

*STING promotes homeostatic maintenance of tissues and
confers longevity with aging*

A Thesis submitted by

Jacob Hopkins

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Immunology

Tufts University

Graduate School of Biomedical Sciences

May 2024

Advisor: Shruti Sharma, PhD

Abstract

Chronic inflammatory disease is a pressing public health issue and believed to be a dominant driver of pathology accompanying all stages of aging. While the bulk of “inflammaging” research has focused on circulating biomarkers of immunity and disease, cell autonomous and innate immunity encompasses the bulk of tissue-centric immune responses and has been largely overlooked. Here, I present a comprehensive integration of unbiased single cell RNA sequencing, spectral cytometry, in situ imaging, and ex vivo profiling of cell intrinsic age-associated immunity and report specific, tissue-intrinsic immune fingerprints spanning inflammation, Type I Interferon, and cellular senescence inherently linked to the Stimulator of Interferon Genes (STING) pathway. Thorough analysis of myeloid and stromal elements confirms that the propagation of these signals is critical to both tissue resident macrophage vitality and organismal longevity, highlighting previously unappreciated mechanisms by which STING signaling maintains tissue homeostasis with age. Together, these data expand upon an intricate inflammatory network that integrates and masks protective immune responses that are vital to the aging organism.

Dedication

This work dedicated first and foremost to my loving wife, Taylor Waggett. Without her love and support I don't know where I would be today.

Acknowledgements

I would first like to acknowledge my mentor, Dr. Shruti Sharma, for an incredible amount of guidance and support over the years, and for staying as excited about this project as anyone could be. I came into this program with an open mind for my project, and Shruti was ready to run with it. Pushing us into single cell sequencing work and sending us away to a “coding bootcamp” for two weeks was, while unbearable frustrating, potentially the most impactful course of my graduate work. She never doubted my potential and helped me grow as a scientist more than I ever thought possible – in part by holding me (and everyone in lab) to the highest standard. One of the more rewarding experiences I had was slowly watching fewer edits being returned, with one of the final comments simply “liked this paragraph”. As one of the better writers I’ve worked with, this was a huge achievement.

While I started alone, this lab has grown with some exceptional people: Kate, Machlan, and Kimberly. Kate and I spent years pushing through the hardest aspects of this project together and was my perfect balance to get through some of those long, long days. Although Machlan and Kimberly joined the lab later, they have both contributed more in a shorter amount of time than I could have imagined. I truly look forward to seeing where all three of these people go with their projects and their careers.

My committee, Drs. Steve Bunnell, Linden Hu, and Caroline Genco pushed hard to reign me (and Shruti) into a more focused project, and while they only partially succeeded, their consistent direction and support made this project possible. I would especially like to acknowledge Dr. Steve Bunnell for the countless hours we spent chatting not inherently about my project but about *science*. There are plenty of times through this project that I questioned my decisions, but the excitement Steve brought (about everything) helped keep my motivation high with the understanding that... science is cool. While Dr. Eric Reed was never formally a part of my advisory committee

but became an integral part of the last two years of my graduate training. Eric brought a formality to my training in bioinformatics and single cell analysis that considerably elevated my work.

My time at Tufts was highlighted by certain people that I know I would never have made it through without them around. My classmates, Hymlaire, Urmila, and Linus: hanging back after lectures to (try to) decompress our first two years, to bouncing ideas off, to just general griping about whatever was happening; grad school wouldn't have been the same without you. My quasi-classmate Matt Zunitch, I am eternally grateful for the love and support you showed me and Taylor in the last few years of my graduate career. I don't know if anyone could quite relate to our situation as well as you did. Giang Nguyen, you were an amazing role model for the beginning of my time at Tufts. I never knew someone to work harder, and she *always* seemed to have the answers I needed. I am so very grateful for Judi Hollander's attempts to get us to relax and leave the building even just for a little bubble tea.

Prior to joining the graduate program at Tufts, I spent three years as a technician for Dr. Laura Haynes (Trudeau Institute, Saranac Lake, NY; UConn Health, Farmington, CT). Laura was unbelievably generous taking me into her lab when I was spending time in Saranac Lake, and not only helped to train me, but continued to offer me employment when her lab moved to UConn Health to join their Center on Aging. I barely knew how to pipette, but she and Dr. Julie Lefebvre taught me everything I needed to know to be a successful technician and build the base to be a successful scientist. I am beyond grateful for everything Laura did for me at UConn; from treating me like another graduate student, allowing me to present at conferences, and her general enthusiasm about science were major reasons I decided to continue a career in research.

However, all of this would not have been possible without Dr. Connie Thompkins. While I was an undergraduate at the University of Vermont, I thought I wanted to

become a physical therapist. Dr. Thompkins graciously gave me an override to take one of the doctoral level classes and even allowed me to work in her research clinic. Dr. Thompkins gave me such an incredible introduction to research science – expanding an exercise clinic in northern Vermont, helping to write an abstract, and even beginning to plan a case study before I left for Saranac Lake.

Without my family, I know I never would have made it where I am in life. My parents held us all to the highest standards and constantly sacrificed everything they could to make sure we had everything we needed. Helping with school projects, teaching me the importance of asking questions and finding my own answers (even if I didn't look for them at the time). My siblings are some of the smartest people I know. While it was frustrating growing up having to follow in the footsteps of two brilliant people, the push to be better than them got me to where I am today.

Last and certainly not least, I must thank my wife, Taylor Waggett, for simultaneously grounding me and reminding me that there really IS more to life than work, lab, and stress. You did everything you could to help me through these years, even when I didn't like it. I could never have imagined one person providing so much love, support, and confidence in me every day – especially when I didn't have it for myself.

Table of Contents

Abstract	ii
Dedication	iii
Acknowledgements	iv
Table of Contents	vii
List of Figures	xii
List of Abbreviations	xiv
Chapter 1: Introduction	1
1.1 Aging	1
1.1.1 Aging and human disease	2
1.1.2 Healthspan and lifespan	2
1.1.3 Hallmarks of Aging	3
1.1.4 Aging and the immune system	4
1.2 The immune system	4
1.2.1 Innate immunity	5
1.2.2 Interferon	7
1.3 Cytosolic DNA Sensing	8
1.3.1 STING is a major sensor of genomic stress	10
1.3.2 STING loss of function is a dominant polymorphism in humans	11
1.4 Senescence	12
1.4.1 Senescence, Aging, and Cancer	13
1.4.2 Consequences of Senescence	13
1.4.3 Senescence and Immunosenescence	14
1.4.4 Senescence-Associated Secretory Phenotype	14
1.5 Tissue Macrophages	15
1.5.1 Origin, function, and ontogeny of tissue resident macrophages	16
Chapter 2: Methods	18
2.1 Ex vivo techniques	18
2.2 Mice	18
2.3 Tissue collection and processing	18
2.4 In vitro techniques	19

2.4.1	Generation of L929-Conditioned Media	20
2.4.2	Generation of Bone Marrow Derived Macrophages	21
2.5	Quantitative Real Time Polymerase qChain Reaction (qRT-PCR)	21
2.5.1	Primer Designing	22
2.5.2	RNA Purification	22
2.5.3	qRT PCR	23
2.6	Single cell RNA Sequencing	23
2.6.1	Methodology	24
2.6.2	Alignment and Preprocessing	25
2.6.3	Clustering and cell type identification	25
2.6.4	Integration of scRNAseq samples	26
2.6.5	Principle Component Analysis	26
2.6.6	Uniform Manifold Approximation and Projection	27
2.6.7	Differential Gene Expression Analysis	28
2.6.8	NicheNet	29
2.6.9	Gene Set Variation Analysis	29
2.6.10	RNAMagnet	30
2.6.11	Polypositive	31
2.6.12	Gene Ontology	32
2.6.13	Network Analysis	33
2.7	Flow Cytometry	33
2.7.1	Flow Cytometry	34
2.7.2	Flow Cytometric Analysis and Gating	35
2.7.3	Optical Compensation	36
2.7.4	Fluorescence Activated Cell Sorting	36
2.7.5	Spectral Cytometry	37
2.7.6	Uniform Manifold Approximation and Projection (UMAP)	38
2.7.7	FlowSOM	39
2.7.8	Identification of tissue resident macrophages	39
Chapter 3: Results		40
3.1	Introduction	40
3.2	Regulation of Inflammation by DNA sensing	40
3.2.1	In vitro stimulation of Bone Marrow-Derived Macrophages	40
3.2.2	Conditioning of the immune response by DNA sensing	43
3.2.3	cGAS/STING-dependent sensing of DNA	47
3.2.4	Aging as a model of chronic activation of DNA sensing	48

3.3	Flow cytometric identification of tissue resident macrophages	52
3.3.1	Introduction	52
3.3.2	Identification and enrichment of tissue resident macrophages	53
3.4	Isolation of tissue-associated macrophages	57
3.4.1	Physical isolation	57
3.4.2	Fluorescence Activated Cell Sorting (FACS)	59
3.5	Age-associated inflammatory profile	61
3.5.1	Introduction	61
3.5.2	Inflammatory cytokines and chemokines are elevated in aging tissues	61
3.5.3	Mononuclear cell infiltrates increase in aging tissues	63
3.6	The effects of aging on transcriptional dysregulation	65
3.6.1	Background	65
3.6.2	Methodology	66
3.6.3	UMAP generation of scSeq datasets	67
3.6.4	Describing the Dataset – Population Identification	69
3.7	Generation of Immune Signatures	72
3.7.1	Immune fingerprinting	72
3.7.2	Relative distribution of age-associated immune fingerprinting	78
3.7.3	Generation of a Network-based description of immunity in aging	79
3.7.4	Senescence is a dominant immune program dysregulated with age	81
3.7.5	Correlating tissue-centric immunity and peripheral cytokines	81
3.8	Characterization of immune pathway enrichment	84
3.8.1	Gene set variation analysis	84
3.8.2	Population specific enrichment of immune pathways	86
3.8.3	Comprehensive dysregulation of immune pathways	88
3.9	Regulation of intrinsic tissue immunity through STING	90
3.9.1	Aging as a model of chronic DNA sensing	90
3.9.2	Inflammatory cytokines are further upregulated in the absence of STING	91
3.9.3	The absence of STING drives stromal and immune dysregulation	91
3.9.4	Comprehensive age-associated immune dysregulation is dependent on STING signaling	95
3.9.5	STING regulated predicted physical adherence of myeloid cells	98
3.9.6	Immune dysregulation determines macrophage responsiveness to stromal anchors	100
3.10	Discussion	103

3.10.1	Age-associated development of immune fingerprints	104
3.10.2	Pathway Enrichment	105
3.10.3	STING-dependent regulation of tissue intrinsic immunity	106
3.11	Transcriptional profiling of tissue macrophages	108
3.11.1	Subsetting single cell RNA sequencing	108
3.11.2	Describing the macrophage dataset	110
3.11.3	Expression of Tmem173 in resident macrophages	112
3.11.4	Immune fingerprinting of resident macrophages	115
3.11.5	Marks of senescence are not globally regulated in resident macrophages	116
3.12	Macrophage Directed flow cytometry	119
3.12.1	Identification of macrophages through spectral flow cytometry	121
3.12.2	FlowSOM to characterize and identify TRM populations	123
3.12.3	FlowSOM to identify macrophage populations – C57BL/6 and STING ^{KO}	127
3.12.4	Tissue macrophages: traditional gating	129
3.12.5	Homeostatic immunophenotype of TRMs.	132
3.13	Ex vivo analyses of TRM viability and vitality	137
3.13.1	Assessment of TRM intrinsic cell viability	137
3.13.2	In vitro induction of intrinsic TRM cell death	141
3.13.3	TRM distribution and disorganization	142
3.14	Contributions	145
Chapter 4: Discussion		147
4.1	General Overview	147
4.2	Significance of the work	152
4.2.1	Aged tissues are marked by immune fingerprints independent of circulating cytokines	152
4.2.2	Comprehensive immune dysregulation	153
4.2.3	STING signaling is a critical component in healthy aging	155
4.2.4	Potential ligands	156
4.2.5	Tissue resident macrophages are distinct immune elements of aging tissues	158
4.2.6	Tissue Macrophages are lost with age and functionally regulated by STING signaling	162
4.2.7	STING calibrates TRM vitality and sensitivity to cell stress	164
4.3	Consequences of Senescence	166
4.4	Incomplete TRM replacement	168

4.5 Future Directions	169
4.5.1 Proposed model	169
4.5.2 Overview	170
4.5.3 STING signaling in human health and disease	171
4.5.4 In vitro assays – STING and IFN responsiveness	173
4.5.5 Flow cytometric characterization of TRMs	174
4.5.6 Integration of immune reactivity	175
4.5.7 STING regulation of senescence progression/maintenance	175
4.5.8 STING regulation of cell death	176
4.5.9 Alternate activation of STING signaling	176
4.5.10 Independent signaling through cGAS, STING	177
4.5.11 Balance of nucleic acid sensing	178
4.5.12 Localization of interferon production	179
4.5.13 Senescence, health, and disease	180
4.5.14 Lineage-specific deletion of STING	182
4.5.15 Maintenance and function of fetal TRMs	183
4.5.16 STING in chronic injury models	184
4.5.17 Regulation of cell autonomous immunity by STING signaling	185
4.6 Final Conclusion	186
Chapter 5: Bibliography	189

