

Abstracts: An abstract was submitted and accepted for the 2020 Radiation Research Meeting planned for Hawaii but changed to a virtual format describing immune associated gene expression in myocardium obtained from the RSC. An abstract was presented at the November 2019 AHA meeting in Philadelphia. Abstracts, presentations, and manuscripts are listed in section 6.

Grants submitted:

1. Program Project Application: Studies supported by the current program led to the submission of a P01 application to NIAID which was submitted in January 2020.
Overall Project Title: *Regenerative Immunology in Radiation Injury*
Project 1 (PI: Chao): *Macrophage dysregulation in radiation survivors*
Project 2 (PI: Doan) *Persistent vascular endothelial progenitor defects in radiation survivors*
Project 3 (PI: Andrews) *Evaluation of chronic neuroinflammation and cerebrovascular injury in NHP TBI survivors*
Project 4 (PI: Register) *Monocyte and Macrophage Reprogramming in Radiation Induced Heart Disease*
Project 5 (PI: Kavanagh) *Development of Insulin Resistance and Type II Diabetes by Pathogenic Macrophages*

This P01 did not receive a sufficient score overall for funding.

2. NIH R01: An R01 entitled *Cellular and Transcriptional Profiling of Radiation Induced Heart Disease and Fibrosis (Register: PI)* was submitted in June 2020 to expand studies initiated in this DOD funded effort and is under review late October 2020.

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

1) Major Activities (Year 5)

- a. In Year 4, the 16 animals in the prospective cohort (X-15-03) were immunized with the 2018/9 quadrivalent seasonal flu vaccine and 23-valent pneumococcal polysaccharide vaccine, and sera were collected before and at multiple timepoints after immunization. In Year 5, we assayed these sera for IgG against the 23 streptococcal polysaccharide antigens and the 4 influenza hemagglutinins in the respective vaccines. We also assayed the sera for influenza neutralizing antibody capability.
- b. We Received and processed blood from all NHP in the X-10-12 long-term survivor cohort. We performed immunophenotyping and froze viable PBMC and plasma for future batch molecular analysis and multiplex cytokine profiling, respectively.
- c. We submitted all flow cytometry and sjTREC data collected from studies of the long-term survivor cohort in Years 1-3 to the Wake Forest University-hosted database (CARS), and collaborated with Dr. Tooze at WFU to analyze these data for significant relationships to age, time since radiation and radiation treatment.
- d. We Completed analysis of all TCR α and TCR β sequencing of the paired baseline and 9-month post-irradiation PBMC collected from the X-15-03 prospective cohort.
- e. We wrote and submitted a journal article describing our observations of the immune system in the prospective cohort.
- f. Using single cell RNA sequencing analysis, we studied how T cells recovered post-irradiation at the molecular level.
- g. Unsupervised clustering analysis revealed unique T cell populations when recovering from radiation injury.
- h. Trajectory inference analysis indicated progressively differentiated phenotypes following TBI exposure.
- i. The analysis of the RNAseq data suggested that T cells rely on OXPHOS rather than aerobic glycolysis to promote survival and homeostatic proliferation following TBI.

2) Specific Objectives (Year 5)

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

Phenotypic analyses in prospective and long-term cohorts

Major Task 2: Murine Studies

Determine the roles of thymopoiesis and peripheral expansion in overall T cell recovery in two mouse radiation injury models

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 Task 3: HGH Mouse Studies

Determine the effect of HGH on phenotypic T cell reconstitution

Major Task 4: IGF-1 Mouse Studies

Determine the effect of IGF-1 on phenotypic T cell reconstitution

3) Results/Key Outcomes (Year 5)

SA1 Major Task 1: NHP Studies (Sempowski/Macintyre, Duke)

Long-term cohort (X-10-12):

At intervals throughout year 5, PBMC and plasma from 157 animals in the long-term survivor cohort (X-10-12) were isolated and stored. Fresh PBMC from all 157 animals were immunophenotyped via flow cytometry using previously described panels for B cells, T cells, NK, NK-T, and dendritic cells. This was the fourth annual round of immunophenotyping for this cohort.

CD4+ and CD8+ T cells were isolated by positive selection from approximately 280 PBMC samples collected from X-10-12 animals during years 4 and 5, and sjTREC were quantified in each isolated T cell population. We are currently collating these sjTREC data and the respective flow cytometry data for submission to the CARS database at WFU.

All flow cytometry and sjTREC data from the survivor cohort studies in Years 1-3 was submitted to CARS in Year 4. In Year 5, we collaborated with Dr. Tooze at WFU to analyze these data. While there was insufficient repeated-measurement data for longitudinal analysis, Dr. Tooze was able use single-timepoint data from each animal to test three models. These models evaluated:

- irradiated vs. non-irradiated animals, with adjustment for sex and age and the interaction of age and irradiation
- Radiation dose category with non-irradiated included, with adjustment for sex and age and the interaction of age and dose category
- Radiation dose category on irradiated animals only, with adjustment for sex and age and time since irradiation, with interaction of category and time since irradiation

Select p values from these models are reported in Tables 3.1-3.3. Initial evaluation of these results suggest that the long-term immunological effects of irradiation are subtle but significant, with a particular impact on the naïve/effector/memory T cell balance. This is analogous to our findings from the prospective NHP cohort, where we observed minimal long-term impacts on dendritic and B cells but sustained differences in the T cell balance over a year after irradiation.

Table 3.1. Select p values from Model 1: irradiated vs. non-irradiated animals, with adjustment for sex and age and the interaction of age and irradiation. p<0.05 bolded.

Population	Main Effect (Irradiation)	Interaction of Age & Radiation	Significance at Age:				
			3-4	5	6-7	8-12	13-20
B cells	0.384	0.124	0.013	0.174	0.095	0.635	0.830
Memory B cells	0.140	0.505	0.735	0.384	0.845	0.038	0.600
Activated B cells	0.401	0.951	0.322	0.871	0.407	0.783	0.885
Myeloid DC	0.180	0.483	0.013	0.989	0.593	0.200	0.943
Plasmacytoid DC	0.578	0.846	0.515	0.668	0.342	0.805	0.588
T cells	0.028	0.238	<0.001	0.575	0.036	0.455	0.527
CD4+CD8+ T cells	0.787	0.662	0.072	0.855	0.994	0.897	0.619
CD4 T cells	0.530	0.680	0.909	0.374	0.986	0.303	0.448
CD8 T cells	0.582	0.460	0.142	0.753	0.682	0.431	0.201
CD45RA+ CD4 T cells	<0.001	0.017	<0.001	0.572	0.042	0.003	0.389
CD45RA+ CD8 T cells	0.008	0.011	0.137	0.045	0.794	0.037	0.029
Effector Memory CD4 T cells	0.063	0.390	0.521	0.191	0.537	0.264	0.229
Central Memory CD4 T cells	0.001	0.170	<0.001	0.844	0.025	0.048	0.828
Naïve CD4 T cells	0.682	0.001	<0.001	0.106	0.223	0.866	0.340
Effector Memory CD8 T cells	0.004	0.010	<0.001	0.054	0.214	0.026	0.084
Central Memory CD8 T cells	0.004	0.530	<0.001	0.371	0.301	0.019	0.809
Naïve CD8 T cells	0.053	0.003	0.034	0.004	0.725	0.149	0.018

Table 3.2. Select p values from Model 2: Radiation dose category with non-irradiated included, with adjustment for sex and age and the interaction of age and dose category. p<0.05 bolded.

Population	Main Effect (Radiation Dose)	Interaction of Age & Dose	Significance at Age:				
			3-4	5	6-7	8-12	13-20
B cells	0.044	0.050	0.003	0.179	0.030	0.086	0.997
Memory B cells	0.301	0.423	0.254	0.243	0.762	0.087	0.789
Activated B cells	0.770	0.903	0.347	0.974	0.327	0.544	1.000
Myeloid DC	0.077	0.886	0.015	0.768	0.590	0.478	0.848
Plasmacytoid DC	0.919	0.457	0.698	0.914	0.047	0.710	0.712
T cells	0.070	0.235	0.005	0.111	0.161	0.744	0.222
CD4+CD8+ T cells	0.969	0.876	0.355	0.617	0.629	0.780	0.937
CD4 T cells	0.948	0.924	0.996	0.597	0.718	0.583	0.807
CD8 T cells	0.960	0.971	0.538	0.991	0.894	0.883	0.605
CD45RA+ CD4 T cells	0.022	0.176	<0.001	0.991	0.159	0.023	0.502
CD45RA+ CD8 T cells	<0.001	<0.001	0.001	<0.001	0.017	0.056	0.059
Effector Memory CD4 T cells	0.020	0.127	0.399	0.150	0.618	0.463	0.026
Central Memory CD4 T cells	0.001	0.335	<0.001	0.532	0.039	0.167	0.070
Naïve CD4 T cells	0.263	0.020	<0.001	0.041	0.313	0.914	0.669
Effector Memory CD8 T cells	<0.001	0.005	<0.001	<0.001	0.059	0.077	0.356
Central Memory CD8 T cells	<0.001	0.332	0.004	0.014	0.043	0.055	0.928
Naïve CD8 T cells	0.016	0.009	0.102	<0.001	0.802	0.444	0.082

Table 3.3. Select p values from Model 3: Radiation dose category on irradiated animals only, with adjustment for sex and age and time since irradiation, with interaction of category and time since irradiation. p<0.05 bolded.

Population	Main Effect (Radiation Dose)	Interaction of Dose and Time Since Irradiation	Significance at Years Since Irradiation:				
			0 to <1	1 to <2	2 to <3	3 to <5	≥5
B cells	0.218	0.375	0.390	0.105	0.546	0.545	0.181
Memory B cells	0.003	0.082	0.098	0.764	0.001	0.023	0.885
Activated B cells	0.165	0.241	0.521	0.150	0.246	0.046	0.854
Myeloid DC	0.975	0.823	0.941	0.827	0.426	0.338	0.979
Plasmacytoid DC	0.192	0.524	0.122	0.693	0.189	0.707	0.768
T cells	0.986	0.561	0.637	0.928	0.086	0.787	0.623
CD4+CD8+ T cells	0.806	0.781	0.692	0.513	0.551	0.262	1.000
CD4 T cells	0.490	0.384	0.502	0.369	0.251	0.361	0.490
CD8 T cells	0.338	0.783	0.691	0.882	0.549	0.783	0.297
CD45RA+ CD4 T cells	0.766	0.122	0.392	0.686	0.113	0.223	0.331
CD45RA+ CD8 T cells	0.860	0.024	0.021	0.149	0.616	0.097	0.815
Effector Memory CD4 T cells	0.186	0.123	0.661	0.591	0.150	0.293	0.047
Central Memory CD4 T cells	0.056	0.080	0.093	0.528	0.992	0.345	0.008
Naïve CD4 T cells	0.051	0.187	0.085	0.105	0.087	0.676	0.108
Effector Memory CD8 T cells	0.924	0.001	<0.001	0.454	0.216	0.806	0.230
Central Memory CD8 T cells	0.327	<0.001	<0.001	0.563	0.275	0.921	0.287
Naïve CD8 T cells	0.203	0.115	0.026	0.772	0.686	0.478	0.086

Once the data from Years 4 and 5 are entered into CARS, we anticipate having sufficient repeated measurements for Dr. Tooze to incorporate longitudinal responses into the mixed-effects models.

Prospective Cohort (X-15-03)

Molecular analysis of samples from Prospective Cohort:

In Year 4 we worked with the Duke Human Vaccine Institute Sequencing Core to develop an in-house pipeline for NHP TCR α and TCR β sequencing. In Year 5, we analyzed TCR sequences from prospective cohort (X-15-03) cryopreserved PBMC collected at baseline and 9-months post-irradiation. PBMC from five control and six irradiated animals yielded sufficient high-quality 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 (**Figure 3.1**).

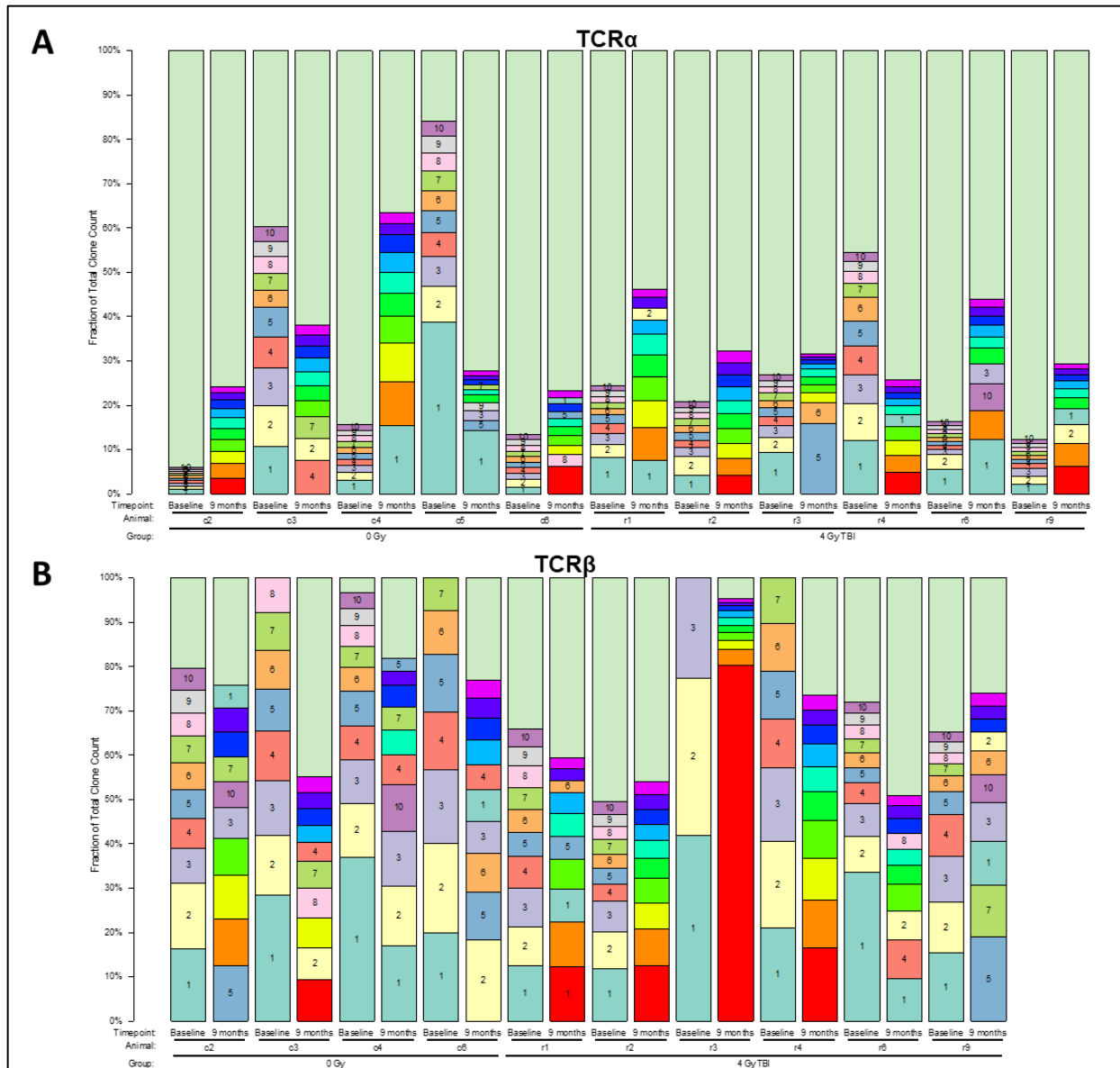


Figure 3.1. TCR clonal distribution before and nine months after irradiation

Plots show cumulative frequency distribution of top (A) TCRα and (B) TCRβ 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 based on rank order at baseline. NB: there is no relationship between numbered clones in different animals.

We then calculated the TCRα and TCRβ sequence entropies, which are highest when clonality and diversity are maximized, for each animal at both timepoints. While entropies in each animal changed over time, there were no consistent trends (**Figure 3.2A, 3.2B**), and mixed-effects modeling did not indicate any effect of time or radiation on the entropies. Next, we determined the overlap in clones between baseline and nine months post TBI for each animal. The cumulative frequencies of overlapping TCRα and TCRβ clones over time were less in irradiated animals, although these differences did not reach statistical significance (**Figure 2C, 2D**). There

was, therefore, a trend for irradiated animals to have a higher proportion of clones at nine months that were not present at baseline.

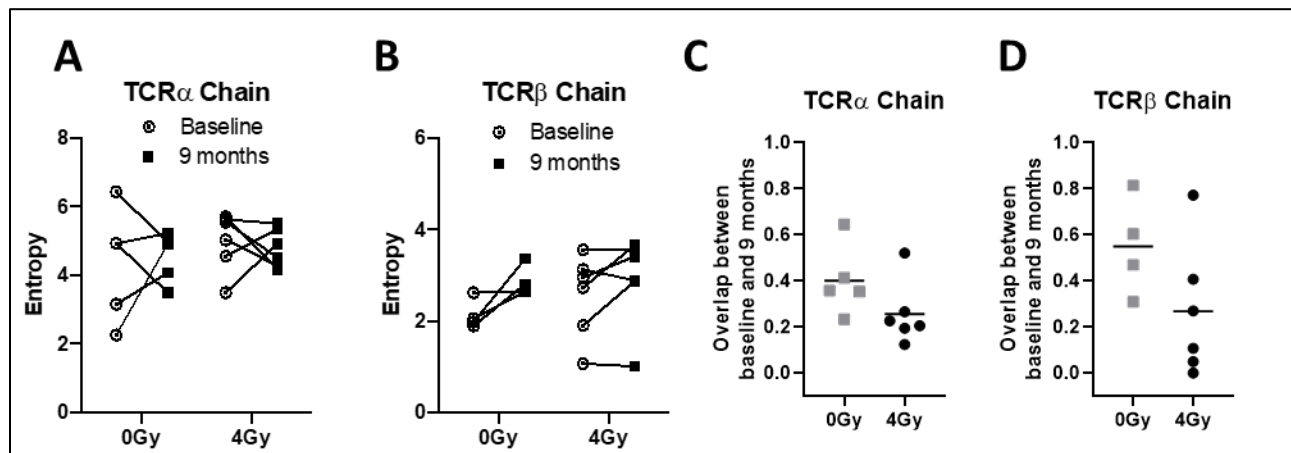


Figure 3.2. TCR Shannon entropy and per-animal cumulative frequencies of overlapping TCR clones

A and B show Shannon entropy in each prospective cohort animal at baseline and 9-months post exposure. Lines link samples from the same animal. C and D show the mean and per-animal cumulative frequencies of overlapping clones between baseline and 9-months post exposure.

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 sequences only arise during thymopoiesis, and so these findings 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.

Immune-response Analysis of Prospective Cohort:

In Year 4 the WFU primate core immunized the prospective cohort with two multivalent vaccines: a 23-serotype pneumococcal polysaccharide vaccine (23vP; Pneumovax 23) 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 (826 days) 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.

For each animal, we assayed IgG titers for each HA in the influenza vaccine by ELISA, normalized the data to baseline and then plotted the results against time. The area under each curve (AUC) was calculated as shown in **Figure 3.3A** for a representative animal and HA. The IgG responses for all animals and all HAs are summarized in **Figure 3.3B**. With the exception of one animal, 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. In addition, there were no significant differences in IgG responses between the control and irradiated groups after the animals had received only the prime dose (**Figure 3.3C**). Thus, irradiation did not affect the amounts of anti-HA IgGs produced in response to vaccine strain HA proteins.

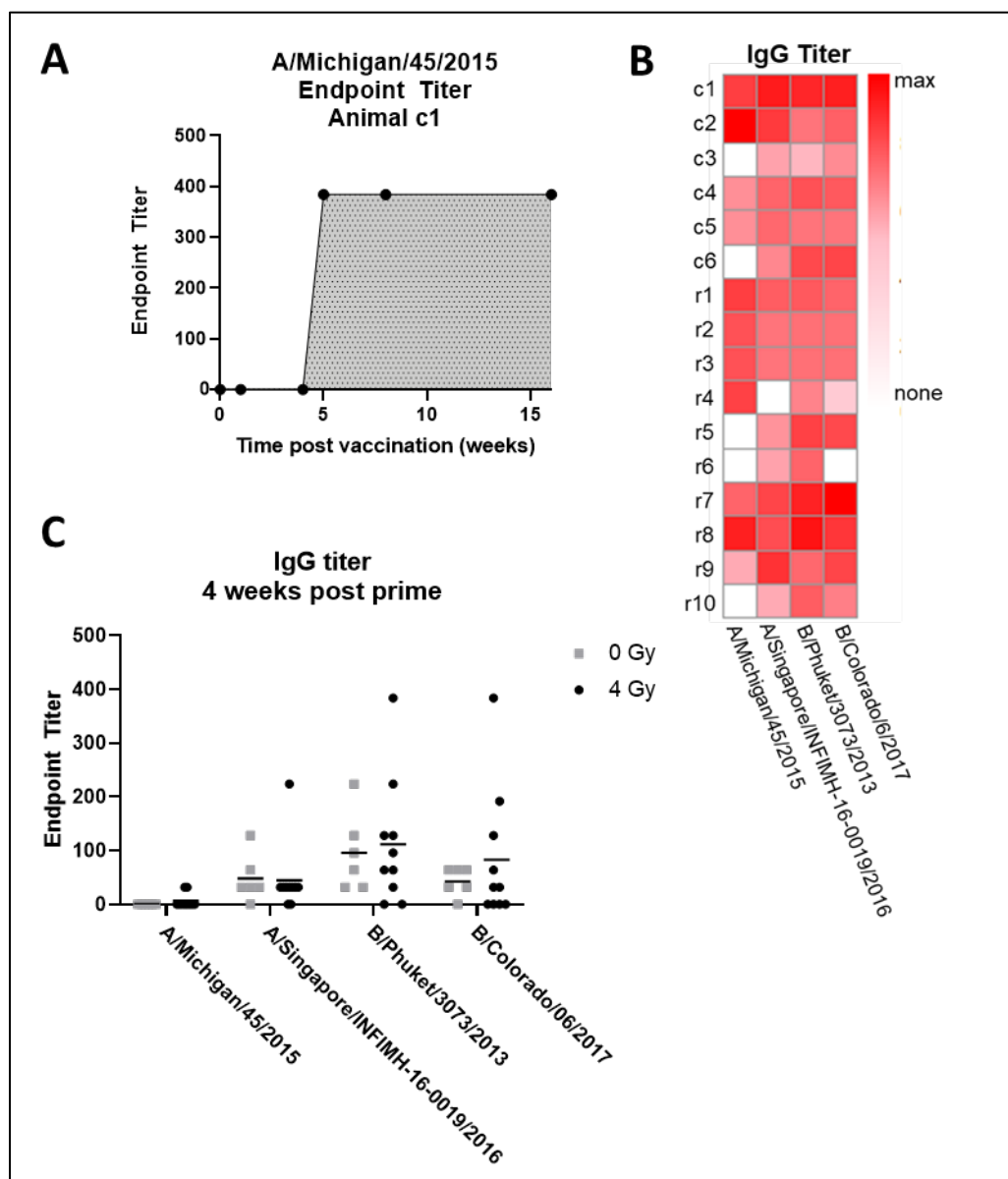


Figure 3.3. IgG responses to Seasonal Influenza Vaccination

Baseline-normalized HA-binding of IgG for each animal and HA was determined by ELISA, plotted as shown in (A) and the Area Under the Curves (AUC - shaded) calculated. (B) Heatmap shows the log-scaled AUC ($\log(\text{AUC}+1)$) for the above-baseline response to each HA over time for all animals and HAs. White indicates AUC=0, i.e. no response above baseline. (C) plot shows post-immunization, pre-booster titers for each animal.

Sera from the vaccination study were then tested for their ability to neutralize the four influenza virus strains represented in the tetravalent vaccine. Neutralizing titers for each virus were plotted against time and AUC calculated as shown in **Figure 3.4A**. The AUCs for all four viruses and 16 animals are summarized in **Figure 3.4B**. Baseline normalization was not required as there were no detectable neutralizing responses prior to the boost.

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. Influenza B does not naturally infect NHPs. The vaccine doses were therefore likely these animals first exposure to type B HA, and so the poor neutralization titers were unsurprising. In contrast, influenza A is zoonotic and the animals in the study were likely to have experienced similar viruses previously. This was borne out by the neutralizing responses to A/Michigan/45/2015 and A/Singapore/INFIMH-16-0019/2016. There were no significant differences in neutralizing responses to influenza A between the irradiated and sham-irradiated animals (**Figure 3.4B**).

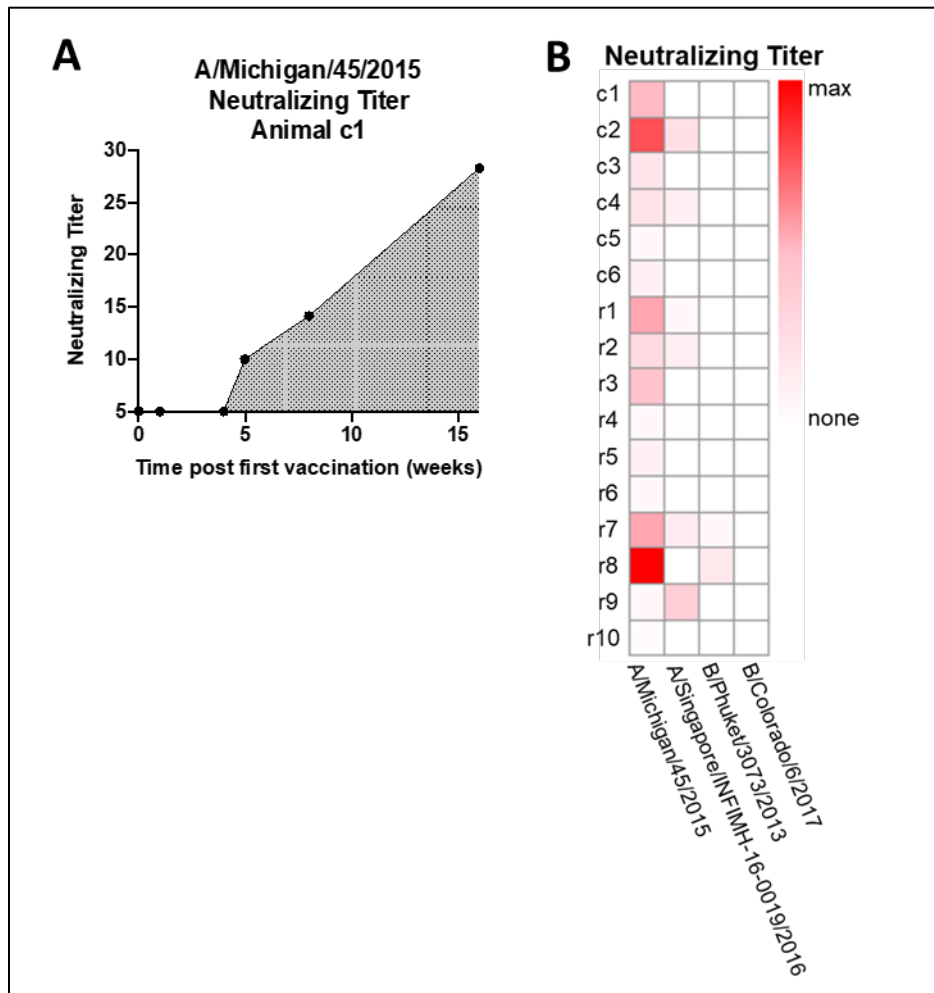


Figure 3.4. Neutralizing antibody responses to Seasonal Influenza Vaccination

Neutralization titers for each influenza strain and animal were plotted as shown in (A) and the Area Under the Curves (AUC - shaded) were calculated. (B) Heatmap shows the AUC for the

neutralization response over time for all animals and influenza strains. White indicates AUC=0, i.e. no response at any timepoint.

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 **Figure 3.5A** and the AUCs for all serotypes and animals are shown in **Figure 3.5B**. Some serotypes, e.g. 3, 2, 10A and 9B, elicited similar IgG responses in control and irradiated 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 studies described in Years 2-4 showing that DC, B and T cell populations returned to their expected setpoints, irradiated animals mounted significantly weaker humoral responses to select bacterial polysaccharide antigens over two years after radiation exposure.

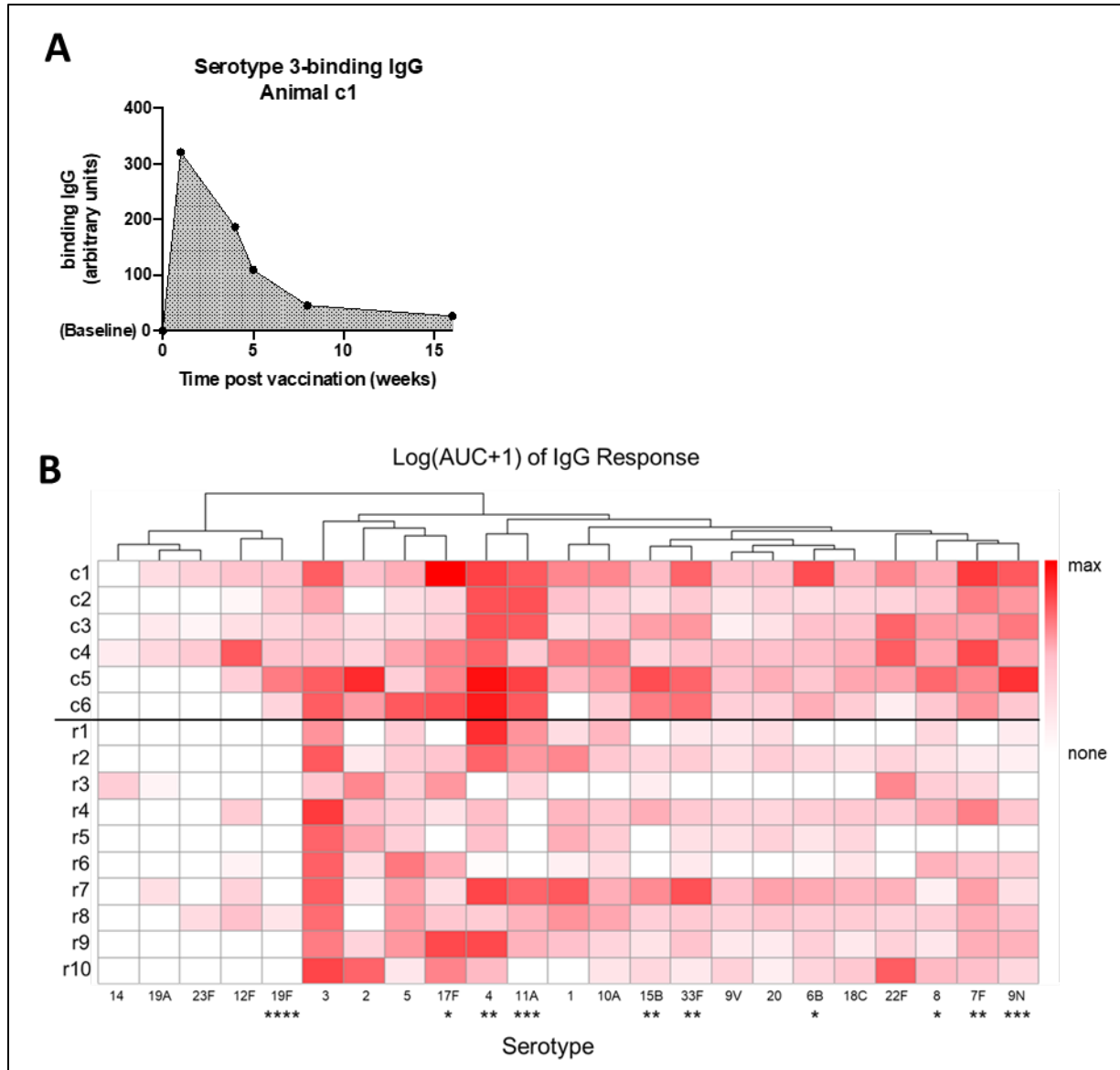


Figure 3.5. IgG Responses to 23-Valent Polysaccharide Vaccine

Pneumococcus serotype-specific IgG antibodies were assessed by multiplex bead array, data were plotted as shown in (A) and Area Under the Curves (AUC - shaded) were calculated. (B) Heatmap shows the normalized, log-scaled AUC ($\log(\text{AUC}+1)$) for the above-baseline response to each serotype over time. White indicates AUC=0, i.e. no response above baseline. Columns are arranged by hierarchical clustering and rows are not clustered. Significant differences in serotype response between the two groups according to two-tailed *t* tests are indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

SA1 Major Task 2: Murine Studies (Chen, Duke)

Milestones 2.2 and 2.3: Determine the roles of thymopoiesis and peripheral expansion in overall T cell recovery

Single cell RNA-sequencing reveals distinct T cell populations following exposure to total body irradiation

The total body irradiation (TBI) model was established by transferring lineage-negative bone marrow cells from syngeneic CD45.1⁺ donors into lethally irradiated (10.5 Gy) wild type C57BL/6 recipients (CD45.2⁺). Splenocytes from recipient-derived (CD45.2⁺) T cells were collected for control (n = 3), 5 days (n = 6), and 21 days (n = 5) groups. The distribution of various lymphocytes prior to purification is shown in Figure 3.7. 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.

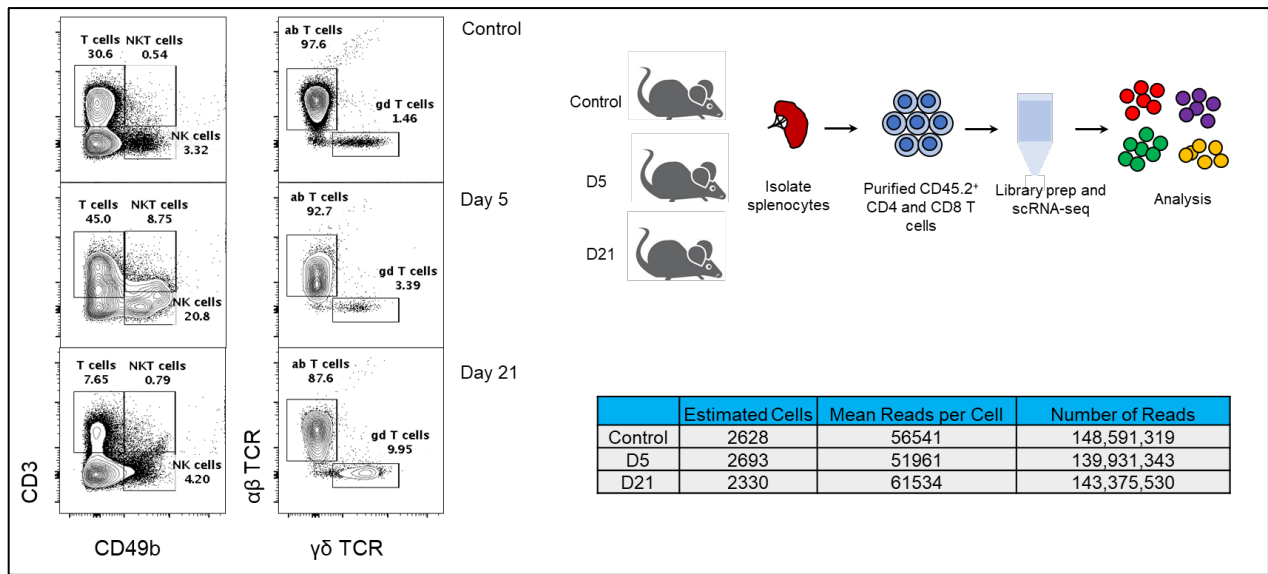


Figure 3.7. Experimental flowchart for single-cell RNA sequencing of splenic T cells from control and irradiated mice. Distribution of distinct lymphocyte cell types prior to T cell purification is shown in the flow cytometry analysis. Splenic T cells were pooled from each group (control, n = 3; day 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.

To characterize the effect of TBI on changes in T cell subsets and gene expression, splenic CD4 and CD8 T cells were enriched 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 depths were greater 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 (2628 cells, control; 2693 cells, day 5; 2330 cells, day 21) (Figure 3.7), including dimensionality reduction using Uniform Manifold Approximation and Projection (UMAP), differential gene expression analysis, and clustering.

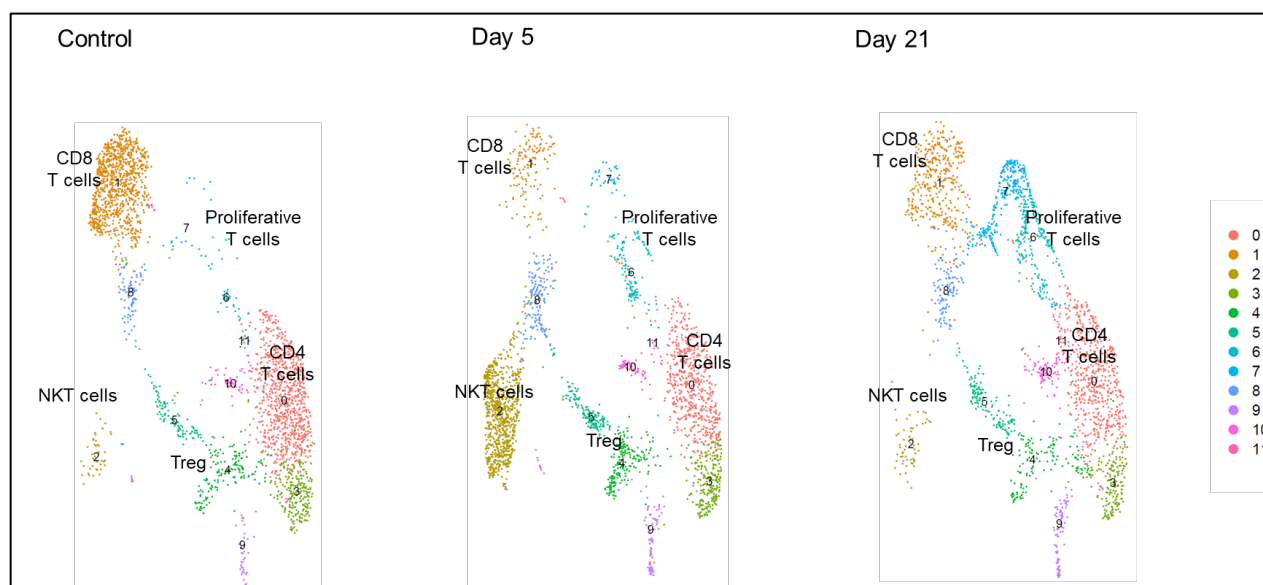


Figure 3.8. Single-cell sequencing of splenic T cells reveals eleven clusters in control, day 5, and day 21 groups. Cells are represented by individual points, assigned to annotated cell types within each cluster.

Previously, we observed eleven clusters based on gene expression signatures, including CD4 and CD8 T cells, two adjacent clusters of proliferative T cell populations, regulatory T cells (T_{reg}), and NKT cells (**Figure 3.8**). In the subsequent analyses, both T_{reg} and NKT cells were removed from the dataset. We performed a second unsupervised clustering analysis using the remaining cells in attempt to further resolve the populations, yielding six distinct clusters (clusters 0, 1, 2, 3, 4, and 6) (**Figure 3.9**). Heatmap of top ten upregulated genes associated with each population showed distinct transcriptional profiles (**Figure 3.10**). To characterize the identity of each cluster, two annotation methods were used. SingleR, an automatic annotation method was first used to label clusters based on the reference cell types using the murine Immunological Genome Project (ImmGen) database.