List of Figures

Figure 1.1 cGAS/STING recognition of cytosolic DNA to propagate an interferon response	10
Figure 3.1 Stimulation of BMDMs with poly(dA:dT) induces IFN-I expression but not IFN-I regulatory programs.....	41
Figure 3.2 Transfection of DNA-analogues induces prolonged <i>Ifnb1</i> transcription with delayed IFN α 4/13.....	43
Figure 3.3 Overnight treatment with poly(dA:dT) maintains the IFN-I response potential of bone marrow derived macrophages greater than IFN α	45
Figure 3.4 Interferon lambda attenuates IFN-I responsiveness but maintains IFN λ 2 induction of the immunosuppressive marker <i>Agr1</i>	46
Figure 3.5 STING is a potent inducer of interferon and interferon stimulated genes with poly(dA:dT) stimulation	48
Figure 3.6 Aging tissues develop a quantifiable inflammatory and interferon signature.	50
Figure 3.7 cGAS/STING signaling contribute to tissue-wide interferon signatures by 6 months of age	51
Figure 3.8 STING contributes to, but is not a dominant driver of tissue interferon signature in 5 week old mice	52
Figure 3.9 Identification of tissue resident macrophages.	55
Figure 3.10 cGAS/STING signaling limits the expansion of tissue-associated macrophages in 6-month-old mice	56
Figure 3.11 Plate-adherence enrichment protocols sufficiently alter the TRM transcriptome	59
Figure 3.12 FACS sorting is not a viable enrichment procedure for tissue resident macrophage bulk transcriptomics.....	60
Figure 3.13 Inflammatory serum cytokines are elevated in aged mice	62
Figure 3.14 Aged tissues are marked by increased cellular infiltrates	64
Figure 3.15 Full cell type annotation of tissue associated cells.	70
Figure 3.16 Descriptive cell type annotation of general cellular populations.....	71
Figure 3.17 Tissue-specific cellular distribution of scRNAseq datasets.	72
Figure 3.18 Aging is marked by tissue-intrinsic gene modules.	75
Figure 3.19 Inflammatory genes can be segregated into age- and tissue-specific gene modules.....	76
Figure 3.20 Aged tissues are enriched with reactive inflammatory gene programs.....	77
Figure 3.21 Age-associated inflammation and interferon-associated genes are distributed among aged myeloid and stromal cells	79
Figure 3.22 Age associated immune dysfunction is best characterized by inflammation, interferon, and senescence programs.....	80
Figure 3.23 Senescence associated genes are not comprehensively regulated at the tissue level with age.....	82
Figure 3.24 NicheNet identifies signaling relationships between similar cell type regardless of tissue of origin.....	84
Figure 3.25 GSVA-enrichment scoring of inflammatory and interferon responsiveness is generally linear.....	86
Figure 3.26 Gene Set Variation Analysis-based distribution of immune signatures	87
Figure 3.27 Comprehensively immune dysregulated (polypositive) cells are conserved as a function of aging.....	90
Figure 3.28 Circulating inflammatory cytokines are elevated in the global absence of STING signaling.....	92

Figure 3.29 Age and STING signaling does not drastically alter general immune or stromal populations in the heart, liver, and kidney	92
Figure 3.30 STING signaling facilitates the maintenance of physical tissue architecture with age.	94
Figure 3.31 cGAS/STING signaling confers longevity	95
Figure 3.32 STING is a key modulator of age-associated immune signatures in the heart, liver, and kidney.	96
Figure 3.33 STING signaling selectively regulates immune dysregulation in stromal and myeloid cells.....	97
Figure 3.34 RNA Magnet predictions of cell-cell interactions	99
Figure 3.35 Polypositive regulation of meloid-stromal interactions are primarily dictated by the polypositive state of the myeloid cell.	102
Figure 3.36 Macrophage subsets from the heart, liver, and kidney scRNAseq datasets	109
Figure 3.37 Identification of tissue macrophage subsets.....	112
Figure 3.38 Descriptive pathway analysis of macrophage subsets.....	114
Figure 3.39 Aged macrophages are enriched in inflammatory gene pathways	114
Figure 3.40 STING expression maintains elevated expression in aged macrophages	115
Figure 3.41 Immune fingerprints are preserved with age in tissue macrophages.....	117
Figure 3.42 Senescence is not universally regulated by STING signaling in aged macrophages.	118
Figure 3.43 Inflammatory SASP-like cytokines are elevated in aged macrophages in the absence of STING signaling.	119
Figure 3.44 Flow cytometry gating strategy to identify tissue resident macrophages from the heart, liver, and kidney.....	121
Figure 3.45 cGAS/STING signaling regulates F4/80 ^{hi} macrophage maintenance with age	122
Figure 3.46 Flow-SOM identification of macrophage subtypes by spectral cytometry .	124
Figure 3.47 FlowSOM populations subdivide distinct macrophage subclusters	126
Figure 3.48 Tissue resident macrophages are decreased with age and in the absence of STING signaling.....	128
Figure 3.49 cGAS/STING signaling mediate F4/80 ^{hi} liver and kidney macrophage maintenance with age	130
Figure 3.50 cGAS/STING signaling regulates the inflammatory composition of F4/80 ^{hi} TRMs.....	131
Figure 3.51 Homeostatic genes are preferentially enriched in TLF-like TRMs	133
Figure 3.52 Homeostatic gene expression is decreased in the absence of STING signaling	135
Figure 3.53 Protein expression of homeostatic receptors are reduced in the absence of STING signaling.....	136
Figure 3.54 Relative rate of change of GSVA activity scores in TRM subsets	138
Figure 3.55 STING signaling regulates apoptotic signaling in aged TRMs	139
Figure 3.56 STING signaling prevents the accumulation of double strand DNA breaks in aging Kupffer cells.	141
Figure 3.57 STING signaling is a critical regulator of TRM distribution in aged tissues.	143
Figure 4.1 Representative model describing the regulation of multiple immune signatures by STING signaling during aging.....	151

List of Abbreviations

AIM2 – Absent in Melanoma 2
ANOVA – Analysis of Variance
B-ME – Beta mercaptoethanol
BCR – B cell receptor
BMDM – Bone Marrow Derived Macrophage
CD – Cluster of Differentiation
CDN – Cyclic di-nucleotides
cGAMP – cyclic GMP-AMP
cGAS – cyclic GMP AMP Synthase
CSF1 – Colony stimulating factor 1
DC – Dendritic Cell
DEGs – Differentially Expressed Genes
DISC – Data Intensive Studies Center
DLAM – Division of Laboratory Animal Management
DMEM – Dulbecco's Modified Eagle's Medium
EDTA – Ethylenediaminetetraacetic acid
EMP – Erythro Myeloid Progenitor
ERV – Endogenous Retrovirus
eWAT – Epididymal White Adipose Tissue
FACS – Fluorescence Activated Cell Sorting
FBS – Fetal Bovine Serum
FlowSOM – Flow Self Organizing Maps
GEMs – Gel Beads in Emulsion
GO – Gene Ontology
GOF – Gain of Function
H&E – Hematoxylin and Eosin
LOF – Loss of Function
LPS - Lipopolysaccharide
GSEA – Gene Set Enrichment Analysis
GSVA – Gene Set Variation Analysis
IACUC – Institutional Animal Care and Use Committee
IFN – Interferon
IFNAR – Interferon Alpha Receptor
INF – Inflammation
IRF3 – Interferon Responsive Factor 3
ISGs – Interferon Stimulated Genes
KEGG – Kyoto Encyclopedia of Genes and Genomes
kNN – K Nearest Neighbors
L929 – L929 Supernatant
LEC – Lymphatic Endothelial Cells
MCSF – Macrophage Colony Stimulating Factor
MHC – Major Histocompatibility Complex
MoDM – Monocyte Derived Macrophage
NFkB – Nuclear Factor kappa B
NIA – National Institute of Aging
NIH – National Institute of Health
OCT – Optimal Cutting Temperature
PBS – Phosphate Buffered Saline
PCA – Principal Component Analysis

PP – Poly Positive
PRR – Pattern Recognition Receptors
qRT-PCR – Quantitative Real Time Polymerase Chain Reaction
RBS – Rodent Breeding Services
RPMI – Roswell Park Memorial Institute
SASP – Senescence Associated Secretory Phenotype
SAVI – STING-associated Vasculopathy with onset in Infancy
SEN – Senescence
SNP – Single Nucleotide Polymorphism
STING – Stimulator of Interferon Genes
TBK1 – TANK Binding Kinase 1
TCR – T cell receptor
TLF – Tim4/Lyve1/Folr2
TMC – Tufts Medical Center
TRM – Tissue Resident Macrophage
tSNE – t Stochastic Neighbor Embedding
TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling
UMAP – Uniform Manifold Approximation and Projection
UMI – Unique Molecular Identifier

Chapter 1: Introduction

1.1 Aging

The concept of aging has been at the forefront of human curiosity for millenia, dating as far back as Greek philosophers considering aging and associated diseases as a natural process, a disease of the body, or the mind. Through the exploration of the New World and the quest for the Fountain of Youth mankind, humanity has yearned for control over the slow descent through aging, seeing this desire spill into the supernatural through works of fiction and horror (Dracula, The Picture of Dorian Grey). In the more modern era, even the age of medical research and biological understanding, a unified theory of aging and the emergence of chronic or debilitating diseases has generally remained a mystery.

In the natural world aging is a certainty – aging is a mark of the passage of time – yet for the vast majority of organisms on the planet, is not a real concern. Most animals live and die in an environment where “old age” is only experienced by a select few. Only since humanity has evolved, settled, and gained a greater understanding of natural medicine has the concept of an aging society even been comprehensible. Every society has referenced “elders” that rule or pass on their wisdom to the future generations, but the crux of humanity has always been disease; be it infectious or cancerous. The most modern advancements of medicine have created such an incredible shift in the median lifespan that the human population, as a whole, is now aging. In fact, the human population is aging so fast that the senior (65+) population in the United States is expected to double between 2015 and 2060. ¹ In fact, within the next decade it is projected that aged adults will outnumber children for the first time in United States History.

1.1.1 Aging and human disease

The progression of aging has forever been linked to the emergence of disease, be it physical, mental, or infectious – even before a true understanding of these mechanisms were known²⁻⁴. Most notable, since the discovery and understanding of the immune system and inflammation, has been the clear, stepwise emergence of a chronic inflammatory response termed “inflammaging” that has more recently been accepted as a true “hallmark” of aging.^{5,6} The identification of these inflammatory markers – most notably IL6 – have long been viewed as biomarkers or even potential drivers of age-associated inflammatory dysfunction.^{7,8} In fact, recent work using longitudinal profiling identified four core “systems” that were predominately affected by aging, included a generalized “immune aging.”⁹ However, the severity of dysfunction across the other three “ageotypes” were all elevated with increasing impact across the immune system. This observation is further supported by work identifying not only an increase prevalence of morbidity with age, but that by the age of 50 half of US adults are experiencing at least one chronic inflammatory disorder, while that prevalence rises to three or more by the age of 75.¹⁰

1.1.2 Healthspan and lifespan

The emergence of these chronic inflammatory disorders and diseases with age is often considered one and the same – aging is disease. However even Plato felt it necessary to distinguish the concept of aging from that of disease, which was focused strongly on aging of the intellect than the body. The distinction between aging and disease is best conceptualized in the terms “lifespan” and “healthspan”; healthspan being a remarkable decrease in quality of life – be it mobility, independence, or other functional abilities. It has even been suggested that the “health adjusted life expectancy”

decreases by as much as 10 years, accounting for the emergence of age-associated morbidities.¹¹

A significant amount of aging research – professional or amateur – highlights the extension of lifespan as an essential need. While the root cause of aging is still uncertain, it is still believed that there is an upper-limit to human lifespan that, while remarkable, is not wholly unattainable.¹² However, the extension of healthspan would be a demonstrably greater increase to human quality of life, decrease burden on the healthcare system, and generate overall greater outcomes for the human population as it would certainly affect millions more that currently are expected to suffer from debilitating disease.

1.1.3 Hallmarks of Aging

While aging is generally categorized as a chronic inflammatory state, there has been a more recent push to understand the core attributes of aging that support or drive all other diseases – the “hallmarks” of aging.^{6,13} While many of these hallmarks can be attributed to a dysfunctional immune system (chronic inflammation, dysbiosis) the majority of these issues are intrinsic defects within a cell with direct impact on the cell’s ability to function normally such as genomic instability, decreased autophagy, or engagement of a senescent phenotype. What remains quite interesting about these hallmarks is very few, if any, of them are unique to immune cells – which have been a major focus of the theory of aging as primary regulators of an inflammatory response. In fact, while somewhat unappreciated, canonically nonimmune cells also retain the ability to participate in immune and inflammatory responses, emphasizing that aging is a biological process at the cellular level with systemic effects.¹⁴

1.1.4 Aging and the immune system

Immune involvement, in the context of dysfunction, is a widely accepted phenomenon in which the immune system not only contributes to the chronic inflammatory syndrome observed in aging patients but generally deteriorates. A clear inability to effectively respond to pathogenic infection, mount a protective humoral response to vaccination, and adequately respond to tissue damage and initiate a wound healing response are common defects cited when discussing the aged immune system.¹⁵⁻²⁰

A competent immune system has evolved over millennia to defend against pathogens, assist in tissue function, and repair physical damage. Modern medicine has effectively filled many, if not most, of the dysfunctional roles of the aging immune system. Robust antibiotics and increasingly effective vaccination strategies can support the aged immune system and most symptoms can be managed through analgesics, anti-inflammatories, steroids, and even functional supportive methods such as forced respiration or transfusions. In fact, a systemic analysis of all-cause mortality has shown that death rates have, generally, progressed away from communicable, or nutritional causes towards non-communicable disease.²¹ This emergence of non-communicable disease is a major aspect of the age-associated decline in quality of life that is such a detriment. Cancer, cardiac dysfunction, aortic stiffening, autoimmunity, kidney or liver failure, and neurodegeneration are so common with ageing they are in fact delineated by “early onset” or “pediatric” when they occur outside of the elderly.

1.2 The immune system

The immune system is a complex, yet delicate network of cells specifically evolved to recognize and respond to danger signals: damage, stress, and infection. Rudimentary immune systems likely emerged alongside early life in a manner to

recognize self from non-self and facilitate organismal duplication. Through the integration of beneficial mutations, duplications, and deletions the mammalian immune system has evolved into a remarkably effective framework to respond to virtually any potential pathogen.

1.2.1 Innate immunity

The innate immune system consists of the earliest responses from canonical immune cells: phagocytic neutrophils, monocytes, dendritic cells, and macrophages involved to varying degrees in the breakdown of pathogens or cellular debris and presentation on major histocompatibility complex (MHC)-I or -II, or basophils, eosinophils, and mast cells: granulocytes critical for responses against extracellular pathogens such as helminths.^{22,23} Cell intrinsic, or cell autonomous, immunity is a largely unappreciated aspect of the immune system, consisting of receptors of cellular health, stress, damage, and pathogenic infection. These pathways are generally conserved regardless of cell type, and as such are not restricted to classical immune cells. The true first line of defense, these pathways are critical to provide initial sequestering of pathogens by inducing “bystander” activation through the action of a family of immune cytokines called “interferon”, limiting viral replication through engagement of cell cycle inhibition, and preventing bacterial replication through directed cellular death pathways to remove a dying cell and begin the recruitment of specialized immune cells.²⁴

Cells of the innate immune system are poised to respond to general pathogenic proteins through the engagement of pattern recognition receptors (PRRs) that recognize conserved repeated structures. Phagocytes, specifically macrophages and dendritic cells, specifically function to “sample” their surroundings for yet-undetected pathogens or damage signals. Neutrophils and monocytes are more generally understood to circulate

and even “patrol” through tissues until certain immune responses are engaged and specific activating cytokines are released at which point these cells “swarm” in an attempt to clear any potential pathogen and protect the tissue.²⁵

A dominant function of the innate immune system is digestion of circulating proteins or phagocytic destruction of pathogens for integration with the adaptive immune system. While phagocytic processes are continuous, upon receiving proper activation signals, phagocytes exit their tissue of residency and migrate to the local draining lymph node. Here, digested proteins are displayed upon MHC-I and MHC-II for the benefit of affinity maturation and initiation of T cell responses by the adaptive immune system.

The most common branch of the immune system people are familiar with are the adaptive immune cells. Primarily T and B lymphocytes, these cells are dominantly engaged during symptomatic infections, driving cellular- and antibody-mediated immune responses. A key attribute of cells of the adaptive immune response is the ability to gain and maintain specificity towards a specific protein. T and B cells are constitutively produced from the thymus and bone marrow, respectively, and constantly circulate through the blood, spleen, and lymph nodes, probing MHC-I and MHC-II on dendritic cells in search of their cognate antigen. Upon recognition of their antigen, and activation, these lymphocytes undergo significant clonal expansion to begin to mount a specific immune response.²⁶

T cells, specifically, are selected for non-self-reactive but functional T cells in the thymus through random recombination of the constant and variable regions of the T cell receptor (TCR), and commitment to the CD4-expressing or CD8-expressing lineage. These randomly specific T cells patrol the periphery and lymph nodes until specific ligation of their TCR with MHC-I (CD8 T cells) or MHC-II (CD4 T cells) at which point they undergo clonal expansion to combat the infection.

B cells, on the other hand, upon engagement of their B cell receptor (BCR) experience clonal expansion with significant replicative promiscuity to allow for random mutations within their specific receptor. While many of these mutations will significantly reduce the quality and specificity of the receptor, the cells that *increase* their specificity will continue to expand, until sufficiently strong enough to exit the lymph node and become antibody-producing cells.²⁷

Both T and B cells can functionally mature into active cells to fight an infection as well as cells that become long-lived (resident) memory cells. These memory cells are the most critical aspect of the adaptive immune system, as the maintenance of memory cells offers the ability to increasingly fast and specific immune responses to re-challenge with an infectious agent.

1.2.2 Interferon

The interferon family of cytokines are a family of cytokines originally identified due to their critical role in the regulation of viral infections, by “interfering” with their replication.²⁸ The interferon family of cytokines consists of three distinct families based on their receptor ligation, Type I (IFN α 1-14 and IFN β through IFN-I alpha receptor (IFNAR) 1 and 2), Type II (IFN γ through the IFN γ receptor) and Type III (IFN λ 1-4 through the IFN λ receptor and IL10 receptor). While IFN-I and IFN-III are still generally considered to induce similar responses, their receptors (and relative distribution of said receptors) are very different.²⁹ However, both of these IFN families induce generalized, robust IFN-I responses in diverse cell types. IFN γ , however, appears to be much more specific to cells of the adaptive immune response, while IFN α , IFN β , and IFN λ can be expressed by many different cells with some regional specificity.³⁰⁻³³

While IFN-I (and IFN-III) is generally understood as an antiviral effector cytokine, its functional role is exceptionally broader. An early effector cytokine, IFN-I is a key

regulator of bystander activation that induces a robust anti-viral response in stromal and parenchymal cells to attempt to restrict viral replication. Further, IFN-I can act to regulate antigen presentation capabilities in myeloid cells, maturation of T cells to effector T cells, and B cells to specialized antibody secreting plasma cells²⁸. As IFN-I is such a critical cytokine at every aspect of the immune response, it is not wholly surprising that the identification of an interferon “signature” – quantification of a cassette of genes downstream of the interferon signaling cascade – can be detected in many acute and chronic inflammatory diseases including cancer and autoimmunity. ²⁴

Intriguingly, but not entirely surprising due to its almost ubiquitous involvement in immune activation, generation of IFN-I is not restricted to active immune responses but are produced constitutively in a manner termed “tonic” interferon signaling. ³⁴ While the function and stimuli that drive this tonic expression of interferon is still debated – the production of constitutive IFN-I is so low it cannot directly be detected and requires the tracking of interferon stimulated genes (ISGs) as a proxy for IFN-I signaling – it is clear that this tonic signaling provides some distinct homeostatic potential for the system. ³⁴

1.3 Cytosolic DNA Sensing

Nucleic acid sensing consists of a highly conserved set of pathways that are functionally capable of sensing DNA or RNA in all compartments of a cell. ^{35,36} These pathways are adept at recognizing misplaced, foreign, and damaged strands of both DNA and RNA to propagate powerful inflammatory and interferon responses. Although commonly considered primary responders and mediators of antiviral immune responses, nucleic acid sensors are not inherently capable of differentiating between self and non-self-nucleic acids, whereby acting as mediators of infection as well as cellular damage and stress.

In a healthy cell, nucleic acids consist primarily as DNA confined to the nucleus, or as one of several types of RNA with functional responses throughout the cell.

Constitutive expression of multiple DNases in the cytosol reinforces the restriction of DNA to the nucleus, suggesting that the presence of DNA in the cytosol is of particular importance as a damage or pathogen associated signal. As such, the role of cytosol DNA, and its sensors, have emerged as a particular point of interest in the realm of infection, autoimmunity, and aging.

The primary sensors of double stranded DNA in the cytosol are AIM2 (Absent in melanoma 2) and cGAS (cyclic GMP AMP synthase).³⁶ AIM2 itself is a potent initiator of the inflammasome, an inflammatory complex that precedes inflammatory cell death. cGAS, however, generates the second messenger cGAMP (cyclic GMP-AMP) to activate the intermediate STING (STimulator of Interferon Genes) to induce broad inflammatory responses through TBK1 (TANK-binding kinase 1) and NFκB (nuclear factor κB), as well as a strong interferon response through IRF3 (interferon response factor 3). (Figure 1.1) The function of AIM2 appears to be dominantly regulating inflammasome-mediated cell death, however STING signaling generates a powerful immune response that has been implicated downstream of DNA damage and mislocalization in the context of infection, cancer, genomic stress, and more recently models of accelerated aging.^{36,37}

1.3.1 STING is a major sensor of genomic stress

Although commonly associated with induction of an antiviral immune state, the primary function of the cGAS/STING signaling pathways is to recognize mislocalized or damaged double stranded DNA. Healthy cells effectively sequester DNA within the nucleus, tightly wrapped along histones to prevent errant transcription. The presence of DNA in the cytosol is markedly a sign of genomic stress, nuclear damage, or mitochondrial dysfunction as cGAS can bind both self and non-self-DNA.

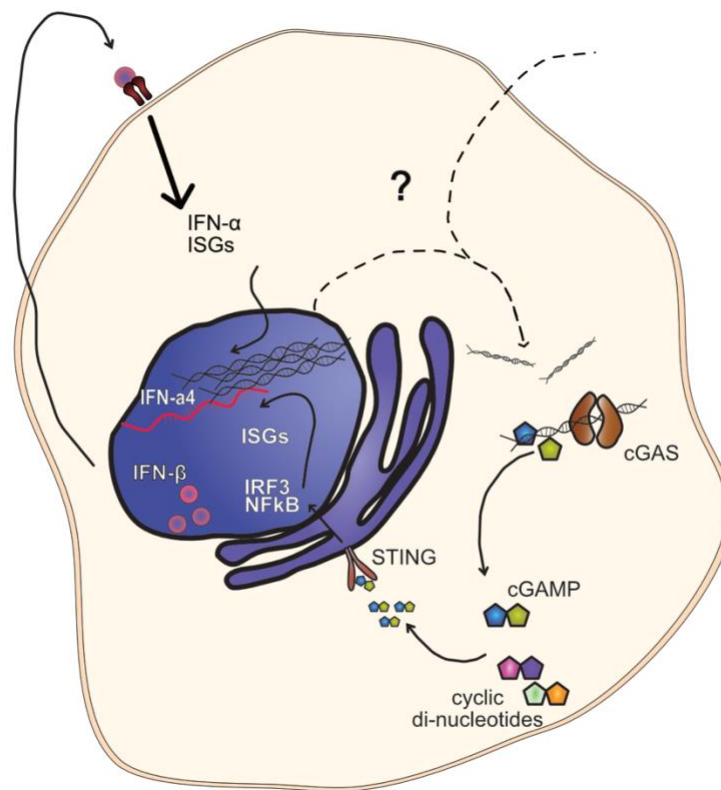


Figure 1.1 cGAS/STING recognition of cytosolic DNA to propagate an interferon response

Common models of cGAS/STING activation, in fact, heavily weigh on the loss of nuclear integrity (LMNA, YAP/TAZ deficiency) and mitochondrial genomic instability (POLG deficiency) that result in transference of nuclear or mitochondrial DNA fragments

into the cytosol.^{38,39} However, more direct models of genomic stress – irradiation – are sufficient to drive potent interferon responses dependent on STING signaling and even induce a senescence-like phenotype.

The hallmarks of aging described previously are incredibly centered around general genomic stress and dysfunction, which are believed to be significant drivers of age-associated pathology. As STING is a major regulator of immune responses to genomic stress, this pathway has often been suggested to be a dominant driver of age-associated senescence and inflammatory pathology. Although supported by models of accelerated aging, the direct role of STING in aging and age-associated pathology remains unclear. Recent work has attempted to solidify STING as a dominant driver of age-associated pathology by further deleting STING in models of accelerated as well as pharmacological blockade of STING in advanced aging.^{38,40} However, models of progeria are, effectively, direct activators of STING and are insufficiently replicated biological aging; and acute pharmacological blockade of STING in advanced aging doesn't adequately address the role of STING throughout the aging process, only after significant pathology has developed.

1.3.2 STING loss of function is a dominant polymorphism in humans

Human-directed research can be a particularly difficult field of study due to extensive genomic variation that can compound biological variability and confound certain analyses. While this genetic variation is difficult to control for during experimental design, large, retrospective meta-analyses can fully leverage the power of this genetic variation. Polymorphisms that exist within the human population can confer both gain- and loss-of-function potential to one or both alleles of a gene to varying degrees.⁴¹⁻⁴⁴ While some of these polymorphisms are exceptionally rare, others persist within the population sufficiently to measure through retrospective analyses.

STING (TMEM173) is a particularly interesting gene in the human population, due to the extensive persistence of multiple loss-of-function polymorphisms: R232 (full function), H232 (R232H, partial loss of function), and “HAQ” (R71H, G230A, R293Q, originally identified as a loss of function, recent work identifies G230A as rescuing mutation that may alter STING sensitivity rather than act as a full loss-of-function mutation).^{42,44} Further, there are rare gain-of-function mutations that result in severe interferonopathy. Although STING signaling is a critical component of the immune response, senescence, and cell death, a remarkable portion of the human population carries H232 or a variation of HAQ.⁴⁵

These loss of function mutations exist in almost half the human population and are enriched to various levels in different ethnic backgrounds. Together, this suggests there existed a selective evolutionary pressure towards a *loss* of STING function. This is particularly intriguing, as STING is a critical mediator of cell stress, bacterial cyclic-di-nucleotide responses, or viral infection and evolutionarily does not seem like a pathway that would be beneficial to lose. Further, with such a dominant deficit in STING signaling in the human population it is critical to further understand the chronic loss of STING signaling on health and disease.

1.4 Senescence

The induction of senescence was first observed in cell culture and was marked as the state at which cells lost their ability to continue dividing – replicative senescence. While senescence is best considered a state of permanent growth arrest, marked by the key factors p16 and p21, more recent research has begun to identify a key role for senescence (or a senescence-like state) in various models of immune activation such as wound healing.⁴⁶⁻⁴⁹

1.4.1 Senescence, Aging, and Cancer

Aging is beset by inflammation and cancer. As unrestricted growth of cells can irreparably harm the host, redundant regulatory steps have evolved with the goal of halting unrestricted cellular division and pre-cancerous growths. As tumors generally occur from inborn errors of the genetic code, any damage to DNA can cause errant repairs, and retained errors can lead to unrestricted activation of oncogenes. A common method of cellular-level protection from oncogenesis is abrupt halting of cellular division to a state of quiescence where nuclear damage is resolved, or the cell undergoes regulated cell death. While a state of quiescence and growth arrest is part of the response, cells can enter a progressive state of senescence while a repair of the damaged DNA is attempted. Senescence, generally an irreversible state of growth arrest, can quickly and assuredly act to restrict the progression of oncogenesis in the host.⁴⁶ The balance of senescence and cancer is an exceptionally fine edge to balance. On one hand, the unrestricted outgrowth of any cells can ultimately cause an entire organ system to cease to exist; on the other hand, extensive propagation of senescence leads to a similarly dysfunctional immune system.⁵⁰ Senescence may impart the vital retention of highly specialized cells that, if lost, would directly affect tissue function. Protection against imminent damage and harm is a priority for the host, which may be the strongest support for persistence of senescence with aging. The progressive loss of organ system function gradually reduces the fitness of the host, but over a longer timespan than sudden metastatic tumorigenesis.

1.4.2 Consequences of Senescence

Progressive accumulation of senescent cells is believed to be a vital regulation against tumorigenesis, but quickly can disrupt the entire functional ability of an organ system. While senescent cells likely serve a vital evolutionary purpose, with the

significant extension of median human lifespan, it appears more likely that senescence cells are becoming detrimental.⁵¹ Substantial research funding is currently allocated towards understanding the methods that drive senescence, but further how to specifically clear senescent cells to allow healthy, functional cells to take their place. Through selective clearance in murine models of age-associated senescent to therapeutic pharmaceuticals (senolytics) specific removal of senescent cells has been shown to be negatively correlated with the progression of disease suggesting that the accumulation of senescent cells is ultimately detrimental to the host.⁴⁸ While true, it is essential that we better understand the role senescent cells play in all organ systems before they are wantonly removed.

1.4.3 Senescence and Immunosenescence

A large portion of research into senescent cells has focused on easily testable structural cells called fibroblasts. Fibroblasts grow easily in culture and have become the bulk of senescence research. Interestingly, although an ever-increasing amount of literature focuses on stromal and parenchymal cells for the induction of senescence, immune cells can undergo another specialized form of senescence termed immunosenescence.