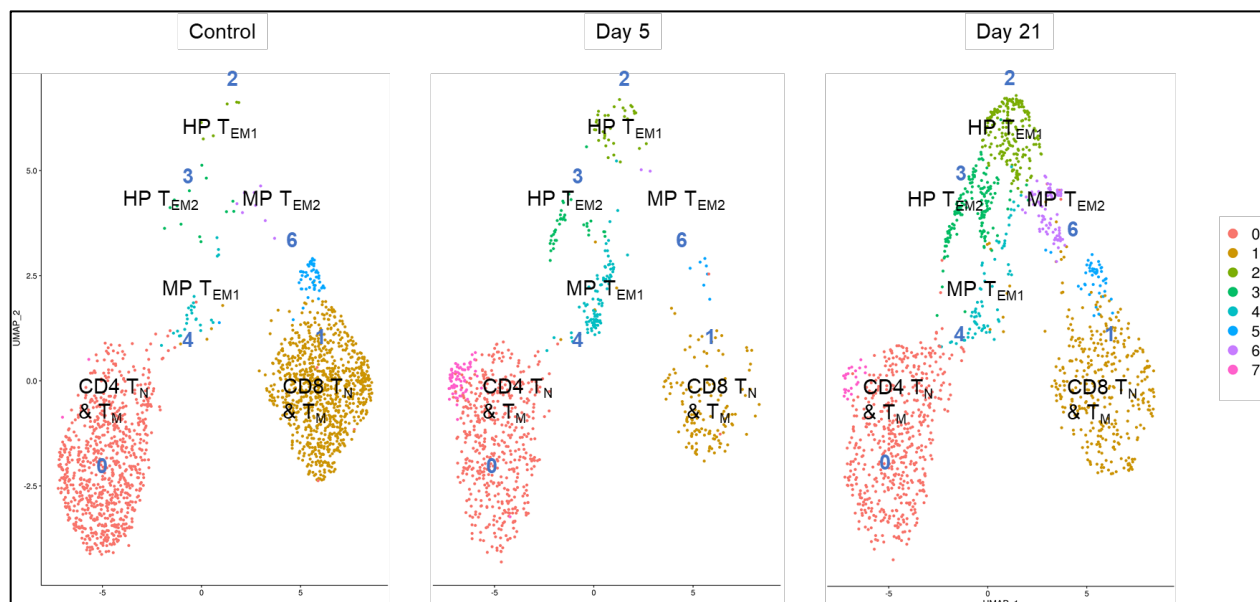


Figure 3.9. Finer clustering analysis reveals six main populations in control, day 5, and day 21 groups. NKT cells and regulatory T cells were excluded from the analyses. Cells are represented by individual points, assigned to annotated cell types within each cluster.

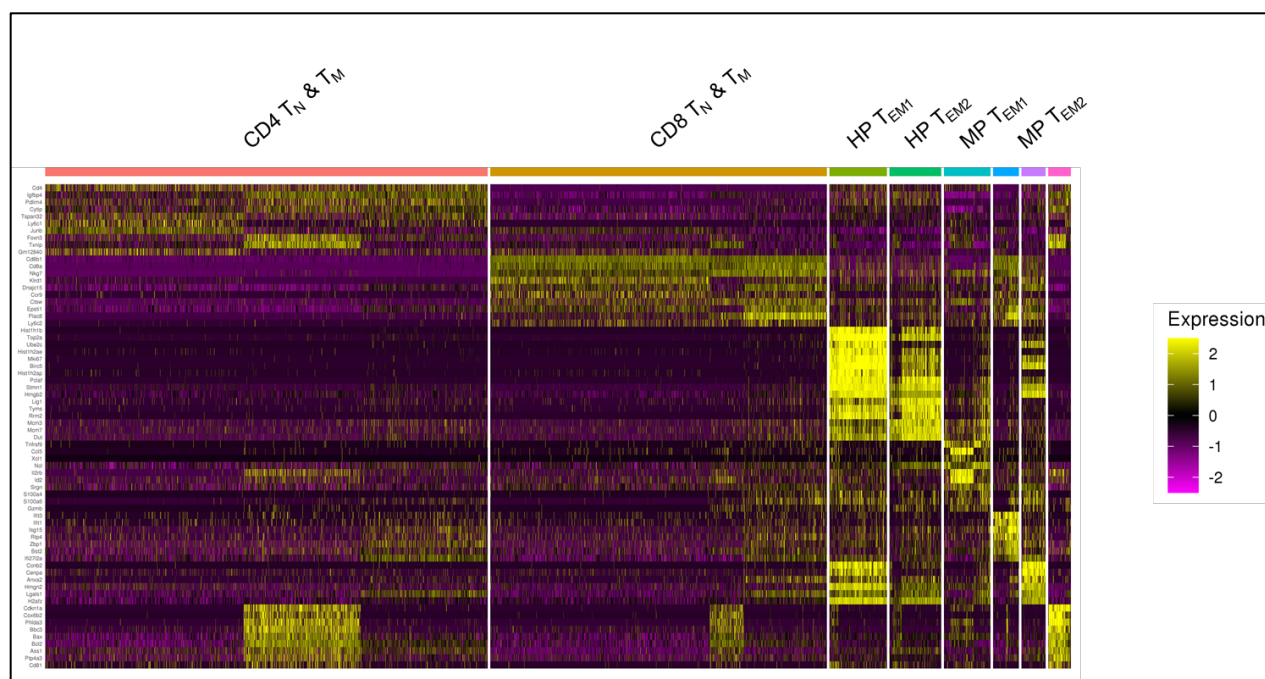


Figure 3.10. Single-cell RNA sequencing reveals distinct gene expression profile in each T cell subset. Heatmap displaying the top ten enriched genes for each T cell subset based on unsupervised clustering analysis. The scale bar indicates expression level based on z-score distribution.

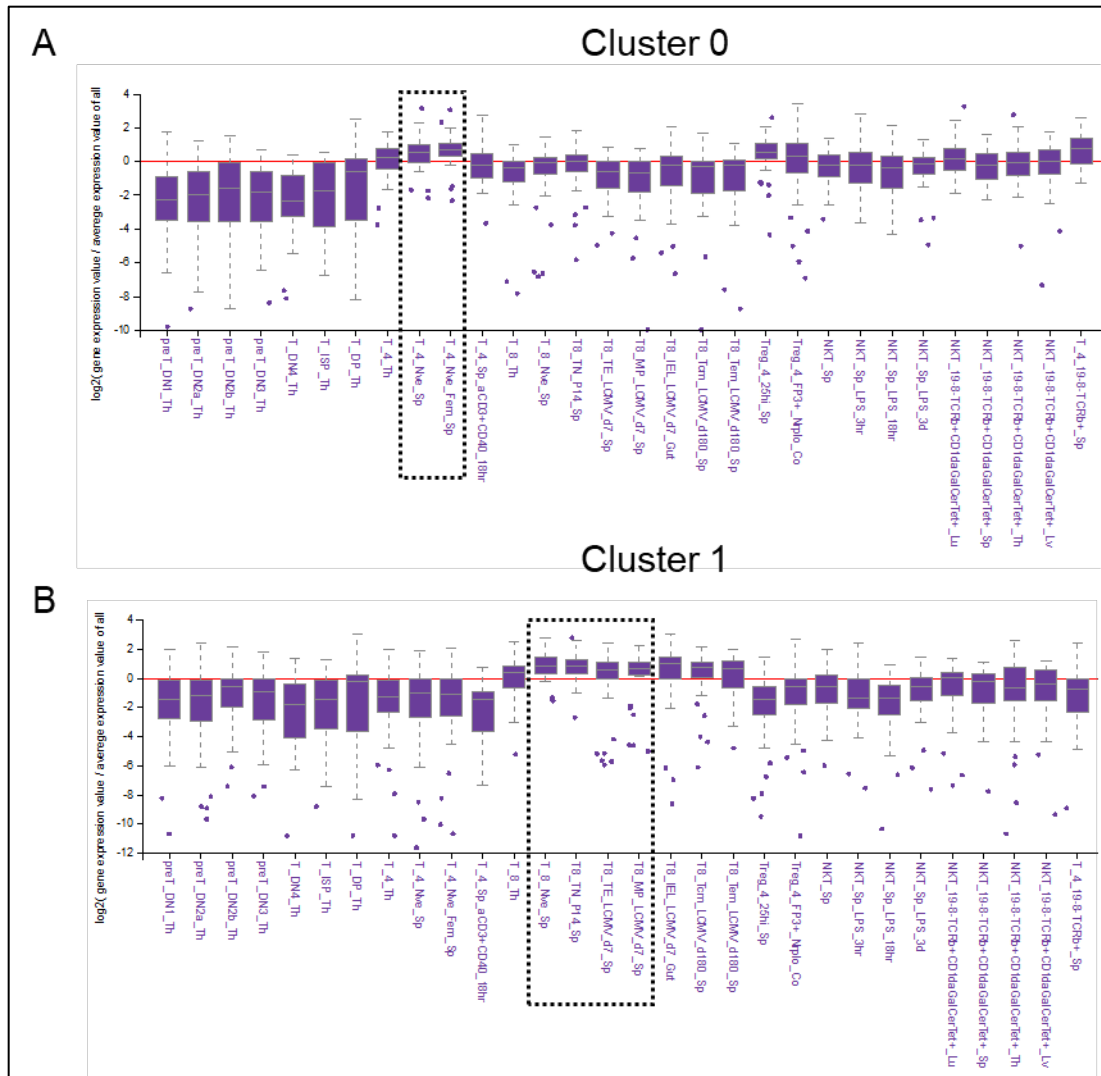


Figure 3.11. Annotation of clusters 0 and 1 indicates the presence of primarily naïve T cells and some memory T cells. Clusters were annotated by SingleR based on the ImmGen reference dataset of murine immune cells.

Clusters 0 and 1 strongly corresponded to CD4 and CD8 naïve T cells (T_N), respectively (Figure 3.11). 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, clusters 2, 3, 4, and 6 corresponded primarily to T_M or T_{EFF} (Figure 3.12). In addition, all four clusters matched strongly with proliferative thymocytes, including DN2b, DN4, and immature single positive thymocytes. The proliferative phenotype of clusters 2, 3, 4, and 6 was further validated by the enrichment of proliferation markers, including Mki67, Top2a, E2f1, and Cdc6 etc. (Figure 3.13). The presence of these proliferative clusters suggests that the two previously identified proliferative populations were further resolved into four distinct clusters. To precisely label each cluster, a second annotation method was applied using representative genes from known T cell subsets, including T_N , T_M , and T_{EFF} (Figure 3.14). 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.

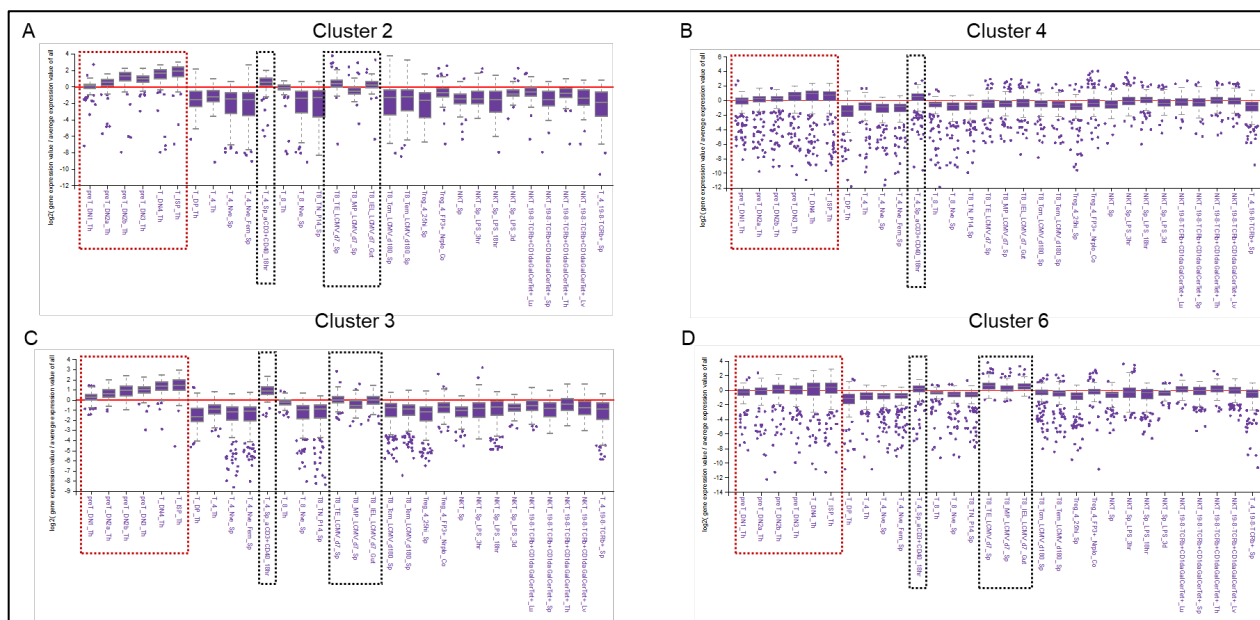


Figure 3.12. Clusters 2, 3, 4, and 6 exhibit a distinct proliferative effector memory phenotype. Clusters were annotated by SingleR based on the ImmGen reference dataset of murine immune cells. Black dashed boxes highlight matches for effector and memory T cells. Red dashed boxes highlight matches for proliferative thymocytes.

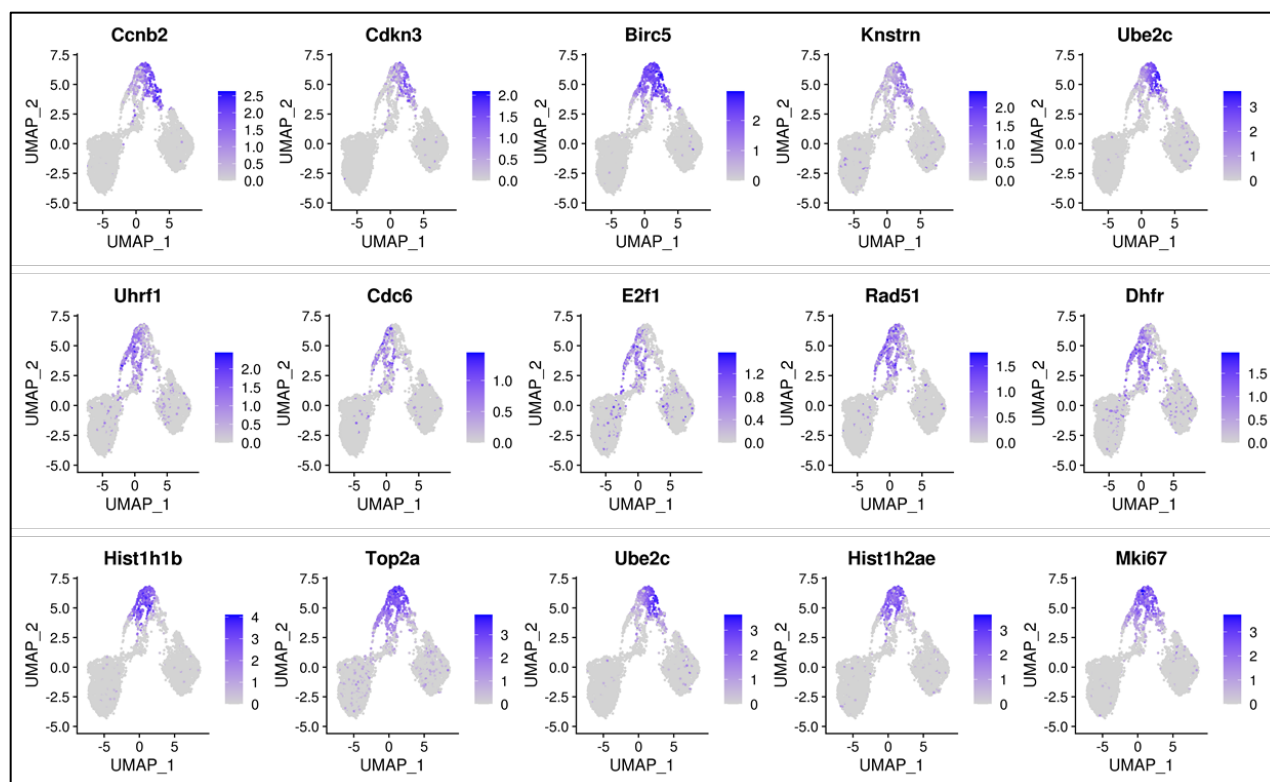


Figure 3.13. Clusters 2, 3, 4, and 6 are enriched for genes associated with cell growth and proliferation. The expression of top enriched genes in clusters 2, 3, 4, and 6 are shown across all clusters.

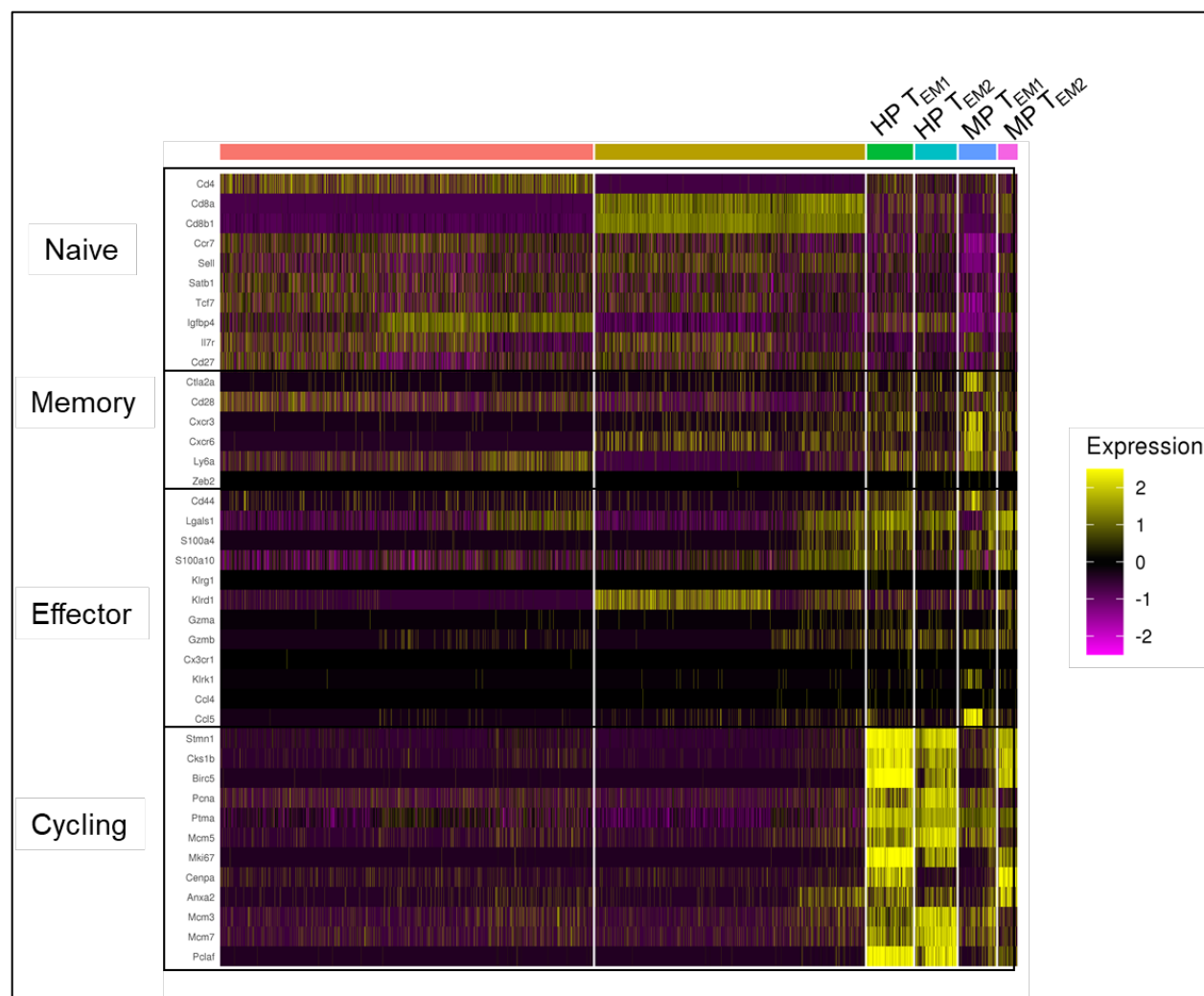


Figure 3.14. Annotation of various clusters using markers associated with known T cell subsets. Expression heatmap showing the relative expression of naïve, memory, effector, and cycling genes. The scale bar indicates expression level based on z-score distribution.