1.4.4 Senescence-Associated Secretory Phenotype

One key attribute of senescent cells fate and function is the progression to SASP, the senescence associated secretory phenotype, an inflammatory state of unclear etiology. While commonly believed to be a state of pathology – result of accumulating and persistent senescence, it is possible that this inflammatory state is specifically engaged as part of a clearance mechanism. Initiation of chemotactic

inflammation to signal local resident phagocytes to stress or damaged cells have progressed sufficiently beyond repair and require clearance.

The immune response has evolved and is primed to deal with very short-term problems. Many immune reactions cause direct, local, damage to the tissue but rely on the functional ability to repair this damage after clearance of the pathogen. However, the persistent exposure to a toxin, pathogen, or stressor continues to engage these systems that are acutely protective, but chronically detrimental or even pathological. Senescence is a prime example of this balance between short- and long-term responses. Acute, sparse initiation of senescence can be critical to limit cell-intrinsic stress and participate in tissue-remodeling processes, but it appears there is a critical mass of cells progressing through senescence towards SASP that contributes to pathology.

1.5 Tissue Macrophages

The macrophage was first identified by Ilya Metchnikoff in 1882 as a critical participant in the host defense against infection.⁵² Since this discovery the understanding of the macrophage in host defense, inflammatory homeostasis, and tissue function has expanded exponentially. The breadth at which these cells can participate in the immune process is astounding, ranging from highly inflammatory or phagocytic, to specialized cells participating in tissue repair and remodeling.⁵³⁻⁵⁵ Originally described based off characteristic *in vitro* assessment, macrophages were shoe-horned into an LPS-derived classically activated “M1” macrophage and IL4-driven alternatively activated “M2” macrophages.⁵⁵ More recently, however, the state of the macrophage, while long understood to retain some reactive plasticity, rather than bifurcating based on a primary stimulus existed along an activation spectrum, balanced between the traditional classical and activated phenotypes.^{53,54}

Traditional macrophages originate from myeloid precursors in the bone marrow, mature through a monocytic intermediate, and ultimately mature into the macrophage during a state of inflammation. Intriguingly, these cells are not found solely in states of active inflammation or immune responses. In fact, macrophages can be found throughout every tissue even in the absence of inflammation, unlike what is seen in resident lymphocytes post-exposure.

1.5.1 Origin, function, and ontogeny of tissue resident macrophages

Tissue resident macrophages (TRMs) are a unique and fascinating cell type. While morphologically a macrophage, these cells originate independent from traditional bone marrow- or monocyte-derived macrophages (BMDM, MoDM). While MoDMs are capable of developing into resident macrophages, canonical TRMs develop in utero and precede the generation of the bone marrow and what is termed “definitive hematopoiesis”.⁵⁶⁻⁶¹ Although not fully appreciated, it has slowly become clear that these fetal-derived TRMs are exceptionally unique and may, in fact, participate in completely novel functions when compared to MoDMs.^{25,62-65}

TRMs were first appreciably discovered through deletions of key transcription factors for myeloid development in the bone marrow, such as Myb or for macrophages specifically (PU.1).^{66,67} Here, these specific deletions identified macrophages independent of the hematopoietic marker Myb yet require the macrophage lineage commitment signals from PU.1. Further, the regulation of fetal macrophages has further been divided into waves of development, where these cells first emerge from the yolk sac and then may exist through a monocyte-like erythro-myeloid progenitor (EMP) from the yolk sac and fetal liver.⁵⁷ Further investigation has led to the general understanding that these macrophages are ingrained into the development and function of the tissue as

a whole – even as essential as acting to regulate the conduction of electrical conduction in the heart for ventricular contraction. ^{60,63,65,68-70}

Incredibly, these TRMs have even been shown to directly orchestrate immune responses and, in effect, tissue homeostasis. Brilliant work from Dr. Stefan Uderhart and Dr. Ronald Germain described a process by which homogeneously distributed TRMs in the peritoneal wall could functionally “cloak” inflammatory reactions downstream of single cell “microlesions” allowing the TRMs adequate time to clear the cellular debris. ²⁵ In the absence of these cells – or in the case of a larger, multicellular lesion, TRMs were insufficient to contact, sequester, and limit the propagation of an inflammatory response inducing a “swarming” reaction by neutrophils and monocytes. These observations truly highlight the ontological differences of TRMs and monocytes (even MoDMs) to the forefront – are these cells poised to perform the same response in the context of tissue damage or infection or do these two lineages specifically exist as part of an immunological threshold.

Chapter 2: Methods

2.1 Ex vivo techniques

2.2 Mice

Male and female C57BL/6 background mice were used for the full set of experiments contained in this body of work, along with cGAS^{-/-}, STING^{-/-}, IRF3^{-/-}, and IFNAR^{-/-} all on the C7BL/6 background. To test time course experiments, mice were grouped into young (2-4mo), early aged (6-8mo), middle aged (12-14mo), late aged (16-20mo), and aged (22-26mo). All mice were aged in house at the Tufts Medical School or purchased as middle aged or aged mice from the National Institute of Aging (NIA) at the National Institute of Health (NIH). Any mice sourced outside of Tufts Medical were housed on site for at least a month before being used in experiments. Mice from all genotypes were assigned to longevity analysis with daily health checks from the Tufts University Division of Laboratory Animal Management (DLAM). Lifespan was allowed to reach a natural end, or was required by DLAM based on humane endpoints, including body condition scoring and disease. All breeding was maintained by the Tufts University Rodent Breeding Services (RBS) with oversight from the Institutional Animal Care and Use Committee (IACUC) and Tufts Medical School. All mice were maintained with standard chow and water ad libidum and housed under specific pathogen free conditions with a 12-h light cycle.

2.3 Tissue collection and processing

All mice were euthanized through the administration of carbon dioxide (CO₂) gas administration at 4.5L/min per IACUC and TMC regulations. After euthanasia, blood was collected through cardiac puncture as a secondary mode of death with a 25-gauge needle and syringe. Blood was allowed to clot at room temperature for at least 30

minutes in an RNase free 1.5mL Eppendorf tube. Clotted blood was spun at 10,000rpm for 10min in a refrigerated centrifuge and serum aliquoted and stored at -80C for future analysis.

Organs were collected by blunt dissection into DMEM, weighed, and either embedded and flash frozen in optimum cutting temperature cryo-embedding medium (OCT) and stored at -80C or dissociated and processed for single cell suspensions for further analyses (flow cytometry, cell culture, or single cell sequencing). Specifically, tissues were finely chopped with a razor blade, incubated with 200U/mL collagenase II and DNase I in serum free DMEM at 37C with intermittent shaking (heart: 20min; liver: 40min; kidney: 45min). After enzymatic digestion, minced tissue was mechanically dissociated through a 70µm filter, red blood cells were lysed with ACK red blood cell lysis buffer per manufacturer's instructions, dead cells were removed using the Miltenyi Debris Removal Kit (following standard protocol). Cells were counted with the Countessa cell counter with Trypan Blue viability stain or manually with a hemocytometer with a Trypan Blue counter stain. Cells were resuspended and aliquoted for experimental use.

2.4 In vitro techniques

In vitro analyses are particularly vital for understanding cellular mechanisms. In vitro techniques are highly specialized and can be performed under both highly specific and controlled conditions, however they are often missing vital aspects of an in vivo experiment. It is incredibly vital that in vitro experiments are designed in such a way that only attempts to address a specific question and doesn't attempt to directly correlate to in vivo biology, without an accompanying experiment.

Further, in vitro techniques, while lacking certain aspects of in vivo work, have the express power of scale. One euthanized mouse can serve to supply multiple technical replicates and experimental conditions – vastly reducing the number of living

animals that must be euthanized. While unable to completely replicate in vivo biology, the advent of new culture systems, transwell devices, or 2D/3D organoids are making these experiments much more powerful with every passing year, furthering the ability of researchers to limit their usage of live animals in experiments.

2.4.1 Generation of L929-Conditioned Media

L929 cells are murine-derived fibroblasts that overexpress the macrophage colony stimulating factor/colony stimulating factor 1 (mCSF/CSF1) protein that is vital for the maturation and expansion of bone marrow myeloid precursors into mature macrophages. While it is possible to generate mature macrophages with recombinant mCSF, the generation of conditioned supernatant from L929 cells adds additional growth factors and pro-survival cytokines to the media that, while not well described, provide extra nourishment, and generate healthier macrophages.

First passage L929 cells were expanded for generation of L929 conditioned supernatant. Cells were quickly thawed and resuspended in complete medium (RPMI, 10% FBS, 0.1% Ciprofloxacin. Live cells were counted and plated in cell culture treated T175 flasks at 1×10^5 /mL in 40mL. Cells were cultured and split every 7 days at which point the entire cell supernatant was collected, any non-adherent cells removed, and stored for future use.

Remaining, adherent, L929 fibroblasts were gently washing with sterile PBS, and resuspended with warm Trypsin/EDTA (5min at 37C). Resuspended cells were washed with complete medium, counted, and replated at 1×10^5 cells/mL in 40mL in cell culture treated T175 flasks at which point the above culture procedure was repeated. Stored L929 conditioned medium was tested for efficacy by differentiating bone marrow derived macrophages at different concentrations. It was found to be sufficiently potent at 10% L929 medium.

2.4.2 Generation of Bone Marrow Derived Macrophages

Mice were euthanized as described above, and full femur and tibia were removed and cleaned of muscle tissue. Using sterile technique, the ends of the femur and tibia were removed, exposing the medullary cavity and marrow. Using an 18G needle and syringe, the bone marrow was flushed with DMEM and passed through a 70µm filter and washed (with 10mL DMEM for 5min at 500xg). Red blood cells were lysed with ACK red blood cell lysis buffer and washed. Bone marrow precursors were then resuspended in 10mL cell culture media (DMEM +10% FBS, 0.1% Ciprofloxacin), and split among 2 large, untreated 10cm plates. Cells were cultured, undisturbed, for 4 days when they were resuspended using a flat cell scraper, fed with additional 10mL culture media, and split into 2 additional (4 total) 10cm plates for an additional 3 days. At day 7, cells were adherent, indicative of mature bone marrow-derived macrophages.

2.5 Quantitative Real Time Polymerase qChain Reaction (qRT-PCR)

qRT-PCR is a classic system that utilizes cellular replication machinery to form homologous pairs of RNA strands and specifically designed primers to logarithmically expand genes of interest. Using a cyclical heating and cooling cycle, double stranded DNA segments can be gently denatured, and as they re-associated with their homologous pairs in the presence of saturating primer pairs, reverse transcriptase enzyme, and sufficient nucleotides, these segments will be available for association with the reverse transcriptase enzyme which will specifically elongate the primer/gene pairing. With repeated cycling of heating and cooling the presence of the primer/gene pairing will exponentially grow. Further, this system is run in the presence of a fluorescent probe that only fluoresces when it intercalates into double stranded DNA. As

the targeted gene segment is expanded, more of the fluorescent probe is sequestered appropriately.

2.5.1 Primer Designing

Nucleic acid oligomers (16-22 nucleotides) were designed complimentary to specific exons located within several genes of interest. These sequences were determined using the NIH NCBI Primer-BLAST online tool. In brief, the accession number of the murine gene of interest was used as an input, and all recommended settings were used. The tool returns a list of possible primers, including possible overlap with other murine genes or gene homologs. All primers were optimized prior to continued usage for specificity.

2.5.2 RNA Purification

Tissues were processed and single cell suspensions generated as described above, and 0.5-1E06 total tissue cells were aliquoted for RNA extraction with Qiagen RNeasy kits. In short, Cells were isolated as described above, either from tissues, bone marrow, or cultured bone marrow derived macrophages. Cells were washed and the pellet was resuspended using the proprietary RLT Buffer with 1% β -mercaptoethanol (β -ME, to denature cell intrinsic RNA degrading enzymes). In the case of bone marrow-derived macrophages RLT Buffer was added directly to the plate and the lysate collected. The cell lysate was added to the RNeasy spin column and centrifuged (washed: 8000xg for 15s) to remove the RLT buffer. Here, an additional DNase treatment was performed to ensure the effective removal of single exon genes, such as Il1b. 10uL of DNase1 + 70uL Qiagen RDD buffer and added the RNeasy column incubated for 15min washed. The RNeasy column was then washed with 350uL of

Qiagen buffer RW1. The sample in the RNeasy column was then washed twice with 500uL Qiagen RPE buffer, with added ethanol to ensure the RNA stays precipitated and bound to the column, for 15s and 2 min, respectively, to dry the membrane of any liquid. As RNA is water soluble, 30uL of RNase-free water was then added to the spin column to elute the purified RNA.

2.5.3 qRT PCR

All qRT-PCR reactions were performed with the ThermoFisher QuantStudio 6 Flex RT-PCR machine and standard settings. A melt curve was included with each run to ensure primer specificity. In brief, the temperature of the qRT-PCR system is slowly increased causing double stranded DNA segments to dissociate, shorter to longer. A majority of the fluorescent probes are sequestered into the gene of interest, and when the temperature is sufficient to denature the gene of interest, the fluorescent probes dissociate and the rate of fluorescent decline spikes. With a unimodal spike in the rate of fluorescent decline it can be presumed that there is one single gene product that has been generated through the RT-PCR system.

2.6 Single cell RNA Sequencing

As biological research has advanced there has been a push towards larger datasets and a more complete understanding of cellular biology. While genomics, genetic sequencing, and bulk RNA sequencing have been staple assays for many years a more recent advent - and quickly becoming a staple in its own right – has been single cell RNA sequencing. While there are multiple general methodologies, generally they all function with a similar structure. The key aspect that makes these technologies possible is the accurate isolation of single cells and the ligation of a unique barcode that, after

pooling and parallel sequencing, allows the computational separation of individual genes from individual cells. This isolation, pooling, and sequencing provides the ability to create high throughput analyses that, depending on the question at hand, can ultimately sequencing many individual samples in parallel to limit inter-experimental batch effects.

The genuine power of single cell transcriptomics can be quite easily demonstrated by just how fast these experiments became commonplace, but an unfortunate aspect of this expansion was that the usage of single cell transcriptomics expanded much faster than the technology, and its limitations, were broadly understood. Single cell transcriptomics offer up a dataset that you can direct almost any question towards – but is not capable of answering every question. It is vital to take extra precaution to properly normalize the data at every step, to include well thought out thresholding of the data as to best approach each question in as rational a manner as possible.

2.6.1 Methodology

This body of work used the 10x Genomics microfluidics system to isolate single cells through a gel emulsification protocol prior to RNA sequencing. All sample processing steps were performed according to 10x genomics standardized protocols following the generation of single cell suspensions as described above. Young, middle aged, and aged heart, liver, and kidney samples from C57BL/6 and STING^{KO} mice were processed with the 10x Chromium Single Cell 3' Gene Expression platform. In short, samples were loaded into the 10x Chromium Controller to generate Gel Beads-in-Emulsion (GEMs). Each GEM is generated to consist of a single cell, a bead with a specific barcode, and reagents necessary for lysis and reverse transcription. After lysis of the cells, transcribed cDNA was ligated with a unique 16-mer barcode (unique molecular identifier, UMI) to distinguish the RNA/cDNA from one cell to another.

2.6.2 Alignment and Preprocessing

Raw sequencing reads were produced by the NextSeq550. This BCL format was used as the input for basecalling, demultiplexing (of sample barcodes), and fastq file generation using cellranger (v3.0.2) mkfastq pipeline. The demultiplexed FASTQ files were aligned to the mm10 transcriptome and filtered. Both barcodes and UMI were quantified using “cellranger count”. All datasets were then aggregated with “cellranger aggr” per the standard pipeline.

With the aid of a bioinformatics expert from the Tufts DISC, pre-processing, normalization, dimensionality reduction, and cluster identification were performed with the Seurat package. Preprocessing and preliminary cell typing were performed separately on each dataset, and any genes that were represented in less than 3 cells were removed as well as cells with less than 200 “features” (genes) or 500 total reads. Filtered datasets were clustered, and any cells that were best characterized by mitochondrial genes were removed from the dataset.

2.6.3 Clustering and cell type identification

Datasets were normalized using the SCTransform function from the Seurat package.⁷¹ While regressing mitochondrial gene percent and total read counts as variables. These normalized values were used for dimensionality reduction using principal component analysis (PCA) with the RunPCA function. The number of principle components to use for cluster-based analysis was determined by the relative standard deviation of each PC, visualized with the Seurat ElbowPlot function. Integration of the individual datasets was performed with the R package Harmony and the RunHarmony function.⁷² After this integration step, clusters were identified using the Seurat functions FindNeighbors and FindClusters. At this point, clusters that were best characterized by high mitochondrial content, identified with the Seurat VlnPlot function, were removed.

Cluster based analysis was visualized using the Seurat function RunUMAP. Cell typing was performed manually using a combination of the Seurat functions FindAllMarkers, FindMarkers, and FindConservedMarkers.

2.6.4 Integration of scRNAseq samples

It has previously been reported that the differential expression of certain genes (mitochondrial or ribosomal genes, Gm42418, AY036118) are more representative in changes in sample quality rather than changes in biological state.⁷³ To account for these potential changes, these genes were removed from the datasets and samples underwent re-normalization (SCTransform) and were combined using the Seurat function PrepSCTFindMarkers. At this point, the R package Rmagic was used to recover low-transcript expression and reduce the relative “noise” of the data.⁷⁴

2.6.5 Principle Component Analysis

Principle Component Analysis (PCA) is used to describe high-dimensional datasets, specifically RNA sequencing, proteomics, or metabolomics. The power of PCA is the ability to reduce an N number of datapoints into one quantifiable variable that represents the majority of the variability of a dataset. With the quantification of the first X number of PCs, control and sample sets can be segregated by certain principal components, and then the principle components can be described, thus more directly describing the *variation* in the dataset (often to describe 85% of the variation). However, PC identification and selection with single cell RNA sequencing is slightly different. Rather than describing the variation of a dataset, as you would to analyze bulk sequencing, single cell RNA sequencing, generally, has a very heterogeneous population. Often, the first principal component of a single cell dataset is high level,

broad markers of general cell types – as segregating a dataset by those markers will “describe” the majority of the difference of a dataset. Much of the power of single cell RNA sequencing relies on the ability to analyze a highly granular dataset; rather than selecting the PCs that describe the traditional 85% of the variability, it is necessary to include all PCs that describe sufficient increases in data variability. This number is highly dependent on the dataset, but generally more PC inclusion results in increasingly granularity of the dataset. The cutoff for PC inclusion relies on the decreasing relative standard deviation of the variability of the dataset with each new PC: rather than looking for the number of PCs that describe the first 85% of the data (such as 30%, 25%, 20%, 10%) it is important to include PCs until the standard deviation of the dataset no longer decreases substantially. Often, this is 10-20 PCs based on the datasets analyzed for this body of work.

2.6.6 Uniform Manifold Approximation and Projection

Uniform Manifold Approximation and Projection (UMAP) is a dimensionality reduction and visualization technique designed to preserve global similarities and data structure.^{75,76} In brief, UMAP generates a high dimensional matrix and attempts to recreate the similar structure in low dimension. UMAP allows the user to regulate the number of “neighbors” for each point, which serves to allow the data to better represent global (high neighbors, large groups) or local (low neighbors, small groups) data structure. Generally, UMAPs are represented as a 2D scatterplot, where more highly related datapoints are clustered closer in 2-dimensional space, and more unrelated points are clustered farther apart. However, due to the multi-dimensional aspect of UMAP being reduced to 2 dimensions, it is difficult, or impossible, to determine if two groups are more, or less, similar than two other groups. When designing and interpreting

UMAP, it is imperative to ensure that the data is not over-clustered, as differences can be “forced” by pushing the algorithm.

2.6.7 Differential Gene Expression Analysis

Designing and selecting appropriate controls during differential gene expression analysis for single cell RNA sequencing is a critical step. Due to the incredible complexity of these datasets, it is important to properly understand what comparisons are being drawn, as opposed to what comparisons are intended to be drawn. All gene expression values are normalized to the dataset, or subset of the dataset, in question – the *background* gene expression of the dataset. If you attempt to compare two clusters across datasets, glaring gene expression programs can be overlooked because their relative expression to the background is close to identical, however if you directly compare these two populations these differences will emerge.

In order to understand the relative change of immune signatures with age and STING signaling in separate tissues, marker genes (sample of interest relative to the remainder of the dataset) for each age/tissue combination were calculated and filtered for only genes with a Bonferroni adjusted p-value < 0.1 and a log2 fold change > 0.2. These differential gene signatures were then sorted for the top 25 genes per sample. These marker gene signatures were then used to calculate the average gene expression profile per sample using the Seurat function `AverageExpression()`.

Generating a profile of the average expression of a gene signature is vital to best understand how individual genes are changing on a global scale. However, with single cell datasets it is possible to fully visualize the scope of the gene signature across multiple cells and cell types. All genes upregulated in an age-dependent manner were calculated per representative cell type and filtered for a Bonferroni adjusted p-value below 0.1. Remaining genes were ranked by their log2 fold change, as described above.

Filtered genes were then used as an input list to generate single cell heatmaps using the Seurat function `DoHeatmap()`. When indicated, relative gene counts per cell were retrieved from the base data with the Seurat function `FetchData()`.

2.6.8 NicheNet

The NicheNet algorithm was designed to investigate and profile intercellular communication networks in single cell sequencing datasets and generate theoretical ligand interaction and signaling networks. In brief, NicheNet uses publically available ligand receptor, signaling, and gene regulatory networks to build a signaling network in single cell datasets that integrated ligands, receptors, transcriptional regulators, and potential gene targets.⁷⁷ This information can be modeled as an “activity score” describing the likelihood that certain ligands are inducing a transcriptional signature through their cognate ligands. The novelty of this system is the ability not only to correlate ligand/receptor expression in two cells, but the integration of induced gene signatures, and the prioritization of receptor activity that promotes those gene signatures.

2.6.9 Gene Set Variation Analysis

scRNA sequencing is an incredibly powerful tool to study any number of genes of interest in a biological sample. The ultimate potential of scRNAseq has yet to be determined and novel methods are being published weekly. While significant genes can be calculated, it is rather difficult to visualize significant changes in larger sets of genes. While different methods have been presented, often for bulk sequencing like GSEA (gene set enrichment analysis) here I elected to use a methodology specifically designed for single cell analysis: the R package GSVA (v1.42.0).⁷⁸ GSVA is a single cell gene set

enrichment analysis that utilizes the relationship between individual gene expression levels, as well as the relative proportion of a gene set that is expressed in each cell. The “enrichment score” that is generated for each cell can then be used for statistical analyses to understand the magnitude and direction of change of a gene set of interest. For this work, I elected to utilize enrichment scores for a number of pathways sourced from Gene Ontology pathways as well as multiple curated pathways partially sourced from Gene Ontology but supplemented with specific genes of interest.

In brief, genes that were not quantified in at least one sample for each tested cell type were removed from the gene set to account for technical variation in read depth of these samples, and gene sets were only tested if more than 25% of the gene set (and 25 total genes) was expressed in the sample of interest. Activity scoring was performed on the log2-transformed Rmagic imputed gene expression values independently for each sample and cell type. Differential analysis of these activity score was performed with linear modeling with the R package LIMMA (v3.50.1).⁷⁹ Specific testing of the rate of change in activity scoring was performed within each genotype (C57BL/6, STING^{KO}) and the magnitude of change between genotypes was performed using LIMMA function `makeContrasts`.

2.6.10 RNAMagnet

RNAMagnet is an algorithm constructed to identify theoretical physical connections between cells in a dataset.⁸⁰ Using a curated dataset of predicted receptor:ligand interactions, RNAMagnet is able to generate a quantification of the relative expression of receptors on clusters designated as “anchors” and cognate ligands on other cells. This quantification, the “strength of interaction” forms the basis for predicted physical interactions in a dataset and is the basis for theoretical niches within a tissue. Here, I am able to leverage RNAMagnet analyses in multiple ways. At baseline,

RNAMagnet analysis is sufficient to predict physical interactions between immune and stromal cells. Further, I can leverage RNAMagnet analysis to understand how specific subpopulations of cells – ie cells that are expressing certain genes or genesets – are interacting with their theoretical environment in experimental datasets. Further, RNAMagnet identified “niches” can be isolated for more specific, and biologically relevant, analyses.

2.6.11 Polypositive

Using the GSVA enrichment scoring I was able to quantify the relative activity of gene sets of interest – specifically Type I Interferon responses, Inflammatory responses, and senescence associated genes. With this activity scoring it was possible to directly scale cells from different populations to understand which populations had the highest activity score and how these scores changed with age. As each cell was assigned a normalized activity score for each pathway of interest, it was possible to calculate the mean score for each cell type as well as the standard deviation of that group. Using this information, it was possible to directly quantify both young and aged cell types from C57BL/6 and STING^{KO} samples to understand which, if any, cells had an activity score sufficiently greater than the mean of the young samples.

Specifically, I selected the gsva enrichment scores from each cell for the three pathways of interest. For clarity, I performed a linear transformation on the activity scores to shift all the scores to positive integers, rather than normalized around zero. For each annotated cluster in the dataset, I calculated the mean (transformed) gsva score from the young control sample to serve as a relative “base” enrichment score. Using this “base” enrichment score, I was then able to normalize each individual cell to the mean gsva score of its respective cluster to better understand the relative relationship between the enrichment of these pathways in each sample.

Using these normalized scores, it was possible to then apply a threshold to each cell and determine which cells were enriched for the pathways of interest *relative to the mean enrichment of the control sample*. This specifically can isolate cells in our experimental datasets that are more active for these pathways. Ultimately, I settled on a relative enrichment greater than 20% (1.2x) higher than the mean of the young cluster for the heart and kidney, while a threshold of 10% (1.1x) was required for the liver, due to the sample size of the experimental datasets. This dataset was uniquely able to identify cells that were enriched in each sample of interest relative to the control dataset, but also cells that were selectively enriched in all three key immune pathways of interest.

2.6.12 Gene Ontology

All Gene Ontology analysis was performed using the R package gprofiler2 and the function gost() to functionally analyze murine gene sets as descriptive pathways. In brief, differentially expressed genes, identified by the Seurat function FindMarkers() or FindAllMarkers(), were used as input gene sets, clustered by either sample or cell type. The output generated supplies the user with multiple types of pathways represented by the input gene set, including GO and KEGG terms.^{81,82} The output was filtered for only GO terms that were annotated under the Biological Processes filter, found to be significantly represented in the dataset, contained greater than 3 genes represented in the dataset, and term size smaller than 2500 terms, irrespective of the dataset. This aimed to restrict the analysis to smaller, more specific terms that are more biologically relevant and interpretable.

2.6.13 Network Analysis

Network analysis was based upon “semantic similarity” scoring by the software Revigo.⁸³ After generating differential gene expression lists and functional pathway analysis descriptions, these pathways were ported to Revigo to generate “semantic similarity” measurements of all the GO pathways represented in the DEG analysis. Revigo uses semantic similarity to reduce redundancy between parent/child terms and create a set of “representative” terms most indicative of the heterogeneity of a dataset. With this semantic representation of GO analysis, Revigo also generates an NxN matrix of similarity scoring of all the input GO terms. This NxN matrix can be visually represented through a Network. In brief, a threshold was applied to normalize and scores less than 0.2 to 0.0. Next, using the ggnetwork, and network packages a network matrix was generated and plotted with ggplot2.

2.7 Flow Cytometry

The basics of flow cytometry rely on the inherent ability of resonance structures within proteins to absorb energy and emit photons. As different chemical structures have differing activation energies before light can be emitted, it is possible to use a combination of lasers of different wavelengths (and intensities) to selectively activate resonance structures at their maximum excitation/emission.

Early use of the flow cytometry system involved a single fluorophore/laser combination for simple detection of the emission spectra. However, incorporation of filters that only allow certain segments of the visible spectra to pass, band pass filters, now allows the detection of multiple fluorophores per laser excitation. Band pass filters are commonly used as long pass (emitted wavelength longer than X), short pass (emitted wavelength shorter than X), or a range around a specific wavelength (i.e. 510/20 – a 20nm region focused around 510nm). The generation of brighter

fluorophores with increasingly “rigid” or narrow excitation ranges have increased the number of fluorophores that can be detected by standard flow cytometric analyses to 12-15 easily, and upwards of 18-24 with a carefully designed system.