Trajectory inference indicates progressively differentiated phenotypes following TBI exposure: Following irradiation, self-peptide/major histocompatibility (MHC) ligands and cytokines induce T cell expansion in the lymphopenic environment. However, the origin of expanded T cell populations remains unclear. To resolve the relationships of proliferative T_{EM} populations and the remaining clusters, unsupervised trajectory analysis for CD4 and CD8 T cells was performed to determine the differentiation lineage, respectively. Following dimensionality reduction, cells were ordered along a pseudotime axis based on the progression of their transcription profile. Cells distributed at the root of pseudotime correspond to relatively immature phenotypes while those distributed at the end correspond to more differentiated phenotypes (Figure 3.15A). The CD4 trajectory analysis revealed 5 differentiation states, with state 1 being the least differentiated, followed by two branches for states 2 and 3, followed by two final branches, states 4 and 5 (Figure 3.15B). To determine the distribution of various T cell subsets, annotated clusters were identified on the trajectory. T_N and T_M are distributed at the root as well as the intermediate section of the trajectory (Figure 3.16). MP T_{EM1} and T_{EM2} are primarily distributed in the latter intermediate section, followed by HP T_{EM1}, then HP T_{EM2} near the end of the trajectory.

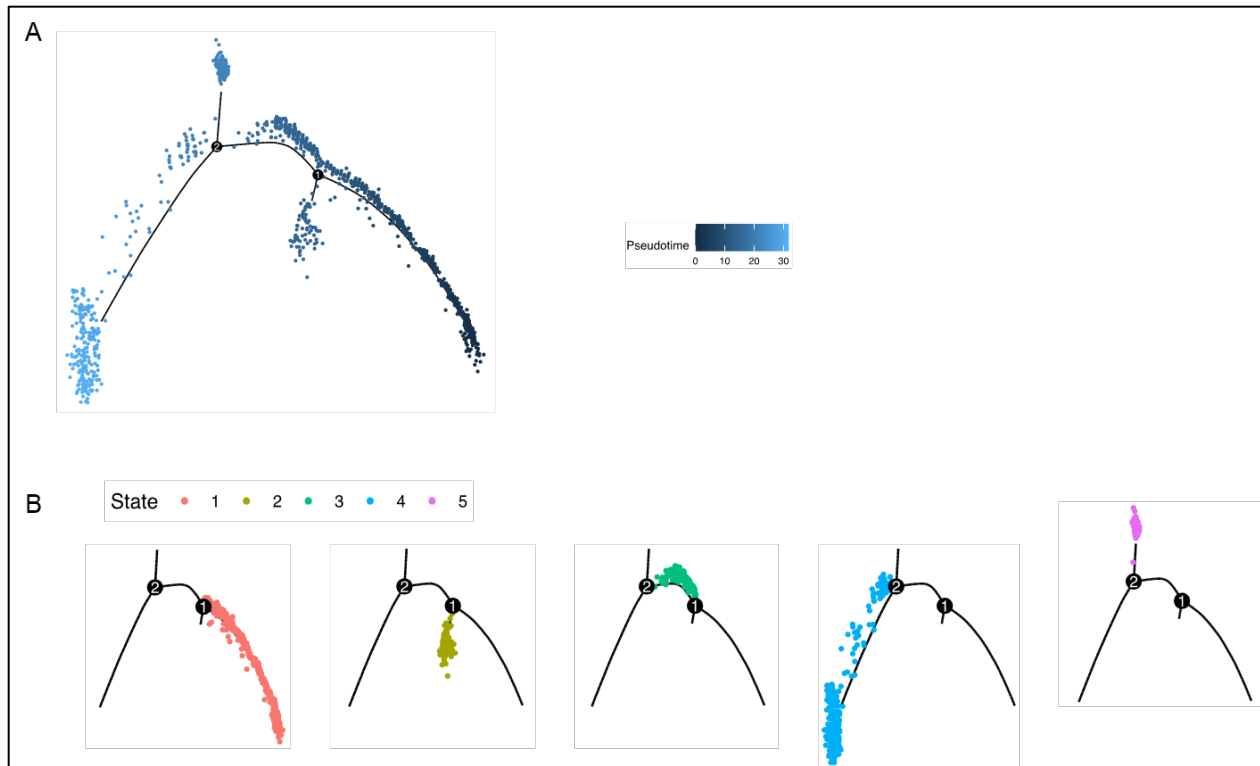


Figure 3.15. Pseudotime analysis of CD4 T cells from control and day 5, day 21 irradiated groups. (A) Monocle pseudotime trajectory of T cells from all three groups. Cells are labelled along pseudotime progression. (B) Trajectory analysis showing five states cells ordered along pseudotime.

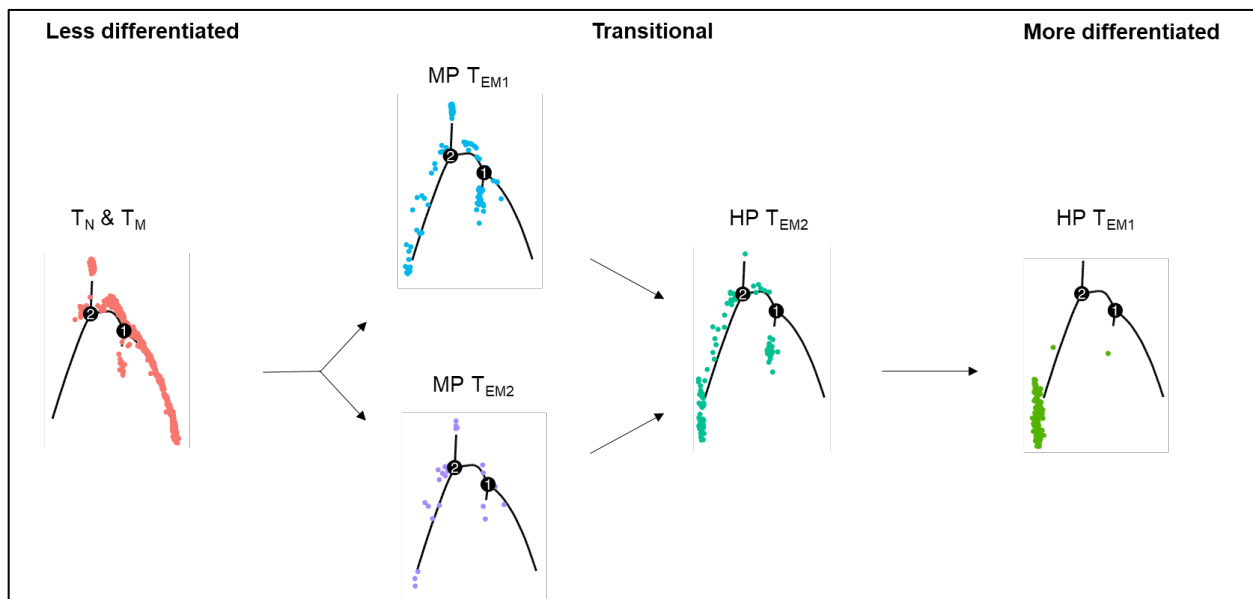


Figure 3.16. Pseudotime analysis reveals sequential progression of distinct CD4 T cell subsets along the trajectory. Distribution of annotated T cell subsets were identified on the differentiation trajectory, ranging from less differentiated, transition, and more differentiated phenotypes.

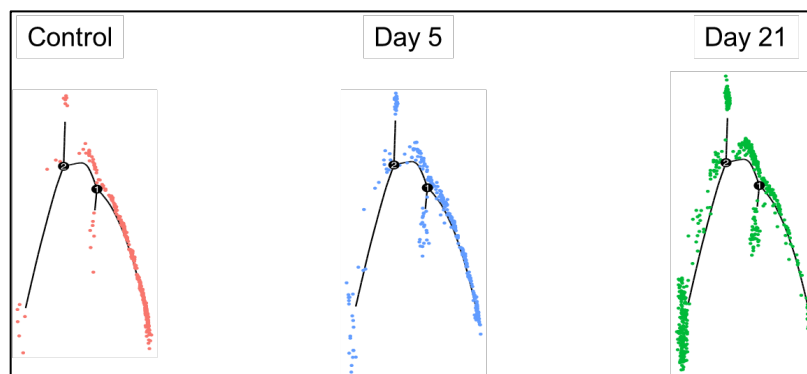


Figure 3.17. Distribution of CD4 T cells from control, day 5 and day 21 after irradiation on the pseudotime trajectory.

Moreover, the analysis showed a sequential progression in trajectory before and after irradiation, with control cells located near the root, followed by day 5 and day 21 (**Figure 3.17**). Notably, the more differentiated cell types, HP T_{EM1} and HP T_{EM2}, were almost exclusively found on day 21. CD8 trajectory analysis revealed three states along pseudotime and similar distribution of T_N, MP and HP T_{EM1} and T_{EM2} subsets on the trajectory (**Figures 3.18 and 3.19**). Control groups also revealed a higher number of less differentiated cells while intermediate and more terminally differentiated cells were found in day 5 and day 21 groups (**Figure 3.20**). The sequential progression of T cell subsets along the trajectory during the course of T cell recovery suggests that both T_N and T_M are potential sources of proliferative T_{EM} subsets during homeostatic expansion, giving rise to intermediate MP T_{EM} and subsequently more differentiated HP T_{EM}.

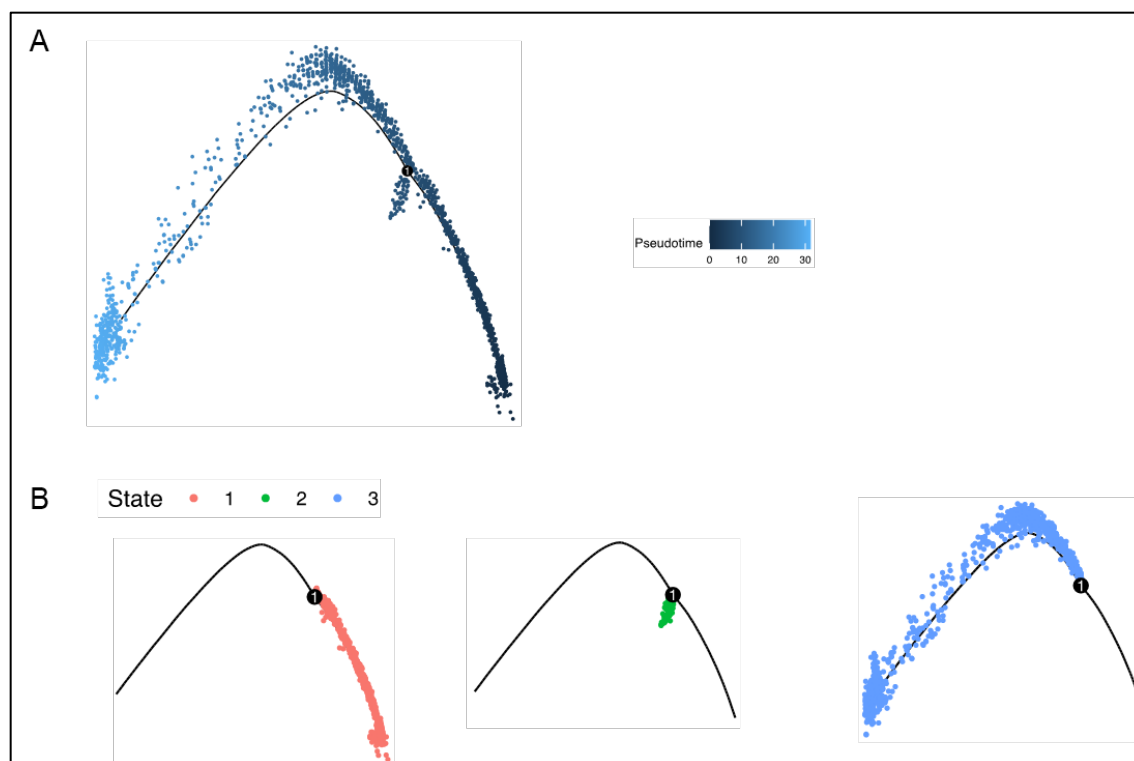


Figure 3.18. Pseudotime analysis of CD8 T cells from control and day 5, day 21 irradiated groups. (A) Monocle pseudotime trajectory of T cells from all three groups. Cells are labelled along pseudotime progression. (B) Trajectory analysis showing three states cells ordered along pseudotime.

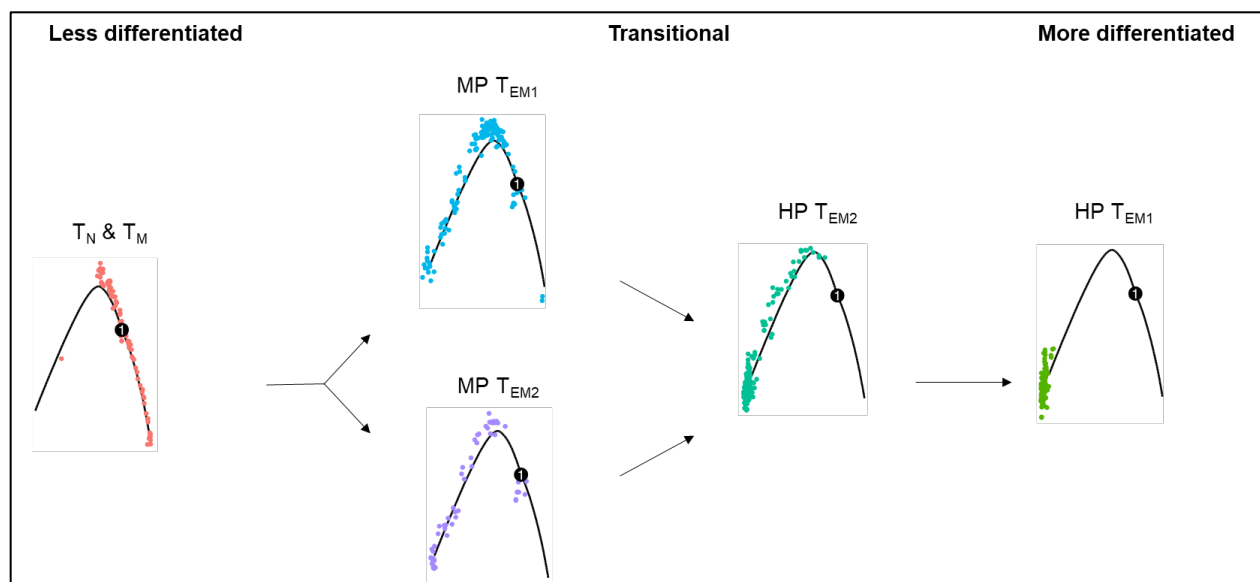


Figure 3.19. Pseudotime analysis reveals sequential progression of distinct CD8 T cell subsets along the trajectory. Distribution of annotated T cell subsets were identified on the differentiation trajectory, ranging from less differentiated, transition, and more differentiated phenotypes.

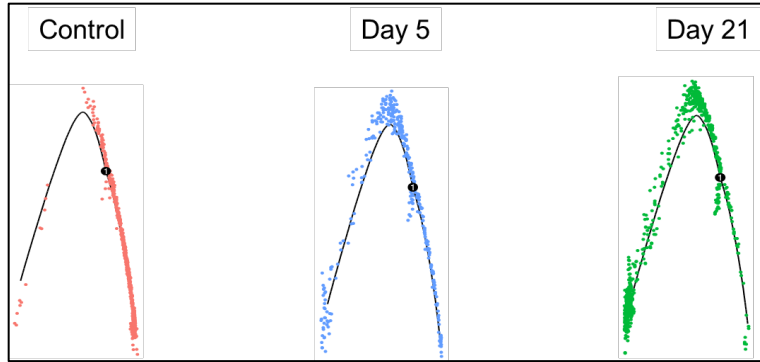


Figure 3.20. Distribution of CD8 T cells from control, day 5 and day 21 after irradiation on the pseudotime trajectory.

Irradiated T cells upregulate mitochondrial activity and oxidative phosphorylation:

The molecular mechanisms of T cell persistence and homeostatic proliferation following irradiation are largely unknown. We therefore performed pathway enrichment analyses to determine pathways upregulated during T cell recovery. Analyses were performed for CD4 and CD8 T cells, respectively, to distinguish subset-specific differences. Early after TBI on day 5, both CD4 and CD8 T cells exhibited upregulation in both intrinsic apoptosis and lymphocyte proliferation pathways (**Figure 3.21**).

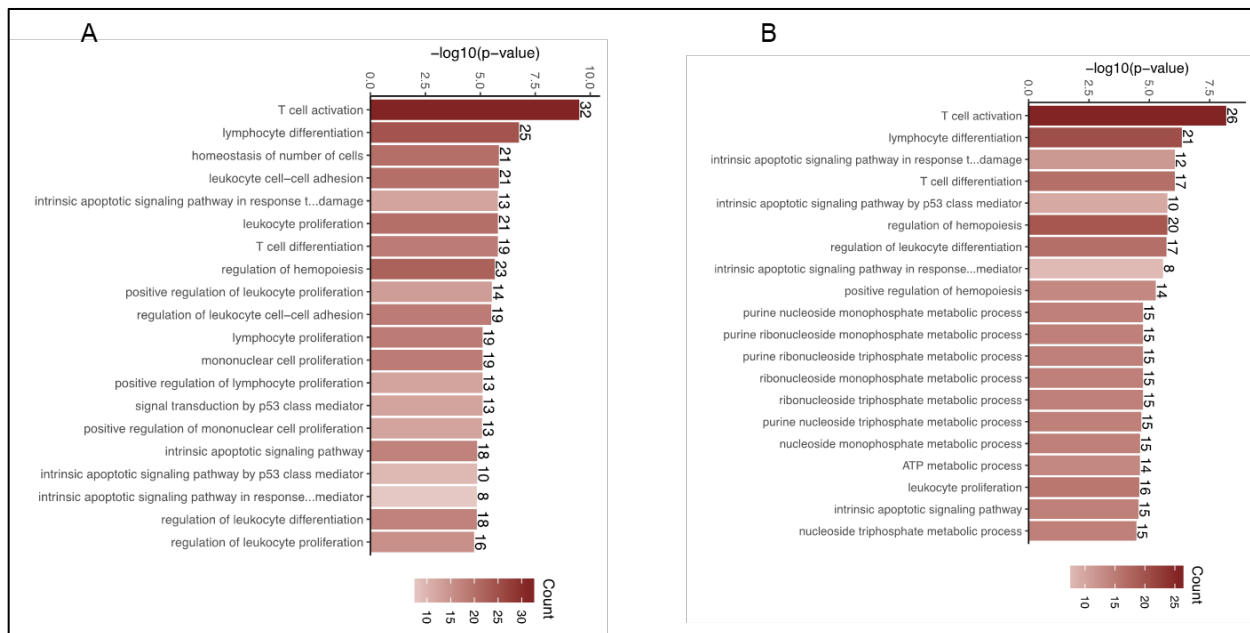


Figure 3.21. T cells are enriched for pathways associated with lymphocyte proliferation and intrinsic apoptosis on day 5. Biological processes enriched in day 5 CD4 T cells (A) and CD8 T cells (B) relative to control T cells. Pathways were ranked by their significance.

In addition, CD8 T cells showed increased ribonucleoside metabolism, which promotes cell growth and proliferation. Day 21 following TBI, pathways associated with mitochondrial metabolism were highly enriched in both CD4 and CD8 T cells, indicating increased dependence on oxidative phosphorylation (OXPHOS) compared to nonirradiated T cells (**Figure 3.22**). Moreover, violin plots for specific genes involved in OXPHOS, Atp5b and Atp5j, showed

the highest expression on day 21, followed by day 5, compared to control (**Figure 3.23**). Notably, both irradiated T_N and T_M , as well as proliferative T_{EM} subsets were progressively enriched for mitochondrial components over time following irradiation. Dependence on mitochondrial metabolism has been shown to be beneficial for anti-tumor T_M persistence, as opposed to T_{EFF} , which are relatively short-lived and rely primarily on glycolysis. It is possible that T cells rely on OXPHOS rather than aerobic glycolysis to promote survival and homeostatic proliferation following TBI.

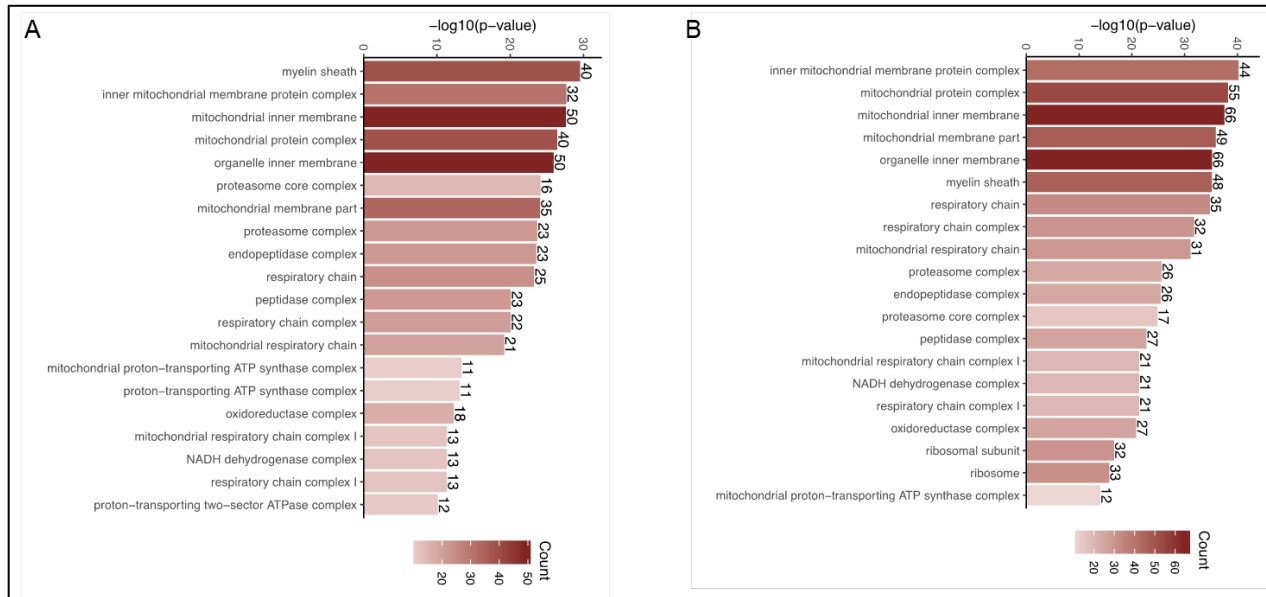


Figure 3.22. T cells are enriched for pathways associated with mitochondrial activity and oxidative phosphorylation on day 21. Cellular components enriched in day 21 CD4 T cells (A) and CD8 T cells (B) relative to control T cells. Pathways were ranked by their significance.

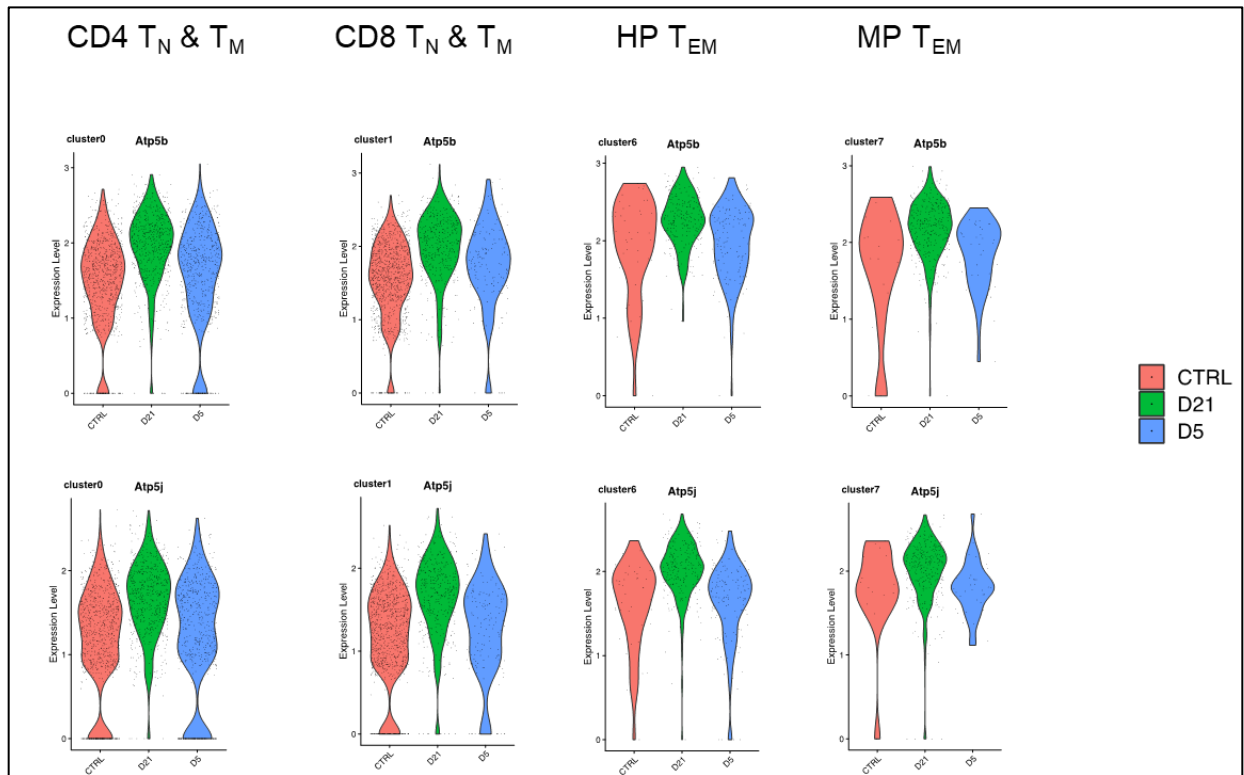


Figure 3.23. Genes associated with oxidative phosphorylation are highly upregulated in irradiated T cells. Violin plots of *Atp5b* and *Atp5j* expression in T_N & T_M, and proliferative T_{EM} subsets in control, day 5, and day 21 irradiated groups.

4) Other Achievements

Nothing to report

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)

In the past year, we finalized our methodologies for DNA whole exome sequencing to define the genetic mutations in rhesus macaques. In the past year, we 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, PMCID [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, PMCID: [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, PMCID: [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.

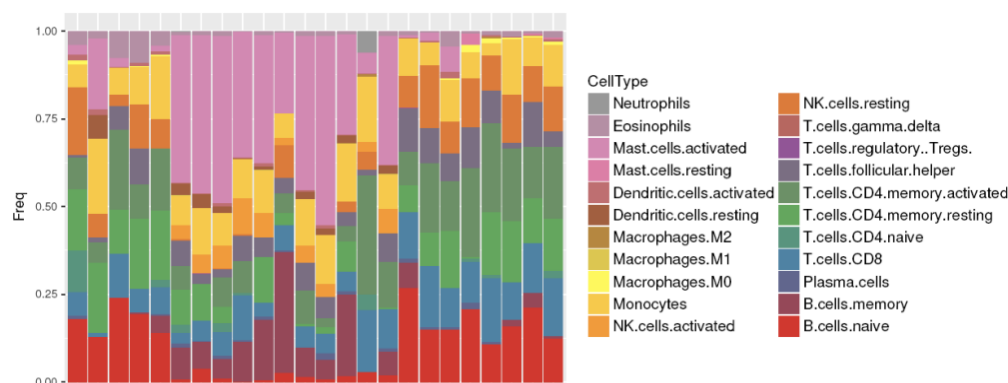


Figure 4.1: Deconvolution analysis of immune populations after exposure to radiation.

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.

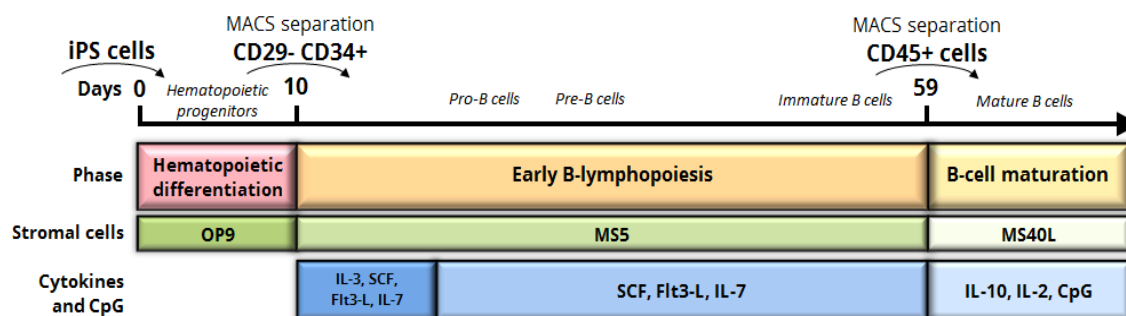


Figure 4.2: Schema for generating B lymphoid cells.

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.

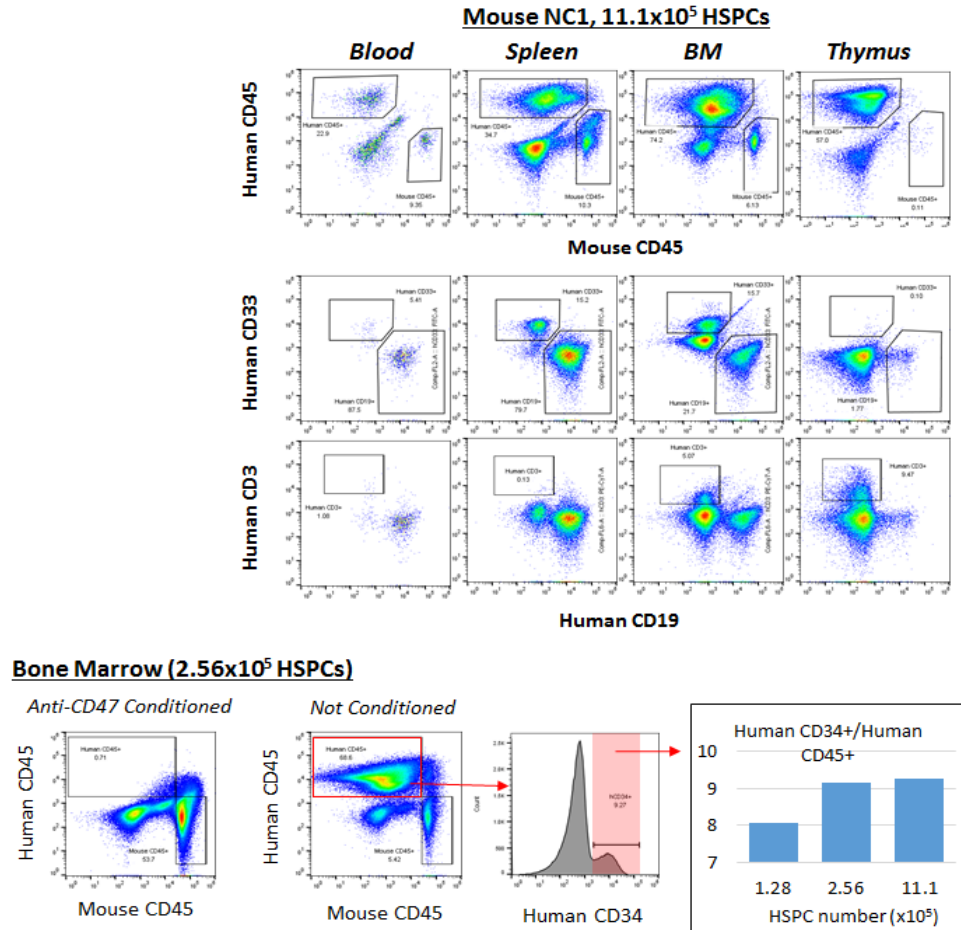


Figure 4.3: Presence of B cells (CD19+), T cells (CD3+), and myeloid cells (CD33+) in blood, spleen, bone marrow, and thymus at 10 weeks post transplantation.

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.

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 Il2ry^{-/-} 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).

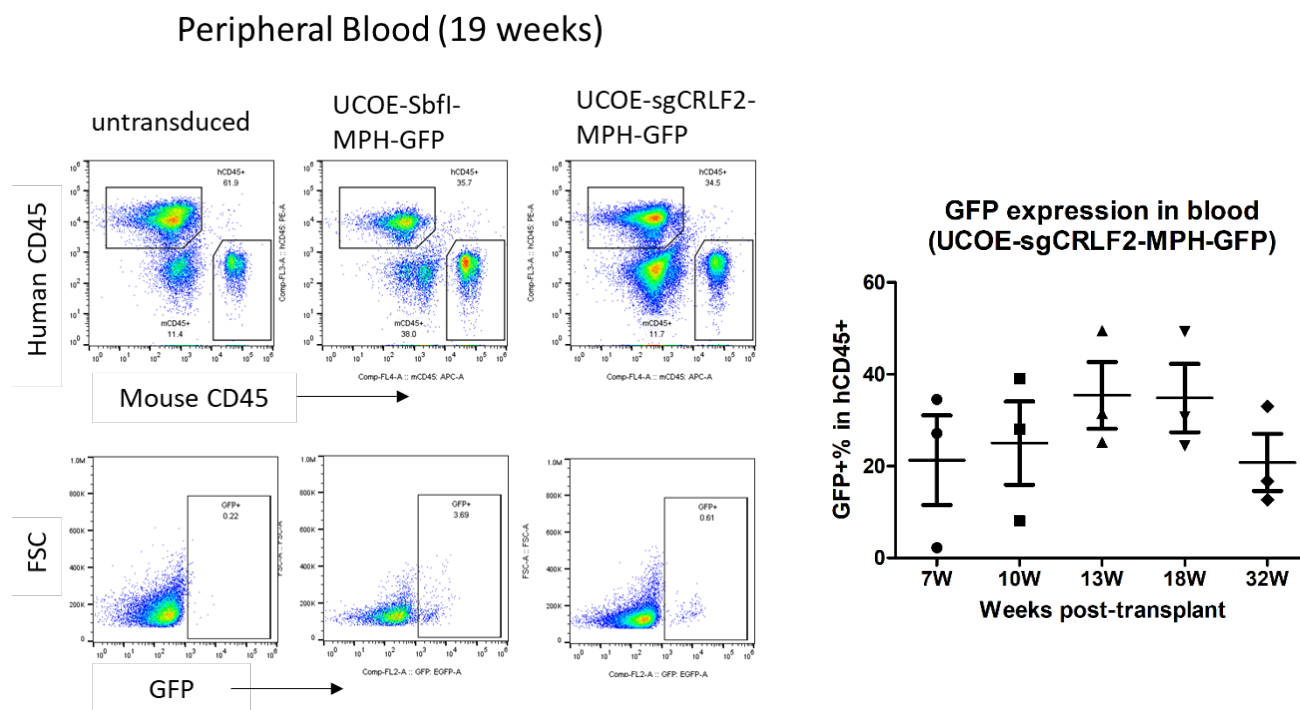


Figure 4.4: Engraftment of B cells occurs successfully in both untransduced and transduced cells, but only the UCOE containing elements have significant numbers of GFP+ cells. Right panel shows long term follow up of GFP expression is maintained in the presences of these UCOE elements.

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.

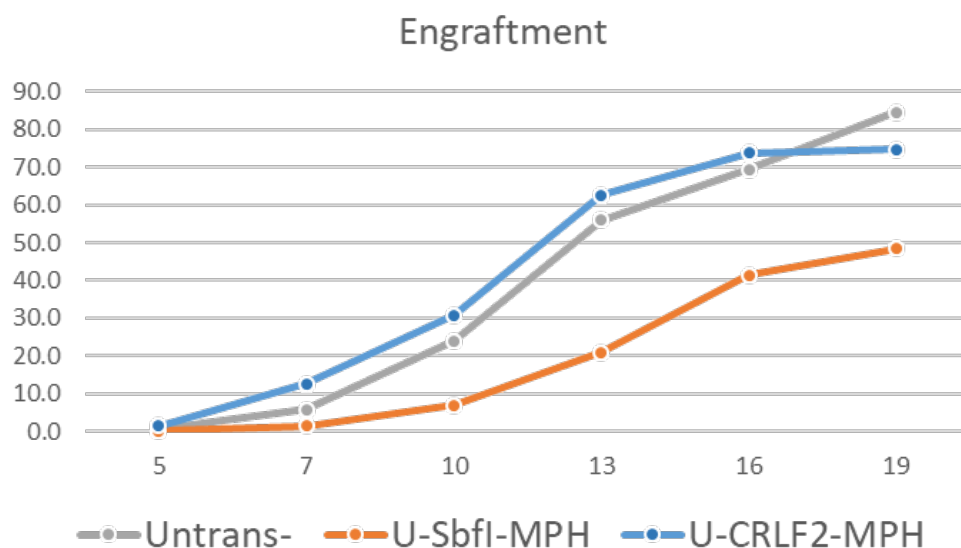


Figure 4.5: Engraftment of B cells occurs successfully in both untransduced and transduced cells and human cells are detectable under all three conditions by 10 days.

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

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 on Oct 16, 2019 and in June 2020.
2. Investigators (Drs Cline, Dugan, and Register) and Fellows (Drs. Andrews, Michalson and Thakur) attended the 2019 Radiation Research Society meeting, which provided education and professional development.

Project 1 Diabetes (PI Kavanagh).

1. Trainees: We have three trainees continuing to work 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. Yujing Zou is a PhD student who is mentored by Dr. Chen. She was involved in many aspects of this study.

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 (San Diego, CA, Nov 3-7, 2019)
- The NIH/NIAID Centers for Medical Countermeasures Against Radiation (Annual meeting Dec 3-4, 2019, and reports during monthly teleconferences with the CMCR Steering Committee)
- 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

What do you plan to do during the next reporting period to accomplish the goals?

Overall (PI Cline):

No Change from Statement of Work.

For all projects we anticipate continued local and national presentations of results at relevant scientific meetings, and publications of results in the peer-reviewed literature.

Project 1 (Diabetes, PI Kavanagh):

No Change from Statement of Work.

Project 2 (RIHD, PI Register):

No Change from Statement of Work.

During the next quarter, we will be

- 1) Finishing analysis of the post-radiation Cardiac MRI and echocardiographic imaging in the PRC
- 2) Continuing RNAseq and miRNAseq of myocardium from deceased RSC and PRC subjects
- 3) Additional immunohistochemical analysis of individual cell types and macrophage polarization in myocardium from deceased RSC and Control rhesus monkeys
- 4) Continuing statistical analyses of the effects of radiation exposure on echocardiographic outcomes in the RSC
- 5) Continuing image analysis of cardiac MR images in the PRC
- 6) Statistical analysis of data
- 7) Analysis of ECM and other biomarkers
- 8) Ongoing molecular and cellular phenotyping of the monocytes in the RSC
- 9) Development and submission of manuscripts containing comprehensive echocardiographic phenotyping of the PRC describing the effects of radiation on the heart
- 10) Processing of PRC myocardial and other samples for detailed molecular and cellular characterization
- 11) Statistical analyses of biomarker, functional, structural, and molecular datasets.

Project 3 (Immune Recovery, PI Sempowski/Chen):

We will work with WFU to commit all data to the shared CARS database, model the results of our survivor-cohort studies and publish a manuscript based on these data. Continue to analyze the single cell RNAseq data. Determine the role of candidate genes in T cell recovery using in vitro and in vivo models. We will test the effect of IL-7 as proposed in the alternative approach of the initial proposal. Presentations, abstracts, meetings and manuscripts as studies mature.

Project 4 (Genomics, PI Dave):

Studies are complete. The genomic data will be uploaded to the program's shared database and utilized throughout the other projects to inform interpretation of experimental studies.