Flow cytometers are a system of microfluidics that only allow a single cell at a time to pass through a series of lasers. As individual cells pass through these lasers the fluorescently tagged antibodies emit light, which is detected and transmitted through fiberoptic cables to a series of spectral filters and detectors. These signals can be deconvoluted and quantified as a measure of specific proteins on the surface of a cell. Further, as cells pass through one of the lasers there are two distinct actions. Firstly, passive detection of the laser intensity is disturbed in a parabolic shape as the cell enters, blocks, and exits the path of the laser. The height, width, and area of the parabola can be used to determine the relative size of the cell passing through the laser, also known as Forward Scatter (FSC; FSC-H, FSC-W, FSC-A, respectively). Secondly, as the cell passes through the laser the laser is non-specifically deflected by intercellular components. This deflection can be quantified similarly to the FSC – height, width, and area of the parabola - however this “Side Scatter” is determinant of the granularity of the cell passing through the laser. The quantification of the forward and side scatter, along with the relative intensity from each laser/fluorescent antibody combination, can be used to progressively identify protein concentration on cells, and the likely lineage or cell type. All cells retain some level of “autofluorescence” so it is vital to compare all samples against cells labeled with individual fluorescent antibodies and unstained/autofluorescent only samples to understand the true positive and negative intensity of each sample.

2.7.1 Flow Cytometry

Single cell suspensions were generated as described above. 0.5-2E06 cells were aliquoted for use in flow cytometry assays, depending on the availability of cells. All

following steps were kept on ice or incubated at 4°C. Aliquoted samples were washed (5min, 500g) with Phosphate Buffered Saline (PBS) and resuspended in 100uL serum-free PBS and incubated with Zombie Near InfraRed (NIR) anti-mouse Live/Dead stain for 15-30 minutes at a dilution factor of 1:1000. Stained cells were protected from light as much as possible and washed with FACS buffer (2% FBS in PBS) and resuspended in 50uL Fc block (anti-mouse CD16/32) to limit Fc-Receptor non-specific binding on the following targeted antibodies for 15 minutes. Without washing, a “master mix” antibody cocktail of curated fluorescent antibodies was added and samples were incubated on ice for 30 minutes. Cells were then washed and resuspended in 200uL of FACS buffer for flow cytometric analysis.

2.7.2 Flow Cytometric Analysis and Gating

Flow cytometric analysis is commonly performed by plotting the intensity of independent fluorescent proteins on an X/Y scatterplot. Cells can be subsetted based on the relative intensity of one or both markers. Traditionally, the first one to three gates are set to remove background debris (FSC-A x SSC-A), “doublets” (cells that are stuck together, FSC-A x FSC-H), or dead and dying cells. Progressive gating of log-transformed intensities of fluorescent antibodies is then used to identify cell types based on the intensity of more broadly expressed proteins, such as the hematopoietic marker CD45, to more specific markers, such as Tim-4 on tissue macrophages. Most commonly, this “gating” is performed on the software “FlowJo” but every year new systems are coming into place, such as the Omiq software used throughout this body of work. The use of these specialized software facilitates drawing of individualized gates, quadrilaterals, circles, or customized polygons, to identify and digitally isolate certain groups of cells for subsequent analysis.

2.7.3 Optical Compensation

Gating strategies should be designed prior to running each experiment, for the most efficient use of fluorophores. Although many fluorophores are highly compatible with narrow primary excitation ranges, high intensity lasers will induce general excitation and non-specific or “auto”-fluorescence. As such, including fluorophores that are not necessary to the final expected analyses will only increase background noise, and reduce the clarity of the dataset.

With rare exceptions, it is generally required to perform some level of spectral compensation to account for autofluorescence of the analyzed cells or spectral spillover – from overlapping or secondary emission spectra. This correction can be performed on the cytometer itself, or locally through FlowJo. In brief, primary compensation is performed by the cytometry based on the relative intensities of the various controls that are used – unstained and singly-colored samples, by artificially lowered the intensity of certain parameters in the dataset. However, these corrections are often too high (or low) and need to be corrected by the user. Creating an NxN matrix of each parameter involved in the experiment can highlight combinations that resulted in the greatest spectral spillover and require user-correction. It is vital here to monitor all parameter combinations, as co-expressed fluorophores can result in larger issues than sequestered fluorophores.

2.7.4 Fluorescence Activated Cell Sorting

Using the principles of flow cytometric analysis, it is possible to specifically identify cells based on the binding of specific fluorescent markers and, using pulsing magnetic charges, to isolate specific populations of interest for further downstream analysis. In brief, microfluidics systems isolate single cells as described above into droplets. Each droplet containing a labeled cell carries a positive charge, while non-

labeled cells remain negatively charged. Magnetic pulses can bend the stream of charged cells away from a central collection tube to perform positive- or negative-selection of the labeled cells.

Cells were prepared as described above, and stained with Zombie viability dye, anti-mouse CD45, anti-mouse Ter119, anti-mouse CD3, and anti-mouse CD19. Cells were selected for the presence of CD45 and the absence of CD3, CD19, and Ter119 to select immune cells with limiting contamination from T and B cells of the lymphoid immune system. Cells were sorted with the Legacy MoFlow (Cytomation; Tufts Medical School) into 1% BSA-PBS for use in single cell sequencing. Alternatively, cells were stained with anti-mouse CD45, anti-mouse CD11b, and anti-mouse F4/80 to select for the presence of CD45, high levels of F4/80, and low levels of CD11b to select for tissue resident macrophages. Macrophages were similarly sorted into 1% BSA-PBS for downstream analysis.

2.7.5 Spectral Cytometry

Traditional flow cytometry relies on unique laser/filter combinations to isolate the peak emission wavelength of individual fluorescent antibodies to identify cells. Most experiments, in this case, are limited to 10-15 markers, or up to 20 markers with an incredible amount of front-loaded work and full knowledge of the proteins of interest and what cells they will be displayed on. While powerful, this is highly limiting for de novo generation of gating strategies and characterization of novel cell types by less than a marker or two at a time. Recently, a system was developed that utilized up to 60 very short band pass filters that could read and quantify the entirety of the emission spectrum from up to 30 unique fluorescent antibodies easily, or 60 with increased knowledge of the proteins of interest. This “Spectral Cytometry” is sufficiently able to “unmix” the signals from each individual antibody, in part based on the peak emission spectra from

each laser, to reliably quantify the relative intensity of each fluorescent antibody, and in effect the protein concentration on the surface of the cell.

Generation of single cell suspensions, fluorescently labelling cells of interest, and analysis for the Spectral Cytometry was described as above. Spectral Cytometry experiments were performed with the Cytex Aurora spectral flow cytometer (Cytex; Tufts Medical School), unmixing was performed using the SpectroFlo software (Cytex) and all data was analyzed using the Omiq software (Dotmatics).

2.7.6 Uniform Manifold Approximation and Projection (UMAP)

UMAP is a dimensionality reduction and visualization technique commonly used in conjunction with single cell RNA sequencing. UMAP uses a k-Nearest Neighbors (kNN) algorithm to quickly and efficiently identify cells (or datapoints) that have similar values across a set of parameters. Here, for spectral flow cytometry, it is possible to input the relative intensity values for each fluorescent probe and “reduce” the data to dataset of 2 dimensions (UMAP-1, UMAP-2) that can visually segregate dissimilar cells. The most common visualization of a UMAP is an X/Y scatterplot, where cells that are most similar tend to cluster closer together, and cells that are more dissimilar tend to cluster farther apart. A common misconception from UMAP (or similar reduction techniques) is that the relative distance between datapoints is indicative of relative dissimilarity. Unfortunately, due to the shift from n-dimensionality to 2-dimensionality, UMAP is unable to maintain global similarity differences and it is difficult, or impossible, to assign relative dissimilarity scores to random datapoints on the plot.

2.7.7 FlowSOM

Traditional flow cytometry analysis uses X/Y scatterplots, as described above. While this has been a standard methodology for quantification of population size and breakdown, as flow cytometric experiments have expanded, especially into high-parameter spectral cytometry described above, bifurcating gates become quite cumbersome and gating strategies overwhelming. Through this body of work, one major research goal was the identification and characterization of novel cell subsets within tissues. As such, it was no longer feasible to manually plot all combinations of fluorescent probes. FlowSOM uses a series of Self Organizing Maps to selectively identify similar cells based on the relative expression of each protein target.⁸⁴ This technique served to identify phenotypically similar cell subsets from these datasets, as well as identify unique markers of each “metacluster”. For this dataset, I was able to include the fluorescent intensities along with the UMAP coordinates and integrate the FlowSOM and UMAP analyses for more specific visualization of the dataset.

2.7.8 Identification of tissue resident macrophages

Tissue resident macrophages are known to originate from a fetal source, establish tissue residency, and self-maintain throughout the life of the organism. These cells are critical for the maintenance of tissue and immune homeostasis and have been described to be highly plastic as a result of their tissue environment. These cells have been traditionally described as having the hematopoietic marker CD45, high levels of the macrophage marker F4/80, but low levels of the myeloid marker CD11b. Further work has attempted to identify more specific tissue macrophage markers and have identified the presence of CD64, Tim4, MerTK, CX3CR1, Csf1r, or CD11c, depending on the tissue of interest. Here, we used these markers, alongside additional markers such as CD103, CD138, CD204, CD206, CD207, CD24, CD68, MHC-II, Ly6C, CCR2, and Ly6G.

Chapter 3: Results

3.1 Introduction

In order to query the role of DNA sensing machinery in the regulation of innate inflammatory responses, I selected to utilize in vitro culture methods to differentiate bone marrow progenitors into mature macrophages using L929 conditioned media as a source of mCSF. Bone marrow-derived macrophages (BMDMs) are a commonly used proxy for macrophages and the innate immune system as they are robust and accessible for in vitro assays. To begin this query, these macrophages were stimulated with specifically curated innate immune ligands: poly(dA:dT) – a DNA homologue to activate the cGAS/STING system; lipopolysaccharide (LPS) – a bacterial carbohydrate; a pan-Type I Interferon (IFN-I) – to model feed forward and feedback mechanisms of the innate immune system; standard culture media –a treatment control for LPS and IFN-I; lipofectamine – a lipid micelle to allow the poly(dA:dT) oligomers to cross the macrophage lipid membrane and enter the cytosol to reach the cGAS/STING system. It was possible to leverage this system, using qPCR analysis to track and quantify changes in transcriptional regulation of inflammatory mediators, to understand how DNA sensing was able to fully modulate the inflammatory response of macrophages.

3.2 Regulation of Inflammation by DNA sensing

3.2.1 In vitro stimulation of Bone Marrow-Derived Macrophages

The primary question this project hoped to answer was if DNA sensing regulated the inflammatory response, or the inflammatory potential, of the innate immune system and macrophages. To begin to answer this, I designed a qPCR primers panel towards immunoregulatory genes to reflect the immune response and potential of macrophages. As the role of DNA sensing was a primary target of this query, many of these primers

targeted the response (interferon receptor subunits), propagation (interferon subtypes), and inhibition (direct immunosuppressive genes) of the interferon (IFN) system. To activate these macrophages I chose to use the lipofectamine transfection mechanism to transfer poly(dA:dT) directly into the cytosol of BMDMs, as well as the extrinsic activation of macrophages with IFN-I and LPS. It was found that the transfection of macrophages with poly(dA:dT) induced robust expression of the IFN-I subtypes *IFN α 4*, *IFN α 13*, and

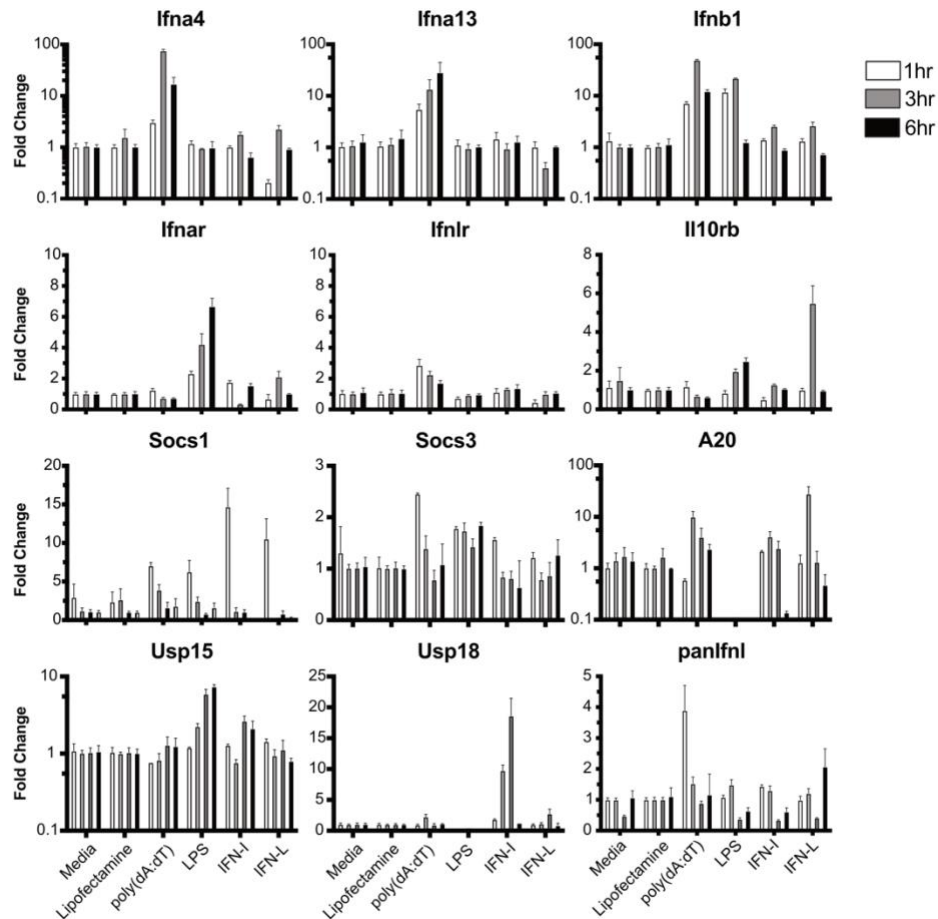


Figure 3.1 Stimulation of BMDMs with poly(dA:dT) induces IFN-I expression but not IFN-I regulatory programs

B6 Bone marrow-derived macrophages (BMDMs) from C57BL/6 mice were stimulated for 1, 3, or 6 hours with culture media, transfection agent lipofectamine, poly(dA:dT), lipopolysaccharide (LPS), Type I, and Type III IFN to measure the temporal production of IFN-I and IFN-I regulatory genes. n = 2 technical, 1 biological replicate.