- 4. IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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, PI Kavanagh):

The work described in this report indicates that radiation exposure produces systemic dysregulation of metabolic functions, leading to altered protein signaling pathways, impaired tissue blood flow and deranged metabolic activity, including overt diabetes mellitus.

Project 2 (Radiation Induced Heart Disease, PI 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.

Project 3 (Immune Recovery, PI Sempowski/Chen):

The results from the single cell RNA sequence study reveal 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, PI Dave):

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

What was the impact on other disciplines?

Overall

The impact of our findings is broad, and spans multiple groups and agencies working to understand and mitigate delayed effects of acute radiation exposure, including the DOD, BARDA, NIH, FDA, and NASA. We anticipate that our data will influence response strategies, biomarker development, and re-adjustment of regulatory assumptions regarding the modeling, prediction and treatment of the long-term consequences described herein.

Project 1 (Diabetes, PI Kavanagh):

The discovery by the Kavanagh lab that radiation-induced insulin resistance and type 2 diabetes mellitus occurs in the absence of the traditional risk factor of obesity has been groundbreaking, and highlights the contribution of oxidative injury, inflammation and extracellular matrix remodeling to the pathogenesis of diabetes.

Project 2 (Radiation Induced Heart Disease, PI Register):

We have been identifying potential new relationships of circulating cytokines with myocardial functional phenotypes which may have broader implications in the scientific community.

Our work with monocyte polarization is the first ever to examine effects of radiation on circulating monocyte polarization (Michalson et al, 2019), receiving the 2020 Editor's Award from Radiation Research. The manuscript describes TBI induced alterations in the proportions of 'classical', 'intermediate', and 'non-classical' monocyte subfractions, and changes in the transcriptional profiles of the intermediate monocyte fraction, which provide supportive evidence that these cells in particular may have pro-fibrotic effects. The work is important in that it provides potential new avenues for intervening on radiation induced fibrosis in the heart and other tissues.

Project 3 (Immune Recovery, PI Sempowski/Chen):

The knowledge learned from the single cell RNA sequence study in radiation injury setting will have impacts in multiple disciplines such as cancer, stem cell transplantation, and immunotherapy. Novel therapeutics developed from radiation studies could potentially be used in many T cell disorder of other causes.

Project 4 (Genomics, PI Dave):

The genomic and transcriptomic datasets produced by this project will enrich public information resources.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

- 5. CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Overall (Cline, PI):

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.

Project 1 (Diabetes, PI Kavanagh):

Nothing to report.

Project 2 (RIHD, PI Register):

Delay in initiating PRC component combined with COVID-19 related delays in the final necropsy and the project in general made finishing the proposed work in Project 2 by the original end date impossible to accomplish. Project 2 is in no cost extension to allow completion of key aims related to the PRC.

Project 3 (Immune Recovery, PI Sempowski/Chen):

Nothing to report.

Project 4 (Genomic, PI Dave):

Nothing to report.

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

Overall:

As noted previously, delays in the acquisition and irradiation of animals at the beginning of the award in 2015 and 2016 have slightly pushed back terminal necropsy studies. These were initially planned for Year 5 Quarter 1 (December 2019), but were pushed back to March 2020, then to June 2020 by the COVID-19 pandemic.

Project 1 (Diabetes, PI Kavanagh):

COVID-19 has led to some delay in data generation, particularly in relation to new liver-outcome data in our treated mice. We are back on track and will complete these in the upcoming months.

Project 2 (Radiation Induced Heart Disease, PI Register)

Nothing to Report.

Project 3 (Immune Recovery, PI Sempowski/Chen)

Plans to complete sjTREC assays by April were delayed due to social distancing measures in response to COVID-19 pandemic. All assays are now complete and we are collating this data with the flow cytometry results for transmission to WFU.

As we reported in the Year 4 report, we do not think HGH and IGF-1 are going to work as initially expected based on the data we have obtained. As an alternative approach detailed in the proposal, we plan to test other new and more potent agents. One of these agents that we are interested in testing is IL-7. IL-7 has been demonstrated to facilitate T cell recovery following hematopoietic cell transplantation. We will obtain prior written approval if significant changes in objectives and scope will need to be made.

Due to the COVID-19 pandemic, the labs were shut down from March to June this year. This has impeded the progress of this project. The lab has been re-opened since June. However, the capacity has not been resumed to the normal level due to social distancing requirements until September. Since then, we have very much recovered back to normal and the progress of this project will be back on track soon.

We will obtain prior written approval if significant changes in objectives and scope will need to be made.

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

Nothing to Report.

Changes that had a significant impact on expenditures

Overall/Primate Core

Nothing to report.

Project 1 (Diabetes, PI Kavanagh)

Nothing to report.

Project 2 (Radiation-Induced Heart Disease, PI Register)

Nothing to report.

Project 3 (Immune Recovery, PI Sempowski/Chen)

We experienced delays in the murine studies due to: a) the two mitigators that we planned to test did not work and we needed to test an alternative mitigator; b) the lab were shut down due to the COVID-19 pandemic from March to June this year. As a result, the fund allocated for murine studies has been spent slower than originally budgeted.

Project 4 (Genomics, PI Dave)

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable - No human subjects

Significant changes in use or care of vertebrate animals

Overview

1. For the Nonhuman Primate Prospective Cohort Protocol PR141508.01, the Wake Forest Protocol number A15-083 was renewed & replaced by Protocol A18-062, without a major change in scope, as part of institutional policy (all Wake Forest protocols are replaced and renumbered at 3 year intervals).
2. For PR141508.2 (Project 2 mice): This protocol was replaced with Wake Forest IACUC protocol number #A18-024 (all Wake Forest protocols are replaced and renumbered at 3 year intervals). The ACURO Assigned Number is unchanged as PR141508.02. The protocol remains identical to that of #A15-068 but the animal number is reduced on account of the work that has already been completed.
3. A new protocol PR141508.05 (Duke IACUC protocol number A010-17-01) was instituted in support of mouse studies in Project 4.
4. A variety of minor protocol amendments were made throughout the year, reflecting personnel changes and minor procedural changes.

Specific details are given below.

Core Primate Studies (Cline)

Protocol 1 of 5 total: [ACURO Assigned Number]: PR141508.04

Title: Radiation Countermeasures Long Term Response of Rhesus Macaques

Target required for statistical significance: 120

Target approved for statistical significance: 132

Submitted to and Approved by:

Wake Forest IACUC original approval 9/17/13 as A13-117

Wake Forest IACUC renewed/reapproved 6/23/16 as A16-094

Wake Forest IACUC renewed/reapproved 6/14/19 as A19-028

Current ACURO approval 11/4/19

Amendments: None

Status

This long-term cohort is under study as planned. No changes in DOD-funded work will be done until pending ACURO approval is obtained.

Protocol 2 of 5 total [ACURO Assigned Number]: PR141508.01

Title: Prospective Radiation Cohort: Cardiac and Metabolic Studies

Target required for statistical significance: 20

Target approved for statistical significance: 28

Submitted To And Approved By:

Wake Forest IACUC (Protocol #A15-083, approved 7/6/15)

ACURO approval (9/3/15, ACURO no. PR141508.01)

Replaced by Protocol #A18-062 as part of routine full renewal every 3 years as per IACUC policy

Amendments: None

Status: Animal work has been completed.

Protocol 3 of 5 total: ACURO Assigned Number PR141508.02

Title: **Prospective Evaluation of Diabetogenesis Post-irradiation in Mice (Metabolic Effects of Radiation Exposure)**

Target required for statistical significance: 200

Target approved for statistical significance: 200

Submitted To And Approved By:

Wake Forest IACUC (A15-068, Approved 5/20/15)

ACURO (PR141508.02, Approved 10/19/15)

Amendments since prior annual report: None

This protocol was replaced with Wake Forest IACUC protocol number #A18-024 (all Wake Forest protocols are replaced and renumbered at 3 year intervals). The ACURO Assigned Number is unchanged as PR141508.02. The protocol remains identical to that of #A15-068 but the animal number is reduced on account of the work that has already been completed.

Amendments: None

Status: Approved studies are in progress.

Protocol 4 of 5 total:

Protocol [ACURO Assigned Number]: PR141508.03

Title: IMMUNOLOGICAL INJURY AND RECOVERY AFTER RADIATION INJURY

Target required for statistical significance: 4184 mice

Target approved for statistical significance: 4184 mice

Submitted to and approved by:

Duke IACUC (A142-15-05, Approved on 5/28/15)

ACURO (PR141508.03, Approved on 3/3/16)

Amendments: None

Status: Approved, work in progress.

Protocol 5 of 5 total:

Protocol [ACURO Assigned Number]: PR141508.05

Title: HUMANIZED MOUSE MODEL OF LYMPHOMA BY ENGRAFTMENT OF CRISPR-CAS9-EDITED HUMAN HEMATOPOIETIC CELLS

Target required for statistical significance: 4184 mice

Target approved for statistical significance: 4184 mice

Submitted to and approved by:

Duke IACUC (A010-17-01, Approved on 1/27/18)

ACURO (PR141508.05, Approved on 8/1/18)

Amendments: None

Status: Approved, work in progress.

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications.

Overall (Cline): See below by project.

Project 1 (Diabetes, PI Kavanagh):

Bacarella N, Ruggiero A, Davis AT, Uberseder B, Davis MA, Bracy DP, Wasserman DH, Cline JM, Sherrill C, Kavanagh K. **Whole Body Irradiation Induces Diabetes and Adipose Insulin Resistance in Nonhuman Primates.** Int J Radiat Oncol Biol Phys. 2020 Mar 15;106(4):878-886. doi: 10.1016/j.ijrobp.2019.11.034. Epub 2019 Dec 2. PMID: 31805366.

Project 2 (Radiation-Induced Heart Disease, PI Register):

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)

Project 3 (Immune Recovery, PI Sempowski/Chen):

Macintyre AN, French MJ, Sanders BR, Riebe KJ, Shterev I, Wiehe KJ, Dugan G, Bourland JD, Cline MJ, Sempowski GD, Long-Term Recovery of the Adaptive Immune System in Rhesus Macaques Following Total Body Irradiation, *Submitted; federal support acknowledged*

Project 4 (Genomics, PI Dave):

Nothing to report.

Books or other non-periodical, one-time publications.

Overall (PI Cline)

Nothing to report.

Project 1 (Diabetes, PI Kavanagh)

Nothing to report.

Project 2 (Radiation Induced Heart Disease, PI Register):

(PS7-25) Myocardial functional, structural, and cellular alterations in Rhesus Macaque survivors of ionizing radiation exposure Kristofer T. Michalson, DVM PhD; Dalane Kitzman, MD; Gregory O. Dugan, DVM; David L. Caudell, DVM PhD; J Mark. Cline, DVM PhD; Thomas C. Register, PhD

Abstract was presented at The 65th Radiation Research Society Annual Meeting 2019
Federal support was acknowledged

Cardiac Functional, Structural, and Cellular Alterations in Rhesus Macaque Survivors of Ionizing Radiation Exposure Thomas C. Register, Kristofer T. Michalson, Dalane Kitzman, Gregory O. Dugan, David L. Caudell, J. Mark Cline.

Abstract. Annual meeting of the American Heart Association, presented in Philadelphia November 2019

Federal support was acknowledged

Project 3 (Immune Recovery, PI Sempowski/Chen):

(PS7-22) Long-term T cell reconstitution in peripheral blood and lymphoid organs following total body irradiation (TBI) Yujing Zou; Xinhua Chen; Yiqun Jiao; Ying Huang; Xiaoli Nie; Xiaoyan Yang; Suijing Wu; Andrew Macintyre; Nelson Chao; Gregory Sempowski; and Benny Chen
Duke University, Durham, NC

Abstract was presented at The 65th Radiation Research Society Annual Meeting 2019
Federal support acknowledged

Project 4 (Genomics, PI Dave):

Nothing to report.

Other publications, conference papers, and presentations.

Overall: (PI Cline):

Project 1 Presentation, Program Annual Retreat, Wake Forest School of Medicine, October 16th 2019. Federal Support was acknowledged.

Rotating monthly presentations of project progress via WebEx to Program Team.

Primate Radiation Survivor Core

J Mark Cline; John Olson; Greg Dugan; Rachel Andrews; Kylie Kavanagh; Daniel Bourland; David Hanbury; Ann Peiffer; Thomas Register; Ryne DeBo; David Caudell; Greg Sempowski and Nelson Chao

Centers for Medical Countermeasures against Radiation Annual Meeting, Rockville, MD, December 3-4, 2019

Federal Support was acknowledged.

Project 1 (Diabetes, PI Kavanagh):

Rotating monthly presentations of project progress via WebEx to Program Team.

Project 1 Presentation, Program Annual Retreat, Wake Forest School of Medicine, October 16th, 2019. Federal Support was acknowledged.

Project 2 (Radiation-Induced Heart Disease, PI Register):

Rotating monthly presentations of project progress via WebEx to Program Team.

Project 3 Presentation, Program Annual Retreat, Wake Forest School of Medicine, October 16th, 2019

Federal Support was acknowledged.

Project 3 (Immune Recovery, PI Sempowski/Chen):

Rotating monthly presentations of project progress via WebEx to Program Team.

Project 3 Presentation, Program Annual Retreat, Wake Forest School of Medicine, October 16th, 2019

Federal Support was acknowledged.

Project 4 (Genomics, PI Dave):

Rotating monthly presentations of project progress via WebEx to Program Team.

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Overall (PI Cline):

Nothing to report.

Project 1 (Diabetes, PI Kavanagh):

Nothing to report.

Project 2 (Radiation Induced Heart Disease, PI Register):

Grant applications submitted and funded

Pathology Internal Pilot Grant Application (funded) Thomas C. Register, PhD. "In Situ Molecular Profiling of Myocardial Responses to Ionizing Radiation in Rhesus Macaques" July 2017-June 2018

RADCCORE Pilot Grant Application (funded)

5U19AI067773-13 (Register-PI) (Year 1) 08/01/17-07/31/18

"Monocyte Polarization in Acute and Delayed Responses to Total Body Irradiation in Nonhuman Primates" This project is designed to determine the effect of total body irradiation on circulating monocyte polarization and transcriptional programming in the acute and chronic phases post irradiation, and to explore relationships of these monocyte phenotypes with radiation induced conditions in male rhesus macaques.

Grant applications submitted and pending

Peer Reviewed Medical Research Program for a Discovery Award (\$200,000, pending)

“Effects of Ionizing Radiation (IR) on Myocardial DNA Methylation Profiles in Relation to Cardiomyopathy in a Nonhuman Primate Model”. (Register-PI 2018)

Project 3 (Immune Recovery, PI Sempowski/Chen):

Nothing to report.

Project 4 (Genomics, PI Dave):

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Core Primate Studies (Cline)

Name:	J. Mark Cline <i>No change</i>
Name:	Michael Benedict <i>No change</i>
Name:	Daniel Bourland <i>No change</i>
Name:	David Caudell <i>No change</i>
Name:	Janet Tooze <i>No change</i>
Name:	Jean Gardin <i>No change</i>
Name:	Matt Dwyer <i>No change</i>
Name:	Russell O'Donnell <i>No change</i>
Name:	McKinley Williams-McDowell <i>No change</i>
Name:	Renae Hall <i>No change</i>
Name:	Patricia Warren <i>No change</i>

Project 1 – Diabetes (Kavanagh)

Name:	Kylie Kavanagh <i>No change</i>
Name:	Ashley Davis <i>No change</i>
Name:	Christina Sherrill

No change

Name: Cristina Furdui
No change

Name: Hanzhi Wu
No change

Project 2 – Radiation/Heart Disease (Register)

Name: Thomas Register
No change

Name: Sujethra Vasu
No change

Name: Craig Hamilton
No change

Name: James Bottoms
No change

Name: Maryanne Post
No change

Project 3 – Immune Recovery (Chen & Sempowski)

Name: Gregory Sempowski
No change

Name: Andrew Macintyre
No change

Name: Ryan Wuerker
No change

Name: Benny Chen
No change

Name: Yiqun Jiao
No change

Name: Yujing Zou

Name: Xiaoyan Yang

Project Role: Visiting scholar

Researcher Identifier (e.g. ORCID ID): N/A

Nearest person month worked: 10

Contribution to Project: Dr. Yang has been involved in cell processing, flow cytometric analysis, and RNA sequencing.

Project 4 - Genomic Sequencing and Stem Cell Lines (Duke Consortium – Dave)

Name: Sandeep Dave
No change

Name: Anupama Reddy
No change

Name: Cassandra Love
No change

Name: Guojie Li
No change

Name: Qiu Qin
No change (supported through non-DOD sources)

Name: Alexander Waldrop
No change (supported through non-DOD sources)

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: The Wake Forest Nonhuman Primate Radiation Survivor Cohort
Change: New Award

Project Title: Wake Forest Preclinical Imaging and Irradiation Facility
Change: New Award

Project Title: Modulating the Breast Microbiome to Prevent ER+Breast Cancer
Change: New Award

Project Title: Centers for Medical Countermeasures against Radiation
Change: Project Complete (Core E)

Daniel Bourland (Co-Investigator)

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

Project Title: The Wake Forest Nonhuman Primate Radiation Survivor Cohort
Change: New Award

Project Title: Wake Forest Preclinical Imaging and Irradiation Facility
Change: New Award

Project Title: Preclinical Assessment of Radiation Skin Injury
Change: New Award

Project Title: Evaluation of a Red Duroc Porcine Model to Address Cutaneous Radiation Injury in Black (Type III/VI) Skin
Change: Project Complete

Project Title: Biodosimetry for Irradiated Blood
Change: Project Complete

Project Title: Centers for Medical Countermeasures against Radiation
Change: Project Complete (Core E)

David Caudell (Co-Investigator)

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

Project Title: The Wake Forest Nonhuman Primate Radiation Survivor Cohort
Change: New Award

Project Title: Centers for Medical Countermeasures against Radiation
Change: Project Complete (Core E)

Christina Furdul (Co-Investigator)

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

Project: Redox-dependent Immunometabolism of HIV/Aging Associated Cancers in African Americans
Change: New Award

Project: Detecting Diverse Nucleic Acid Biomarkers of Cancer with Solid-state Nanopores
Change: New Award

Project: 3D Culture Systems of Urine-Derived Stem Cells for NTRI-Induced Mitotoxicity Assessment
Change: New Award

Kylie Kavanagh (Co-Investigator)

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

Project Title: Enabling Accurate Identification and Quantification of Brown Adipose Tissue Mass by Xenon Enhanced Computed Tomography

Change: New Award

Project Title: Copper is a Host Effector in Protection against Urinary Tract Infection
Change: Project Complete

Project Title: Assessment of ARCUS Nuclease Driven Gene Editing Following Lipid Nanoparticle
Change: Project Complete

Thomas Register (Project 3 PI)

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

Project Title: The Wake Forest Nonhuman Primate Radiation Survivor Cohort
Change: New Award

Project Title: Modified Ketogenic Diet Effects on Alzheimer's Disease Biomarkers and Cognition in MCI
Change: New Award

Project Title: Amyloid Beta Oligomer Induction of Alzheimer's Disease in Non-human primates
Change: New Award

Project Title: Centers for Medical Countermeasures against Radiation
Change: Project Complete (Core E)

Janet Tooze (Co-Investigator)

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

Project Title: Modulating the Breast Microbiome to Prevent ER+ Breast Cancer
Change: New Award

Project Title: Detecting Diverse Nucleic Acid Biomarkers of Cancer with Solid-state Nanopores
Change: New Award

Project Title: The Wake Forest Nonhuman Primate Radiation Survivor Cohort
Change: New Award

Project Title: Quantitative CT and MRI-based Assessment of Dynamic Vertebral Strength and Injury Risk Following Long-Duration Spaceflight
Change: Project Complete

Greg Sempowski (Project 3 Co-PI - Duke)

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

No changes to report

Benny Chen (Project 3 Co-PI - Duke)

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

Project Title: Endothelial Cell-Derived Extracellular Vesicles for Mitigation of the Hematopoietic Acute Radiation Syndrome

Change: New grant

Project Title: Mitigation of radiation-induced hematopoietic toxicity by rhIL-7-hyFc

Change: New grant

Project Title: DNA binding polymers

Change: closed

Sandeep Dave (Project 4 PI - Duke)

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

No changes to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

Not Applicable

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

Appended

9. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

1) **Quad Chart**

- 2) Bacarella N, Ruggiero A, Davis AT, Uberseder B, Davis MA, Bracy DP, Wasserman DH, Cline JM, Sherrill C, Kavanagh K. **Whole Body Irradiation Induces Diabetes and Adipose Insulin Resistance in Nonhuman Primates.** Int J Radiat Oncol Biol Phys. 2020 Mar 15;106(4):878-886. doi: 10.1016/j.ijrobp.2019.11.034. Epub 2019 Dec 2. PMID: 31805366.
- 3) Michalson KT, Kitzman D, Dugan GO, Caudell DL, Cline JM, Register TC **Myocardial functional, structural, and cellular alterations in Rhesus Macaque survivors of ionizing radiation exposure.** Abstract (PS7-25), Radiation Research Society, 2019.

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.							
	\$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

Biology Contribution

Whole Body Irradiation Induces Diabetes and Adipose Insulin Resistance in Nonhuman Primates



Nicole Bacarella, MLAS, VMD,^{*} Alistaire Ruggiero, BA,[†]
Ashley T. Davis,[‡] Beth Uberseder, BA,[§] Matthew A. Davis, MS,^{||}
Deanna P. Bracy, BS,[¶] David H. Wasserman, PhD,[¶]
J. Mark Cline, DVM, PhD,[§] Chrissy Sherrill, BS,[§]
and Kylie Kavanagh, DVM, MS, MPH^{§, #}

^{*}Animal Resources Program, Wake Forest University School of Medicine, Winston-Salem, North Carolina; [†]Molecular Medicine and Translational Science, Wake Forest University School of Medicine, Winston-Salem, North Carolina; [‡]Translational Imaging Program, Wake Forest University School of Medicine, Winston-Salem, North Carolina; [§]Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, North Carolina; ^{||}Department of Internal Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina; [¶]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee; and [#]College of Health and Medicine, University of Tasmania, Hobart, Australia

Received Sep 9, 2019. Accepted for publication Nov 15, 2019.

Purpose: Diabetes mellitus is a delayed effect of radiation exposure in human and nonhuman primates. Diabetes mellitus is characterized by peripheral tissue insulin resistance, and as a result, irradiation exposure may cause important changes in insulin-sensitive tissues such as muscle and adipose.

Methods and Materials: We prospectively investigated changes in response to irradiation (4 Gy whole body exposure) in 16 male rhesus macaques. We evaluated changes in body composition and glycemic control for 2 years. Insulin responsiveness, lipolysis, inflammation, and fibrosis were evaluated at study end.

Results: Irradiated animals accumulate less fat and significantly increased percent glycation of hemoglobin A1c over time, such that 40% of irradiated monkeys had values that define them as diabetic at 2 years. Subcutaneous (SQ) adipose tissue was insulin resistant, as evidenced by reduced phosphorylation of the insulin receptor substrate-1 in response to insulin challenge and had increased basal lipolysis despite comparable insulin exposures to control animals. Irradiated SQ adipose tissue had more macrophage infiltration and adipocytes were larger. The observed hypertrophy was associated with decreased glycemic control and macrophage infiltration correlated with decreased adiponectin, signifying that inflammation is associated with worsening health. No evidence of SQ adipose fibrosis was detected.

Corresponding author: Kylie Kavanagh, DVM, MS, MPH; E-mail: kkavanag@wakehealth.edu

Nicole Bacarella and Alistaire Ruggiero made equal contributions to this study.

Sources of support: Funding for this project was provided by the US Department of Defense grant number W81XWH-15-1-0574.

Disclosures: The authors certify they have no affiliations with or involvement in any organization or entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this manuscript.

Acknowledgments—The authors wish to acknowledge the aforementioned funding source, all technicians involved in the care and evaluation of these animals, and the nonhuman primates that made this study possible.

Conclusions: Our study is the first to prospectively illustrate that sublethal irradiation exposures directly propagate metabolic disease in the absence of obesity in nonhuman primates and implicate SQ adipose dysfunction as a target tissue. © 2019 Elsevier Inc. All rights reserved.

Introduction

Survivors of acute effects of radiation exposure are faced with the burden of developing delayed adverse health effects, which continue to be characterized. For example, limb shortening, osteonecrosis, and hypothyroidism are seen in adults who received radiation therapy for childhood cancers.¹ A newly recognized consequence of irradiation is development of diabetes mellitus (DM) and metabolic disease.²⁻⁵ The survivors of childhood cancer who received total body irradiation were 1.8 times more likely to report DM later in life compared with their siblings.⁴ The occurrence of DM in these children was highly correlated with total body radiation therapy and unrelated to body mass index or physical inactivity.² Similarly, nonhuman primates also develop type 2 DM years after irradiation, even when traditional risk factors, such as obesity, are absent.⁶

Prior studies of irradiated nonhuman primates describe peripheral tissue insulin resistance in the absence of obesity and loss of fat depots.^{6,7} Excess adipose tissue is typically associated with insulin resistance; however, ectopic adipose tissue or adipose deficiencies are known to be equally strong drivers of metabolic dysregulation. Relevant to this are lipodystrophic animals and people, which have metabolic disease associated with complete or partial loss of adipose tissue.⁸ Few studies have evaluated irradiated adipose tissue, but Poglio et al reported that in irradiated mice, there were significantly decreased adipocytes in inguinal fat pads and a loss of proliferating preadipocytes.⁹ Fibrosis and loss of capillarization may also contribute to irradiation-exposed adipose tissue insulin resistance, and is observed in irradiated mouse and monkey skeletal and cardiac muscle.^{7,10,11} The accumulation of collagens in adipose tissue likely creates a hypoxic environment. Insulin resistance is believed to result of increased inflammation, which is often observed postirradiation.^{10,12} Hypoxia is thought to drive local tissue insulin resistance and recruitment of inflammatory cells and cause the release of circulating proinflammatory cytokines that induce insulin resistance locally and in remote tissues.

In this study, we prospectively evaluate the changes in body composition and glucose metabolism after irradiation in nonhuman primates, which are an excellent translational model for studies of adipose tissue because tissue architecture, distribution, lipogenesis sites and rates, and inflammatory and immune responses resemble that of humans more closely than rodents. We hypothesized that irradiation would induce inflammation in the subcutaneous space, which would limit normal adipose expansion and responses to insulin. Our study demonstrates that whole

body irradiation increases diabetogenesis and reduces adipose mass, highlighting that peripheral tissue dysfunction is a key mediator of this important delayed effect of radiation exposure.

Materials and Methods

Animal study

Sixteen male rhesus macaques of Indian origin, aged 5.8 ± 0.5 years old and prescreened for tuberculosis and simian retroviruses, were studied. Age of animals were intended to match a postpubertal young adult, approximating 15 to 20 human years of age. Animals were fed a Western-like diet (5L0P; LabDiet, St Louis, MO) and randomized to receive 4 Gy total body irradiation (IRRAD, $n = 10$) or sham irradiation (CTL, $n = 6$). The diet provided 45% of calories as carbohydrates with significant levels of simple sugars, 36% of calories as fat, and 18% of calories as protein, with the majority of protein and fat from animal sources. Irradiations were conducted under general anesthesia (ketamine and isoflurane) on a clinical linear accelerator (Varian, Palo Alto, CA) using 2 lateral fields of 6MV x-rays (174 cm source-axis-distance, 32×40 cm² field size at 100 cm from the source, nominal dose rate of 0.8 Gy/min at 174 cm source-axis-distance, 2.7 min irradiation time per field). After sedation, each animal was situated in a supine position between 2 plastic build-up screens, with knees up and arms crossed at the lower abdomen, with water-bolus compensation for thinner body regions (head and lower legs). First, the left side was irradiated to 2 Gy. The subject was then rotated 180° to irradiate the right side to 2 Gy, yielding a 4 Gy total. Dose calculations were performed for 2 Gy to midplane per field, based on anatomic measurements (nominal depths of 8-10 cm). Sham-irradiated animals experienced all procedural steps aside from irradiation, including sedation, positioning, and observation from outside the radiation room for the elapsed time of irradiation for each field. Monkeys underwent daily monitoring for clinical illness, including observations of food consumption, urination, and defecation. Methods modified from the Children's Cancer Group Clinical Toxicity Criteria were used to classify illness,¹³ and interventions were governed by clinical pathologic findings.¹⁰ All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals, in compliance with the Institutional Animal Care and Use Committee, at our Association for the Assessment and Accreditation of Laboratory Animal Care International accredited institution, which operates in compliance with the Animal Welfare Act.

Supportive care offered in the postexposure period included crystalloid replacement fluids, antibiotics, whole blood transfusions, supplemental nutrition, and analgesics. All interventions ceased by 6 months after irradiation.

Data were collected from the animals before radiation exposure and from 6 and 24 months postexposure, only after the acute effects of irradiation had resolved. The majority of results are presented at 24 months, which was an a priori designated time point at study initiation. Two years in a rhesus macaque lifespan approximates a 5 human year duration.

Blood parameters

Subjects were fasted for ≥ 12 hours before assessments. Blood samples were obtained via percutaneous venipuncture of the femoral vein into ethylenediaminetetraacetic acid and placed on ice until processed for plasma, which was stored at -80°C until analyzed. Fasting blood glucoses were determined by the glucose oxidase method, and fasting insulin concentration was determined by enzyme-linked immunosorbent assay (ELISA; Mercodia, Uppsala, Sweden). Whole blood was used to measure glycated hemoglobin using high-performance liquid chromatography methodolo Gy (Afinion Alere, Waltham, MA). Triglyceride, high density lipoprotein cholesterol, and total cholesterol concentrations were measured enzymatically. Adiponectin and lipopolysaccharide binding protein 1 levels were evaluated via ELISA (R&D Systems, #DRP300, Minneapolis, MN; Cell Sciences, #CKH113, Newburyport, MA). Cytokine panels were measured (Procarta NHP 37-plex, Invitrogen, Carlsbad, CA) on a Luminox platform.

Body composition

Computed tomography (CT) scans were used to measure fat and muscle tissue volumes. The pre-exposure scans were performed on a Toshiba 32-slice Aquilion scanner (Toshiba America Medical Systems, Tustin, CA), and the 2-year scans were performed on a GE 0.625-mm slice Discovery MI DR scanner (GE Health care, Waukesha, WA). The CT images were reconstructed with TeraRecon Aquarius Intuition software (TeraRecon, Foster City, CA) and then converted into a dicom format for analysis. As previously described,⁶ fat- and lean-containing voxels were used to calculate volume results (then corrected for density) and expressed as a percentage of the animal's total body weight. Abdominal fat distribution of intra-abdominal and subcutaneous fat was assessed from regional analysis between the thoracolumbar junction and the fourth lumbar vertebrae using automated differentiation of deposits within and outside the body wall. Subcutaneous fat depth and attenuation values were measured directly above and below the linea alba, across 10 evenly spaced slices within this defined abdominal region. Fat mass and ratios for this body

region were calculated after correction for tissue density. Attenuation values were collected in triplicate for the liver and psoas muscle at the L4 level. Average psoas area was calculated by tracing tissue circumference. Body composition was additionally confirmed by dual energy x-ray absorptiometry at pre-exposure and 24 months (Hologic Discovery A Dual x-ray Bone Densitometer, Bedford, MA). Fat and lean mass values were calculated as the percent of whole-body mass.

Adipose tissue

Adipose tissue biopsies were collected under ketamine and midazolam anesthesia, at baseline and at 24 months after irradiation. The animals were positioned in lateral recumbency, and adipose tissue was collected from the ventral aspect of the caudal abdomen, adjacent to the umbilicus. The samples were collected before and 20 minutes after insulin bolus, flash-frozen in liquid nitrogen, and stored at -80°C . Regular insulin was infused into a peripheral vein (0.2 U/kg) to increase the median circulating insulin concentration to approximately $100 \mu\text{IU/mL}$.¹⁴ Tissue was homogenized using a bead beater and extracted protein from biopsy samples was quantitated by bicinchoninic acid assay before being analyzed for total and phosphorylated insulin receptor substrate 1 (IRS1) by ELISA according to the manufacturer recommendations (Cell Signaling Technologies, Cat# 7328, Danvers, MA). Data are reported as the ratio of activated IRS1 from pre- and postinsulin results. Basal triglyceride breakdown was measured from adipose explants. Tissue was incubated in Krebs-Ringer buffer with 3.5% bovine serum albumin and 0.1% glucose at 37°C on a shaking platform. Release of nonesterified free fatty acids and glycerol (Abcam, #ab65431, Cambridge, UK; Sigma-Aldrich; #MAK117, St Louis, MO) were measured in triplicate from tissue supernatants.

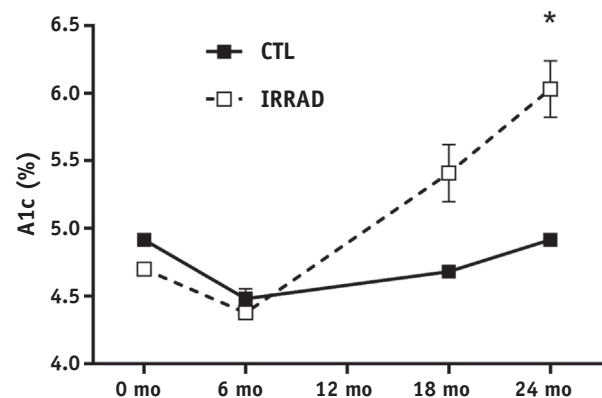


Fig. 1. Glycemic control, as percent glycosylation of hemoglobin A1c chain (A1c), remained stable in the control group (CTL; $n = 6$) while significantly increasing over time in the irradiated (IRRAD; $n = 10$) group ($P = .0003$).

Adipose tissue protein was extracted and immunoblotted as previously described.¹⁵ Using commercially available primary antibodies, the membranes were probed for phosphorylated-SMAD2/3 (Abcam, #ab63399, Cambridge, United Kingdom). As a loading control, glyceraldehyde 3-phosphate dehydrogenase antibody (1:2000; Sigma-Aldrich, #G9545) was used. Each immunoreactive product was quantified for density and normalized to glyceraldehyde 3-phosphate dehydrogenase.

Histology

Paraffin-embedded adipose tissues were used to create histologic sections. Adipocyte size was measured by 100 individual cell diameters from 10 representative fields viewed at 10× magnification with 10 adjacent cell diameters recorded per field. Pan-macrophage marker CD68 (Agilent, #M081401-2, Santa Clara, CA) was applied to determine classically activated macrophages. The numbers of CD68 positive cells were calculated as normalized by both tissue area and total cell numbers, as there were differences in adipocyte size across samples and thus differences in overall tissue density. Total tissue fibrosis was estimated by Masson's Trichrome staining with results reported as percent positive staining area. Collagens type I, III, and IV were evaluated as previously described.⁷ Analysis of all histologic images were conducted using a custom automated macro in VisioPharm (Version 2019.02.1.6005, Hoersholm, Denmark). An investigator

blinded to group identity performed all image acquisition and analyses.

Data analysis

Statistical analyses were performed using Statistica version 13 (StatSoft, Tulsa, OK), and *P* values of $\leq .05$ were considered significant. A trend was considered as $P \leq .10$. Logarithmic transformation was applied to endpoints not meeting normality assumptions. Metabolic and phenotypic variables were analyzed for differences by analysis of covariance using baseline values, if available, and current body weight. Repeated measures of ANOVA was used for body weight and A1c values measured during the 2 years of study. Fisher exact test was used to measure differences between proportions, and Pearson's correlation coefficients were calculated to measure strength of associations.

Results

Radiation exposure significantly worsened glycemic control, as average A1c steadily climbed beginning 6 months after radiation exposure (Fig. 1). At 2 years, 4 of the 10 irradiated animals met the American Diabetes Association criteria for DM. This finding represented a significant increase in the incidence of diabetes as a delayed effect of irradiation compared with no disease development in the control group ($\chi^2 P < .05$). The metabolic disease manifested without obesity and was characterized by

Table 1 Basic demographic and additional body composition details of the study groups at the 2-year assessment point

	CTL	IRRAD	<i>P</i> value
Crown-rump (m)	0.59 (0.01)	0.56 (0.01)	.05
Body mass index (kg/m ²)	47.55 (3.99)	45.71 (2.94)	.35
Arm circumference	23.02 (0.68)	21.55 (0.68)	.08
Leg circumference	33.31 (1.42)	30.03 (1.24)	.05
Waist circumference (cm)	52.67 (3.77)	47.62 (3.05)	.15
Systolic blood pressure (mm Hg)	135.43 (3.14)	133.61 (7.08)	.44
Diastolic blood pressure (mm Hg)	81.38 (6.54)	79.53 (6.16)	.42
HOMA (AU)	10.87 (2.01)	15.33 (4.63)	.53
Fasting insulin (μIU/mL)	59.48 (12.54)	80.53 (24.07)	.56
Fasting glucose (mg/dL)	75.88 (5.80)	77.08 (14.09)	.53
Total cholesterol	173 (20.9)	203 (15.6)	.29
Triglycerides	31.7 (18.3)	58.8 (14.0)	.28
Basal nonesterified free fatty acids* (Free fatty Acids/mg adipose)	0.191 (0.189)	0.5212 (0.145)	.10
CT liver attenuation (HU)	58.2 (1.93)	54.9 (1.47)	.21
CT psoas muscle attenuation (HU)	62.2 (0.90)	61.2 (0.70)	.42
CT abdominal SQ fat area (cm ²)	28.9 (4.40)	21.7 (3.41)	.11
CT abdominal VIS fat area (cm ²)	67.7 (11.2)	47.9 (8.63)	.09
SQ:VIS fat area	0.48 (0.13)	0.54 (0.10)	.72
Dual energy x-ray absorptiometry lean mass (kg)	11.0 (0.43)	9.47 (0.33)	.02
CT lean mass (kg)	10.1 (0.45)	9.47 (0.35)	.14

Data shown are means adjusted for baseline/preirradiation values and standard errors of the mean (SEM), with analysis of variance *P* values, unless baseline data was not available (*).

Abbreviations: CT = computed tomography; CTL = control group; HU = hounsfield units; IRRAD = irradiated group; SQ = subcutaneous, VIS = visceral.