IFN-III subtype *Ifnl2/3*, while LPS induced much stronger expression of the IFN receptor component *Ifnar1*, and the immunoregulatory components *Il10*, and its receptor *Il10ra* that are capable of suppressing cytokine signaling, and the ubiquitin peptidase components *Usp15*, and *Usp18* that directly antagonize interferon signaling^{85,86} (Figure 3.1). The induction of immunoregulatory components downstream of immune activation is well described and serves as a safeguard against unnecessary immune responses.

While poly(dA:dT) induced a more robust IFN-I response with an absence of IFN-I repressors, and LPS appeared to directly antagonize the IFN-I system it suggested that the IFN-I system was a key component that may regulate the inflammatory potential of poly(dA:dT) when compared to LPS. I then performed a similar stimulation, to compare the role of nucleic acid sensing more directly with direct stimulation with IFN-I. An intriguing component of the IFN-I system is a powerful feedback loop IFN-I induces many immune intermediates but also robustly induces different IFN-I components which can then participate in auto- or paracrine signaling to propagate the immune response. This is often viewed as the immune system evolving to induce an immune response in neighboring cells that may not have come into contact with pathological or damage signals – but it is also likely that these signals are involved in a more intricate communication network to regulate their own activation.

Using the components poly(dA:dT), IFN-I, IFN- λ 2, and IFN- λ 3, I measured the relative activation of early, late, and regulatory components of the IFN-I system such as *Mx1* (an early response factor to IFN-I), IFN β (often the first IFN-I subtype to be induced), *Oasl2* (an inhibitor of IFN-I), and *Socs1/3* (suppressor of cytokine signaling 1 and 3) (Figure 3.2). Interestingly, while poly(dA:dT) and IFN-I appeared to induce similar levels of *Mx1*, *Oasl2*, and *Ifnb1*, the relative kinetics were a primary difference in these responses (Figure 3.2). IFN-I induced these factors consistently earlier than poly(dA:dT) with maximum production at 3 rather than 6 hours, likely due to regulation of these IFN-I

components as a direct result of the activation of the IFN-I system itself. However, poly(dA:dT) appears to maintain robust expression of the *Ifnb1* locus through a 6 hour timepoint, while *Ifnb1* has returned to baseline expression after IFN-I treatment. Further, *Socs1* expression peaked between 4-5-fold higher than IFN-I treatment, suggesting some immunoregulatory potential downstream of poly(dA:dT) that is independent of the activation of the IFN-I system.

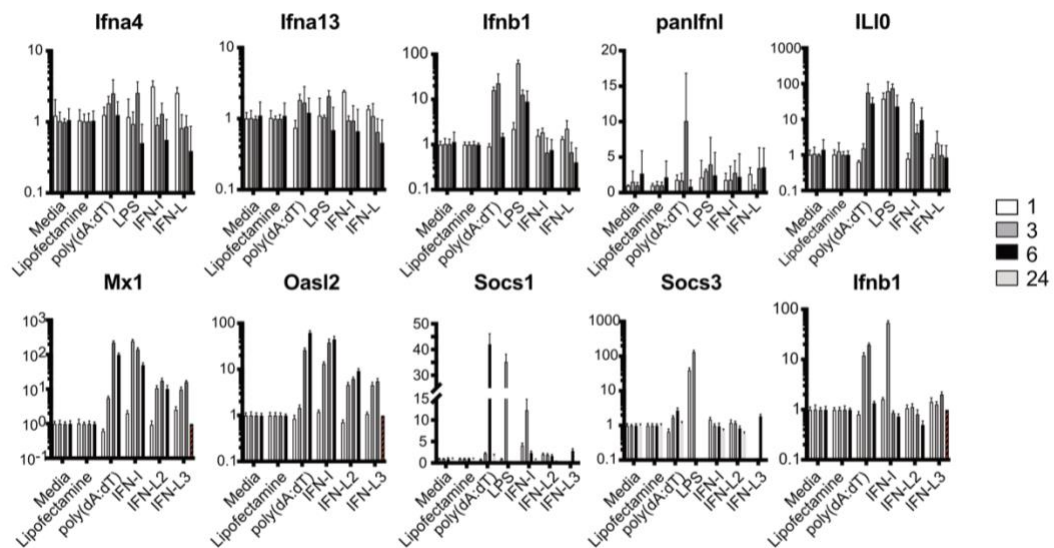


Figure 3.2 Transfection of DNA-analogues induces prolonged *Ifnb1* transcription with delayed IFN α 4/13

Bone marrow-derived macrophages were stimulated with culture media, lipofectamine, poly(dA:dT), pan-Type I IFN, IFN λ 2, and IFN λ 3 to test the relative capacity of DNA analogues, Type I and Type III Interferon to induce a robust interferon signature. $n = 1$ biological, 2 technical replicates.

3.2.2 Conditioning of the immune response by DNA sensing

Data suggests an innate ability of DNA sensing to control and regulate the immune response. However, the extent to which DNA sensing through the cGAS/STING pathway regulates specific immune profiles of macrophages remains unknown. In an attempt to understand how DNA sensing regulates inflammatory immune responses I elected to perform an extended overnight treatment of BMDMs with IFN α , LPS, or poly(dA:dT) prior

to a 6 hour stimulation with IFN α , IFN λ 2, or IFN λ 3. While inflammatory induction downstream of primary IFN α stimulation has been described, a primary stimulation misses the priming effect of these pathways as can be expected in situ. Here, overnight pretreatment of BMDMs with IFN α completely attenuated the ability of IFN-I to drive the expression of multiple inflammatory transcripts such as *Il6*, *Tnfa*, *Ccl2*, while the expression of *Ifnb*, *Il12*, and *Il1b* (data not shown) remained unchanged (Figure 3.3A).

While overnight treatment with the TLR4 agonist LPS was sufficient to drive potent and uniform inflammatory reactions, there appeared to be a unique priming towards inflammatory responses downstream of IFN λ 2, specifically expression of *Il6*, *Tnfa*, and *Arg1* transcripts which may suggest a spatial necessity for a combined sensing of LPS and IFN λ 2 in barrier tissues (Figure 3.3B). However, when pretreating with poly(dA:dT), BMDMs in fact became more sensitive to IFN α , producing increased levels of *Ifnb*, *Il12*, *Arg1*, and *Ccl2* while limiting the production of *Il6* (Figure 3.3C). As mentioned previously, IFN-I induces a robust mechanism to cause a cell to become “refractory” to further IFN-I responsiveness^{87,88}. Interestingly, this overnight-treatment of poly(dA:dT) appears to limit the overall refractoriness of the macrophage to poly(dA:dT)-driven IFN-I responses, suggesting an intriguing method by which DNA responses could regulate the inflammatory phenotype of the macrophage. Notably, these data do not include a measure of the macrophage response profile after overnight stimulation with IFN-I, LPS, or poly(dA:dT). However, previous stimulation experiments show many of these factors returning to baseline between 6 and 24 hours.

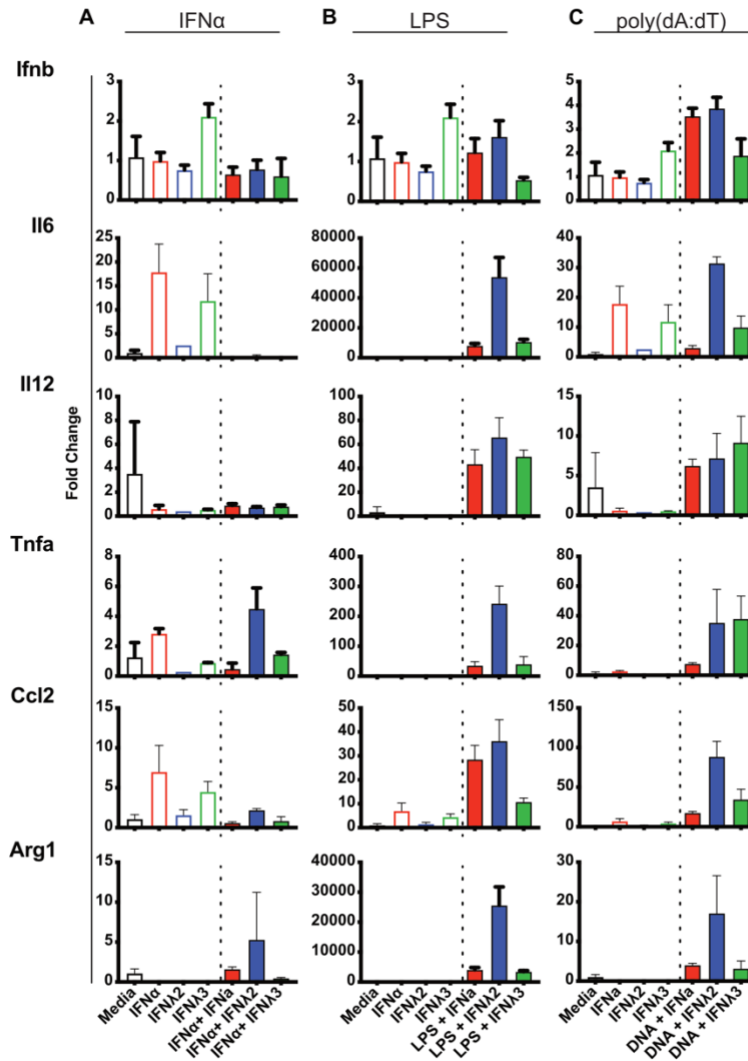


Figure 3.3 Overnight treatment with poly(dA:dT) maintains the IFN-I response potential of bone marrow derived macrophages greater than IFNα.

Bone marrow macrophages were grown for 7 days with 10% L929 conditioned media. Mature macrophages were then treated overnight with IFNα (100U, **A**), LPS (10ng/mL, **B**), or poly(dA:dT) transfection (**C**) prior to a 6h acute stimulation with IFNα (red), IFNλ2 (blue), or IFNλ3 (green). Macrophages were harvested for qPCR analysis for inflammatory transcripts *Ifnb1*, *Il6*, *Il12*, *Tnfa*, *Ccl2*, and *Arg1*. Open bars; primary stimulation. Filled bars; pretreatment + stimulation. n=1 biological, 2 technical replicates.

Additionally, I tested the ability of extended exposure to IFNλ to further regulate IFN responsiveness (Figure 3.4A-B). IFNλ generally generates responses in BMDMs similar to IFNα, however its full activation potential is not completely understood. Here,

overnight treatment of BMDMs with either IFN λ 2 (Figure 3.4A) or IFN λ 3 (Figure 3.4B) imparted a similar IFN α responsiveness as to overnight treatment with IFN α .

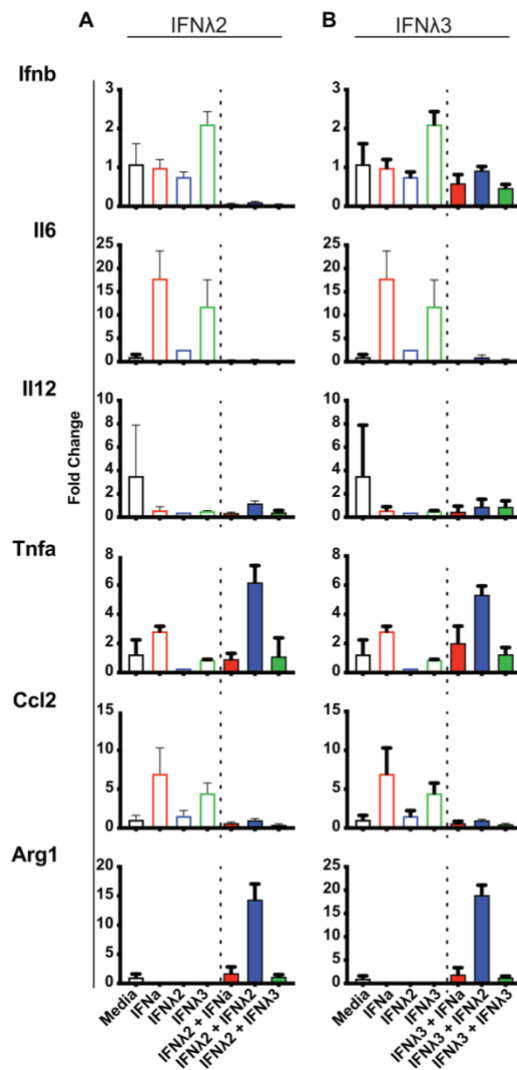


Figure 3.4 Interferon lambda attenuates IFN-I responsiveness but maintains IFN λ 2 induction of the immunosuppressive marker *Arg1*

As in Figure 3.3, BMDMs were cultured overnight with IFN λ 2 (**A**) or IFN λ 3 (**B**) prior to 6h stimulation with IFN α (red), IFN λ 2 (blue), or IFN λ 3 (green) to test the interferon regulatory potential of the IFN λ family of cytokines. Open bars; primary stimulation, filled bars; pretreatment + stimulation.

However, these BMDMs were much more prone to the production of *Arg1*, the canonical marker for “alternative activation” of reparative macrophages after IFN λ 2 but not IFN λ 3

secondary stimulation. The IFN λ family has previously been implicated in the negative regulation of IFN-I responses, however here these data suggest there is further modulation and amplification of certain signaling cascades after IFN λ stimulation.^{29,89}

3.2.3 cGAS/STING-dependent sensing of DNA

DNA sensing is exceptionally redundant, with mechanisms of sensing DNA in the cytosol and endosomes. While many immune pathways have some sense of ingrained redundancy, DNA sensing has retained close to ensured activation. Here, following the work of Sharma et al. I elect to focus the bulk of this work on the cGAS/STING signaling pathways as a more probable mechanism of damaged or mis-localized self-DNA in the cytosol.⁹⁰

Using an in vitro stimulation of C57BL/6 and STING^{KO} BMDMs with IFN α , IFN λ 2/3, and poly(dA:dT) I found a robust (>10 fold) reduction in *Ifnb* and *Cxcl10* production by 2h post-stimulation and a complete lack of *Ifna4* at 4h demonstrating a significant role of STING-signaling in the generation of IFN-I and key interferon stimulated genes (ISGs) downstream of poly(dA:dT) stimulation (Figure 3.5). Intriguingly, by 4h many of these effects were normalized, suggesting a secondary method of DNA sensing independent of protein products from the *Tmem173* locus. Furthermore, there was little to no effect on the response to IFN-I stimulation in the absence of STING suggesting that the basic IFN-I response machinery is functional, even in the absence of DNA-priming responses.