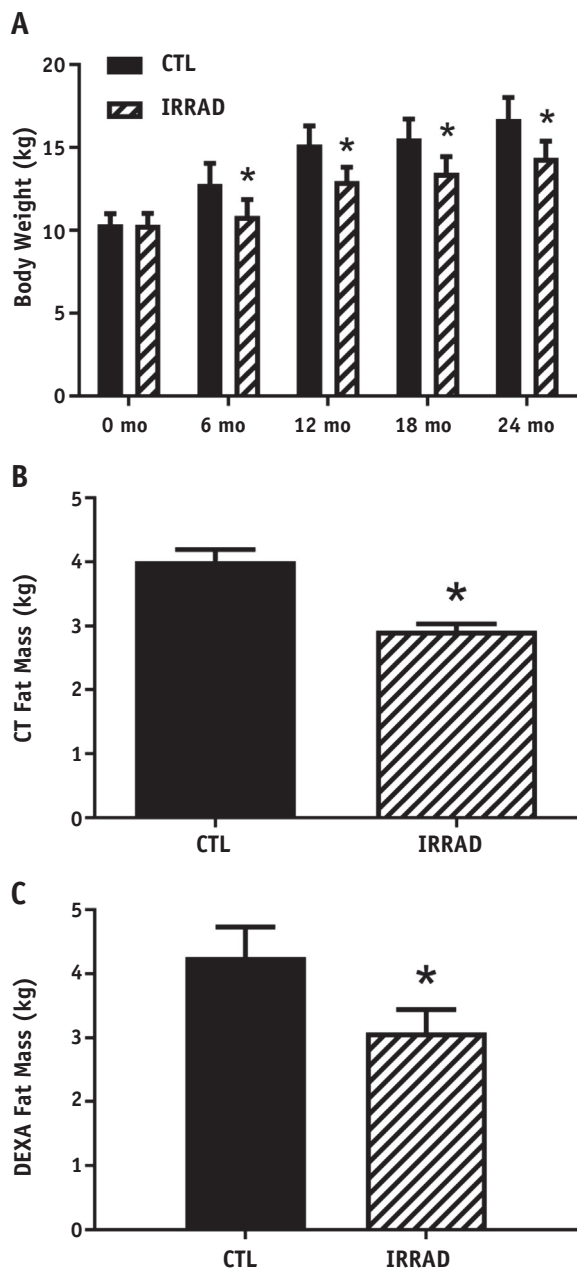


Fig. 2. (A) Mean ($\pm$ SEM) body weights measured in kilograms (kg) demonstrate that irradiation retards growth (repeated measures analysis of variance $P < .0001$; $*P < .05$ for post hoc testing). (B) Computed tomography (CT) measures of total fatness at 2 years confirm that adipose mass was lower postirradiation. Data shown are means adjusted for baseline values ($\pm$ SEM). $*P = .0008$. (C) Dual energy x-ray absorptiometry measures of total fatness at 2 years confirm adipose mass was lower postirradiation. Data shown are means adjusted for baseline values ($\pm$ SEM). $*P = .0007$.

hyperglycemia (Table 1 and Fig. 2). Although the body weight of the all animals increased during the course of 24 months, the irradiated animals gained weight at a slower rate (Fig. 2A) and were significantly lighter at all times. CT

values of total fat mass in the irradiated animals were significantly lower compared with that of the control group, in spite of the animals consuming the same high-fat diet. The total fat mass was 4 kg in the control animals compared with 3 kg in irradiated animals (Fig. 2B). Similarly, dual energy x-ray absorptiometry values for fat mass in the irradiated animals were lower compared with control animals (Fig. 2C). Adipose deposition was lower in both visceral and subcutaneous compartments of the irradiated animals (Table 1). This reduction in tissue is consistent with an overall stunting of radiation exposed animals, evidenced by smaller stature and limb circumferences. We found no evidence for ectopic fat deposition in common sites such as liver and muscle (Table 1).

The IRS1 activation in response to insulin challenge in SQ adipose tissue was significantly lower in irradiated animals compared with that of control animals (Fig. 3A). This tissue insulin resistance is consistent with the trends toward increased basal lipolysis (Table 1). Accordingly, higher lipolytic rates were strongly associated with the animals' A1c values ($r = 0.69$, $P = .003$). Adiponectin levels were not significantly different in the irradiated subjects compared with control subjects, despite having less fat mass. This may be explained by the larger adipocyte cell size (Fig. 3B) and the higher density of macrophages (Fig. 3C and D) in the irradiated monkeys' subcutaneous adipose tissue. Larger fat cell size was strongly associated with glycemic control, as measured by A1c levels ($r = 0.61$, $P = .01$) and the macrophage infiltration was negatively associated with adiponectin levels ($r = -0.56$, $P = .02$; Fig. 3E). These local adipose tissue changes and the related decline in glucose metabolism did not induce a general proinflammatory state. Circulatory cytokines measured showed no significant differences between the 2 groups (Table 2).

Radiation is a widely appreciated stimulus that induces fibrosis, although we detected no differences in collagens or levels of activated smad2/3, which reflects transforming growth factor β signaling activation (Table 3). The amount of transforming growth factor activation in adipose was related to total tissue fibrosis measured by Masson's trichrome staining ($r = 0.55$, $P = .03$; data not shown). With 6 measures relating to fibrosis in adipose tissue, there were no detectable differences.

Discussion

This is the first prospective and controlled study to show diabetes development results of radiation exposure in nonhuman primates and to examine adipose tissue as a target organ. Our data indicate that male rhesus macaques receiving whole body radiation 2 years prior exhibited: (1) less weight and fat mass gains, (2) larger fat cells with a higher abundance of macrophages, (3) Adipose tissue insulin resistance, and (4) increased incidence of DM. Our study is translationally relevant and has implications to

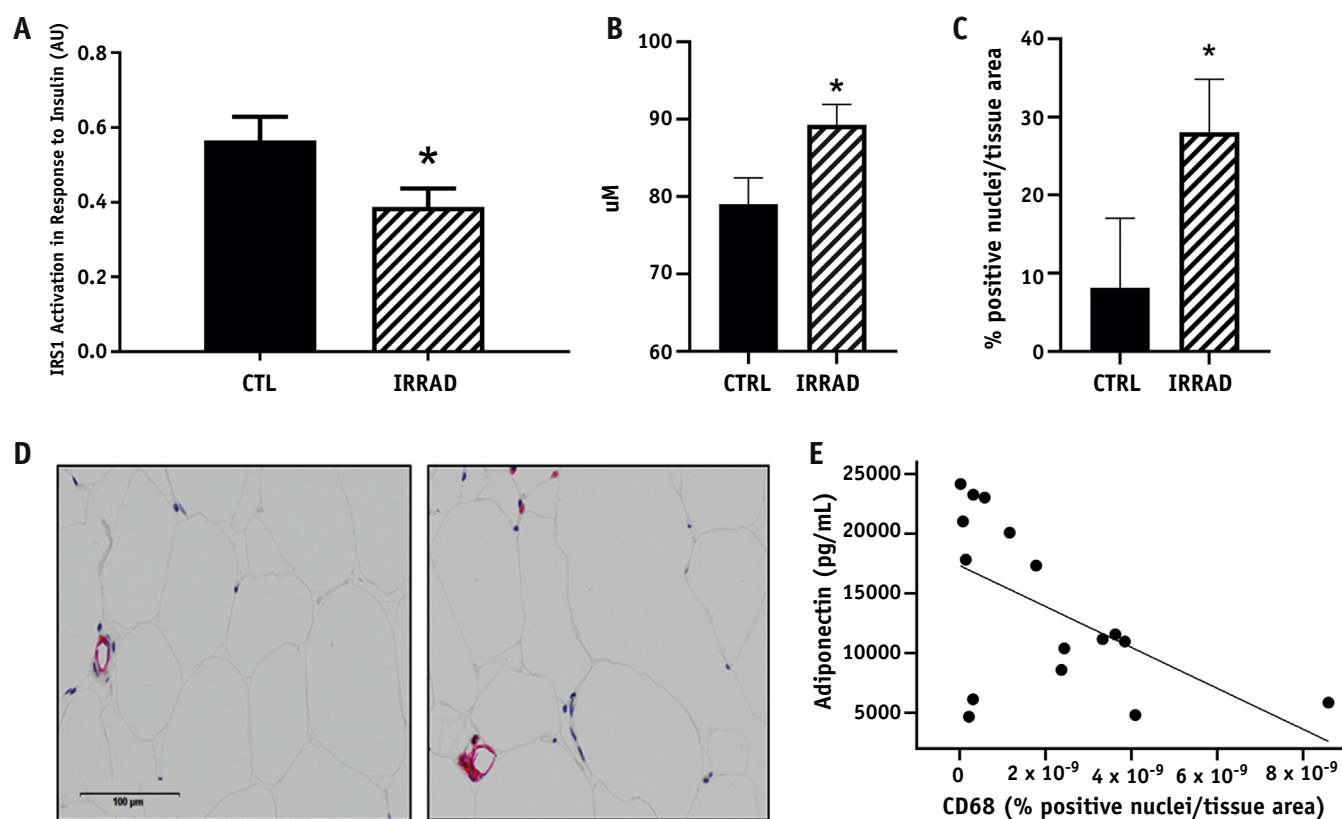


Fig. 3. Subcutaneous adipose tissue insulin receptor substrate 1 (IRS1) activation in response to insulin was significantly lower in irradiated monkeys ([arbitrary units; AU] $*P = .004$). Mean ratio of phosphorylated of IRS to total IRS, postinsulin to baseline ($\pm$ SEM) shown. (A) Subcutaneous adipocyte cell size is larger (analysis of variance $*P = .03$) in previously irradiated monkeys. Data shown is mean adipocyte diameter adjusted for total body weight ($\pm$ SEM). (B) Macrophage density, measured as CD68 positive staining cells, is increased in irradiated monkeys ($*P < .05$). Data shown is means adjusted for total body weight ($\pm$ SEM). (C) Representative histologic sections from a control (left) and irradiated (right) subcutaneous adipose tissue. CD68-expressing macrophages are stained positively. Ten times magnification; scale bar = 100 μ m. (D) Macrophage density of subcutaneous adipose tissue negatively associates with circulating levels of adiponectin ($r = -0.56$, $P = .02$). Adiponectin tended to associate with glycemic control as expected (data not shown; $r = -0.48$, $P = .06$).

humans exposed to radiation, as our study animals developed type 2 diabetes mellitus in a time span of just 2 years after radiation, in contrast to typical diabetes development in this species, which takes an average 4 or more years.¹⁶ Our controlled study is consistent with clinical outcomes that show DM is a late complication that occurs in childhood cancer survivors.³ Case reports include patients who developed severe insulin resistance and hypertriglyceridemia after whole body irradiation in childhood.¹⁷

Mecham et al⁴ showed that a higher percentage of childhood cancer survivors who underwent whole body irradiation develop diabetes mellitus later in life compared with those who only underwent focal abdominal radiation.⁴ This finding highlights the potential importance of adipose tissue in driving increased risks for diabetes postradiation exposure. The diabetes incidence observed in our study is high, and the duration of observation only approximates 5 human years. We attribute the high-risk to the whole body exposures, which is uncommon in human radiation therapy; however, children with whole body irradiation have 28%

diabetes prevalence in just over 10 years since exposure.⁴ We feel our study is dramatic but demonstrates a translationally relevant physiological outcome that deserves attention. Space flight (cosmic radiation) has also been implicated in the development of diabetes.¹⁸ This is relevant, as we consider the varying type and energy of exposures as a result of medical, malicious, accidental, and other sources of irradiation.

The irradiated monkey model has demonstrated premature onset of many age-related diseases, including cardiovascular disease,¹⁰ diabetes,⁶ cellular senescence, and related loss of regenerative capacities.^{19,20} Senescent cells are known to accumulate in adipose and be related to multiple age-related functional declines in both human and animal models.^{21,22} Data from our study confirm that irradiated animals exhibited less adipose tissue 2 years post-irradiation compared with controls. Although displaying a leaner phenotype, irradiated animals exhibited significantly larger adipocytes and increased basal lipolysis compared with controls. This indicates that adipose of irradiated

Table 2 Inflammatory biomarkers measured from the circulation at 2 years in control (CTL) and irradiated (IRRAD) monkeys

	CTL	IRRAD	P value
IL-1 β (pg/mL)	32.45 (19.38)	34.22 (7.85)	.92
IL-6 (pg/mL)	64.4 (52.7)	60.4 (38.1)	.96
TNF- α (pg/mL)	45.0 (21.9)	16.9 (14.3)	.28
IL1-RA (pg/mL)	697 (313)	284 (189)	.38
IL-4 (pg/mL)	36.9 (10.2)	18.2 (5.88)	.17
IL-23 (pg/mL)	42.7 (18.71)	54.39 (24.59)	.78
BLC (pg/mL)	102.40 (32.33)	101.97 (16.83)	.99
Eotaxin (pg/mL)	128.05 (35.51)	182.18 (25.86)	.20
IL-8 (pg/mL)	122.08 (75.63)	90.22 (45.22)	.69
MCP-1 (pg/mL)	210.82 (35.78)	275.43 (50.76)	.35
SDF-1 α (pg/mL)	708.53 (172.30)	627.30 (70.73)	.59
sCD40-ligand (pg/mL)	46.35 (8.44)	200.93 (181.08)	.42
G-CSF/CSF3 (pg/mL)	87.95 (36.13)	121.59 (24.66)	.40
IL-7 (pg/mL)	6.9 (3.45)	11.05 (3.59)	.42
VEGF-A (pg/mL)	55.18 (30.99)	58.42 (17.45)	.91
VEGF-D (pg/mL)	17.73 (14.32)	15.17 (3.58)	.83
Adiponectin (ng/mL)	14902.52 (2795.67)	13150.65 (2132.84)	.64
Lipopolysaccharide binding protein 1 (ng/L)	605 (265)	1012 (293)	.45

Cytokines IL-5, IL-10, interferon γ , and macrophage inhibitory protein were below the assay limits of detection and data for these is not shown. Data are means ($\pm$ SEM) adjusted for total bodyweight, and baseline if available.

Abbreviations: IL = interleukin; TNF- α = tumor necrosis factor alpha; BLC = B lymphocyte chemoattractant; MCP = monocyte chemoattractant protein; SDF = stromal derived factor; CSF = colony stimulating factor; sCD40 = soluble cluster of differentiation 40.

animals is less healthy, as adipocyte hypertrophy has been associated with metabolic dysfunction.²³ The increased adipocyte size despite less fat mass supports the notion that irradiated animals have decreased adipocyte abundance and may have lost the ability to generate adipocyte precursors. This results in the storage of excess lipids into remaining adipocytes, causing them to swell. In metabolically healthy animals and humans, peripheral fat is able to take up excess circulating nonesterified fatty acids, leading to a favorable metabolic profile.²³⁻²⁵ Normal subcutaneous fat function is required for metabolic responses to changes in nutritional state, with intact responses to insulin for lipogenesis and responses to adrenergic stimuli for lipolysis. Adipose tissue must be able to expand and remodel itself to store excess glucose as triglyceride. However, our data shows that irradiated animals are unable to do so and become lipodystrophic with decreased capacity for subcutaneous fat

expansion through differentiation and hyperplasia, resulting in type 2 DM. Ablamunits et al and others have also observed an association between failure of adipose tissue to expand and insulin resistance in laboratory mice, and that insufficient storage for excess energy results in DM.^{26,27} However, in the present study, irradiated animals had decreased ectopic fat in the liver, psoas muscle, abdominal subcutaneous tissue, and visceral tissue. CT and DEXA scan data from the present study confirm that irradiated animals exhibited less adipose density 2 years post irradiation compared with controls, suggesting that irradiation leads to smaller, leaner animals, and it is unclear where excess energy is used or stored.

Fibrosis has been shown to be increased in skeletal muscle tissue from previously irradiated monkeys as a sequela from injury, chronic inflammation, and exposure to reactive oxygen species.⁷ Additionally, fibrosis and tissue

Table 3 Histologic measures of subcutaneous adipose tissue from control (CTL) and irradiated monkeys (IRRAD) at 2 years post-exposure and immunoblot quantitation of pSMAD2/3, which is the key activator signaling increase gene transcription of fibrosis pathway genes, such as collagens

	CTL	IRRAD	ANCOVA P value
CD31 ⁺ cell density (cell/um)	0.0000434 (0.00011)	0.0000341 (0.000013)	.58
MTC fibrosis (% area)	2.23 (0.89)	2.46 (0.67)	.81
Collagen 1 (% area)	17.7 (1.53)	18.9 (1.17)	.55
Collagen 3 (% area)	14.5 (1.37)	15.0 (1.05)	.77
Collagen 4 (% area)	11.5 (1.25)	11.7 (0.95)	.92
Fibronectin (% area)	8.69 (0.77)	9.21 (0.62)	.61
pSMAD2/3 (AU)	0.369 (0.031)	0.481 (0.084)	.35

Data shown are means adjusted for total bodyweight ($\pm$ SEM).

remodeling is critical to diabetogenesis. Studies in rodent models have demonstrated that skeletal muscle extracellular matrix expansion is associated with the development of insulin resistance,^{28,29} and similar changes have been reported in irradiated nonhuman primates.⁷ The vascular endothelium is a particularly important target as radiation damage leads to inflammation, reduced perfusion, and progression of the fibrotic response in nonhuman primates,^{30,31} further limiting perfusion and access of insulin to cells such as myocytes and adipocytes.^{32,33} However, changes in fibrosis or extracellular matrix accumulation in adipose tissue were not observed, which may be an adaptation to allowing hypertrophy rather than constraining adipocyte enlargement while exposed to nutrient excess.

Increased macrophage abundance is thought to be due to the presence of larger, unhealthy adipocytes and more recruitment of macrophages.³⁴ We hypothesize that radiation damages adipocyte precursor cells, resulting in diminished normal adipocyte cell turnover. Adipose tissue has multiple precursor populations able to give rise to new adipocytes,^{35,36} and these cells may be radiosensitive. This is an area for future study, and the concept is supported by a case report that successfully treated irradiated insulin-resistant individuals with insulin-sensitizing agents (thiazolidinediones) that function in part by expanding adipose tissue.^{17,24} Irradiation is a standard procedure for inducing cell senescence, and thus we expect that persistence of senescent adipocytes may be in part an inflammatory signal and limit adipose expansion.

This is the only study of its kind to use a uniquely relevant animal model in a nutritionally relevant context, which increases the translatability of our findings. Prior nonhuman primate studies of irradiation and diabetes risk^{6,7} were cross-sectional and included animals that did not have pre-exposure data, variable origins and age at exposure, irradiation protocols and duration since exposure, diet, and environmental conditions. These studies suggested irradiation increased risk for accelerated diabetogenesis but could infer causation. Our study did present some limitations; our sample size was small, and we only evaluated male monkeys. Sex is an important biologic variable, and future studies will be necessary to determine whether irradiation differentially affects females. Females generally have higher proportions of subcutaneous adipose and thus differences in adipose effects of irradiation may exist. In summary, this report establishes that diabetes is a significant delayed effect of radiation when exposure occurs over a large surface area and highlights the importance of adipose tissue as a causative and target tissue for intervention in exposed people.

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(PS7-25) Myocardial functional, structural, and cellular alterations in Rhesus Macaque survivors of ionizing radiation exposure

Kristofer T. Michalson, DVM PhD; Dalane Kitzman, MD; Gregory O. Dugan, DVM; David L. Caudell, DVM PhD; J Mark. Cline, DVM PhD; Thomas C. Register, PhD Wake Forest School of Medicine, Winston-Salem, NC

Heart disease is a serious delayed/late effect for survivors of ionizing radiation exposure. In the present study, we investigated the delayed effects of acute total body irradiation (TBI) on cardiac function and myocardial structure at the histologic and cellular level in male rhesus macaques, a translational non-human primate (NHP) model. Transthoracic echocardiography including tissue Doppler imaging was performed on survivors of a single dose (6.4-8.5 Gy) of TBI and non-irradiated controls. Subjects were subdivided into short term (4.7-6 years old, 2-3 years post-TBI) (TBI) and long term (12.6-20 years old, 9.7-11.6 years post-TBI) survivors and age-matched controls (CTL). Short-term TBI animals had 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') ratios compared to age matched CTLs (all $p \leq 0.05$), indicative of diastolic dysfunction. Long-term TBI also had higher transmitral early filling velocities (E) and E/e' ratios and lower e' and e'/a' compared to long term CTL (all $p \leq 0.05$). Long term TBI also had lower apical 4 chamber-derived stroke volume, cardiac output, and end-diastolic volume compared to age-matched controls (all $p \leq 0.05$) which were not observed in the short term TBI group. Myocardium from deceased irradiated animals exposed to a single dose (6.9-8.05 Gy) TBI exhibited reductions in left ventricular and interventricular septal capillary density, and higher levels of left ventricular fibrosis, pan-cardiac fibroblast, macrophage, and M1 macrophage staining, and left ventricular and interventricular septum M2 macrophages (all $p < 0.05$) relative to age-matched controls. The findings support the hypothesis that LV diastolic dysfunction associated with radiation exposure may be a pathological consequence of decreased capillary density, increased myocardial fibroblast and macrophage populations, and increased myocardial stiffness due to fibrosis. These findings provide additional insights into the effects of ionizing radiation exposure on the primate heart and suggest that inhibition of macrophage regulation of fibrosis may be a potential pathway to target for therapy. Funding: DOD W81XWH-15-1-0574, U19 AI67798, U19 AI067773, R01 HL122393, T32 OD010957, and P30CA012197.

This abstract was the basis for a Scholar In Training Award for Dr. Michalson.