Oddly, this acute stimulation of C57BL/6 and STING^{KO} BMDMs with IFN λ 2 or IFN λ 3 only induced very low levels of these key ISGs, with the exception of the *Ifnl* loci itself. IFN λ has been shown to induce a robust IFN signature so it is unclear if these specific ISGs are weakly induced by IFN λ or if the stimulation itself was poor.

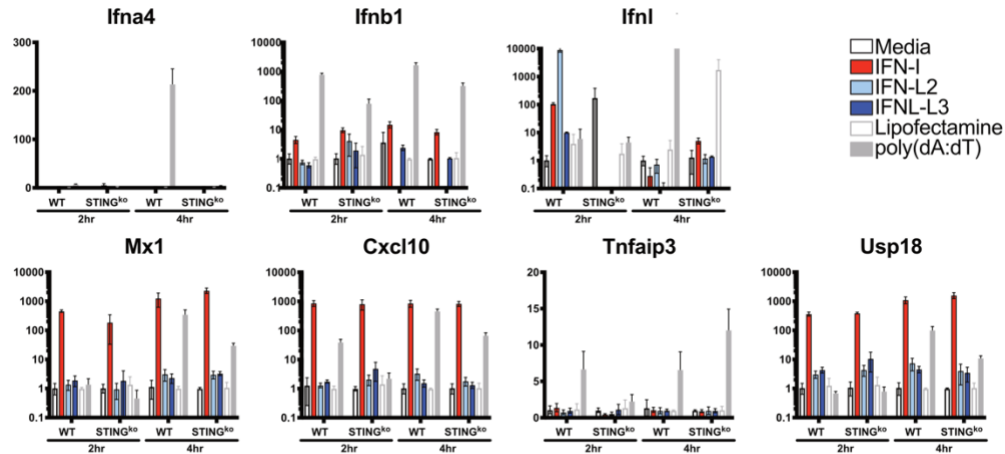


Figure 3.5 STING is a potent inducer of interferon and interferon stimulated genes with poly(dA:dT) stimulation

Mature BMDMs from 6mo C57BL/6 and STING^{KO} mice were stimulated with panIFN α , IFN λ 2, IFN λ 3, and poly(dA:dT) to test the role of STING signaling on the transcription of interferon and interferon stimulated genes (ISGs). BMDMs were stimulated for either 2h or 4 to identify early and later-stage induction of ISGs. Black, open; media, red; panIFN α , light blue; IFN λ 2, dark blue; IFN λ 3, grey, open; lipofectamine, grey; poly(dA:dT). N=1 biological replicate, 2 technical replicate.

3.2.4 Aging as a model of chronic activation of DNA sensing

Here, I describe a method by which pre-stimulation with DNA analogues has the capacity to regulate macrophage refractoriness to IFN-I. DNA sensing has been implicated in the pathology of certain viral infections, oxidative stress, and cancer as a mechanism of responsiveness to the indicated stressor. However, similar to the work by Sharma et al., these data suggest that there is a more intricate downstream signaling network from DNA sensing than simply pro-responsive and pro-pathological mechanisms. To build off this in vitro pre-stimulation model, it was necessary to build a model of *chronic* DNA recognition and signaling to understand the depth and control of this immunomodulation. Chronic DNA sensing is regularly studied in the context of cancer and oxidative stress, however many of these models require surgical, chemical, or viral manipulation of a biological system which, by definition, introduces secondary or

tertiary external stimuli which can confound the understanding of DNA sensing mechanisms.

Here, I elected to use a sterile model of chronic inflammation that is generally believed to rely on, if not be caused by, chronic DNA damage and DNA sensing – Aging. This chronic inflammation – Inflammaging – precedes systemic organ dysfunction and is believed to be due to pathological DNA sensing. While there is significant dysfunction within the immune system with age, the primary observed morbidities are tissue-centric. As such, this project began to pivot towards tissue-intrinsic immunity as a more relevant readout for age-associated changes.

Generally, the presence of interferon is difficult to detect outside of a robust immune response; as such, in order to understand the extent of interferon production in aged tissues it was necessary to quantify the expression of interferon stimulated genes (ISGs) as a proxy of interferon signaling.

First, I compared the expression of a number of canonically inflammatory (Figure 3.6A) and interferon associated (Figure 3.6B) genes from young (3mo) and aging (18mo) C57BL/6 liver and epididymal white adipose tissue. Adipose tissue was included here since adipose is generally considered an inflammatory tissue during obesity and the shift from lean to fat mass accumulation is a common pathology with aging which is similarly observed in mice.

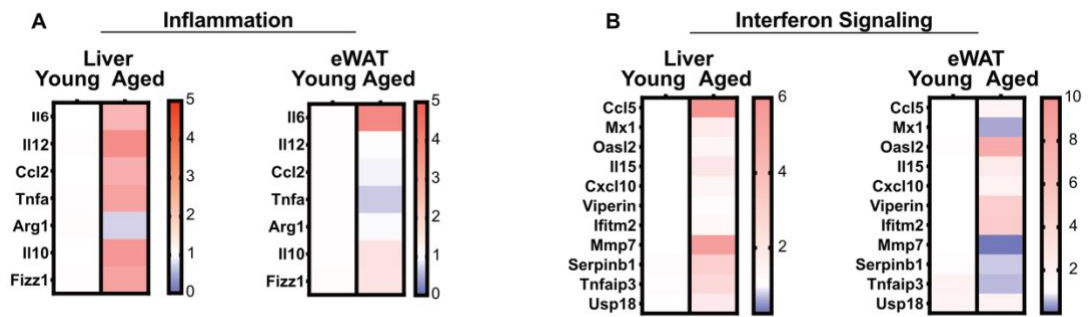


Figure 3.6 Aging tissues develop a quantifiable inflammatory and interferon signature

qPCR analysis of inflammatory (**A**) and interferon stimulated (**B**) genes from young (3mo) and aging (18mo) C57BL/6 liver and epididymal white adipose tissue (eWAT). Fold change normalized to relative expression of housekeeping genes (Hprt1) and media stimulation. Blue; downregulated, red; upregulated relative to young tissues. N = 3-5, pooled from two experiments.

The induction of a robust interferon response is a well-defined anti-viral response associated with nucleic acid sensing. However, the presence of an interferon signature is not inherently indicative of nucleic acid, DNA, or STING signaling in aged tissues. To test the dependence of tissue interferon signatures on STING signaling I expanded the scope of this project to create a more general profile of tissue-centric interferon signaling. I began these assays with 6-month-old C57BL/6, STING^{KO}, and cGAS^{KO} heart, eWAT, lung, liver, and bone marrow samples to understand the role of DNA sensing through the cGAS/STING signaling pathway to the development of tissue interferon signatures. While not inherently aged, these mice will help to inform on the presence of a basal STING-dependent signature which could be exacerbated during aging.

Intriguingly, there were several ISGs that were generated in the presence of cGAS and STING signaling, but this response was rather heterogeneous and there remained ISGs elevated in the absence of cGAS or STING signaling at this time point (Figure 3.7). These data are indicative of a role of STING in the tissue-wide role of

STING signaling but demonstrate that STING is not the sole and dominant driver in the expression of these genes at this age.

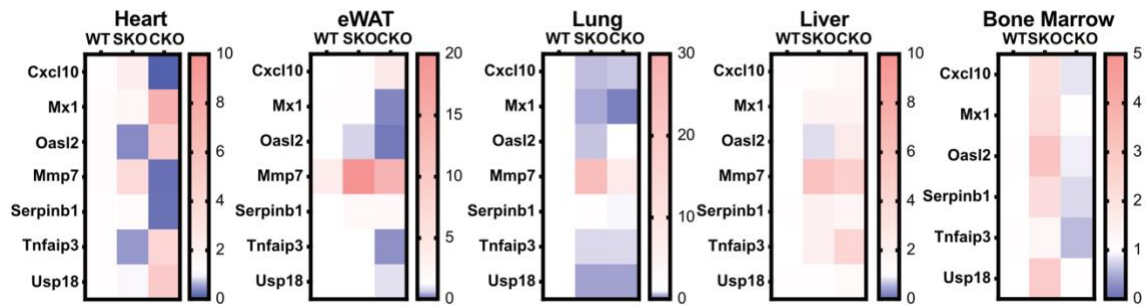


Figure 3.7 cGAS/STING signaling contribute to tissue-wide interferon signatures by 6 months of age

qCPR analysis of tissue-intrinsic regulation of interferon signaling in 6mo heart, epididymal white adipose tissue (eWAT) lung, liver, and bone marrow from C57BL/6, STING^{KO}, and cGAS^{KO} mice. Fold change as indicated: red; upregulated, blue; downregulated relative to C57BL/6 tissues.

With the identification of a potential role of STING signaling in mice as young as 6 months, it was intriguing to consider the fact that STING signaling may contribute to a tonic interferon signature and not be restricted to an age-associated phenotype. As such, I attempted to profile the STING-dependent interferon signature in the heart, liver, and kidney in mice as young as 5 weeks (Figure 3.8). While not entirely conclusive, these data suggest some integral role of the cGAS/STING signaling cascade in the generation of tissue-intrinsic interferon signatures.

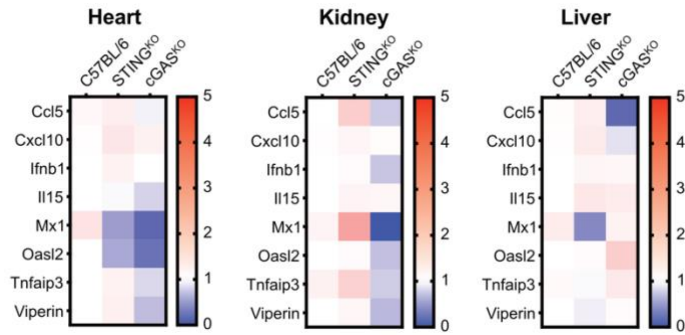


Figure 3.8 STING contributes to, but is not a dominant driver of tissue interferon signature in 5 week old mice

qPCR analysis of whole heart, liver, and kidney tissue samples from 5 week old C57BL/6, STING^{KO}, and cGAS^{KO} mice. Fold change calculated relative to the C57BL/6 sample and housekeeping gene Hprt1. Red; upregulated, blue; downregulated. n = 3.

3.3 Flow cytometric identification of tissue resident macrophages

3.3.1 Introduction

Bone marrow macrophages are an ideal system for investigating the mechanism of action of macrophages in a controlled setting. However, as these experiments are primarily querying the role of DNA sensing and IFN-I signaling in the regulation and control of inflammatory activation, it appeared much more pertinent to focus these investigations on specialized subsets of macrophages that take up residency in tissues. These tissue resident macrophages (TRMs) are long lived, self-renewing macrophages that serve as primary responders to tissue damage, stress, and infection and are a critical focal point to regulate immune activation and propagation. These have been shown to develop unique transcriptional profiles that are intertwined with their tissue of residency – however as specific as these signatures are, they remain plastic. An elegant piece of work described the effects of transplanting these cells between two organs (in this case the peritoneal cavity and the lung) and while there appeared to be a core set of

genes that were retained post-transplant the bulk of the transcriptional profile adapted to the new tissue of residency.

Further, while uniquely adapted to their tissue of residency, these cells are of a lineage entirely distinct from bone marrow or monocyte-derived macrophages (MoDMs) such as the type used in in vitro cell culture experiments. BMDMs or MoDMs can enter solid organs and develop resident-like attributes, but through the use of specialized lineage tracing and genetic knockout experiments it has been determined that TRMs, in fact, develop prenatally from the fetal yolk sac and fetal liver prior to the establishment of the bone marrow and hematopoietic system entirely. Here, it appears that these cells develop unique traits, genetic landscapes, or signaling cascades that allow them to 1) develop long-term residency 2) integrate functionally with their tissue of interest and 3) maintain functional independence from macrophage from the hematopoietic system. With this shift in thinking, it became imperative to better understand the role of DNA sensing and IFN-I in TRMs, along with a clearer understanding of how these pathways regulate inflammation and immunity in a biological setting.

3.3.2 Identification and enrichment of tissue resident macrophages

A base of the field of tissue resident macrophages has focused on differentiation and seeding of tissues during fetal development, with intricate genetic models and specific lineage tracing experiments to identify individual subpopulations of TRMs. While years of work was spent identifying development specifics – and the differentiation potential of yolk sac, fetal liver, and primitive hematopoietic progenitors – very little work had identified the immune trajectories of these mice, and the extent to which they were altered during chronic inflammation. Originally, since the majority of these models were identified using lineage tracing models, it wasn't necessary to employ an intricate immunophenotyping protocol – the presence of the tracer, or macrophages on their own

– was sufficient. However, these groups did employ basic identification with flow cytometry and were able to consistently and effectively identify fetal-derived TRMs as expressing the hematopoietic marker CD45, high expression of the macrophage antigen F4/80, and low expression of the common myeloid integrin CD11b⁹¹⁻⁹⁴. Using this very simple strategy it appears possible to differentiate fetal-derived macrophages from hematopoietic-derived macrophages at least in late development and early life. An open question that remained in the field and spurred my investigation was less about the developmental trajectory of TRMs and focused primarily on the high level of plasticity of TRMs transcriptional state – are these cells susceptible to stark shifts in their inflammatory potential due to DNA sensing, IFN-I, or generalized chronic inflammation and how do these transcriptional alterations affect their viability and vitality.

In order to begin an assessment of TRMs in tissues and the impact of STING signaling with age I first began to profile TRMs in various tissues based on this traditional CD11b^{low} F4/80^{hi} TRM gating as well as integrating other specific tissue macrophage gating strategies. (Figure 3.9A-B) While the traditional TRM gating of CD11b^{low} F4/80^{hi} has proven sufficient to generally classify TRMs across multiple tissues in early life, the development and expansion of hematopoietic-derived cells serves to introduce a number of confounding factors. Specifically, in the heart and liver these CD11b^{low} F4/80^{hi} cells can be further differentiated through the common macrophage antigen CD68 in the liver and CD64/CD68 in the heart (Figure 3.9B). With the proper identification of these TRMs I attempted to understand if STING signaling played a significant role on the maintenance or expansion of these populations. STING was shown to contribute to both the inflammatory and interferon signature for whole tissues; as TRMs are well characterized to respond to and integrate signals from their local

environment, it was very intriguing to understand if these immune signals from STING contributed to the basal makeup of these TRMs.

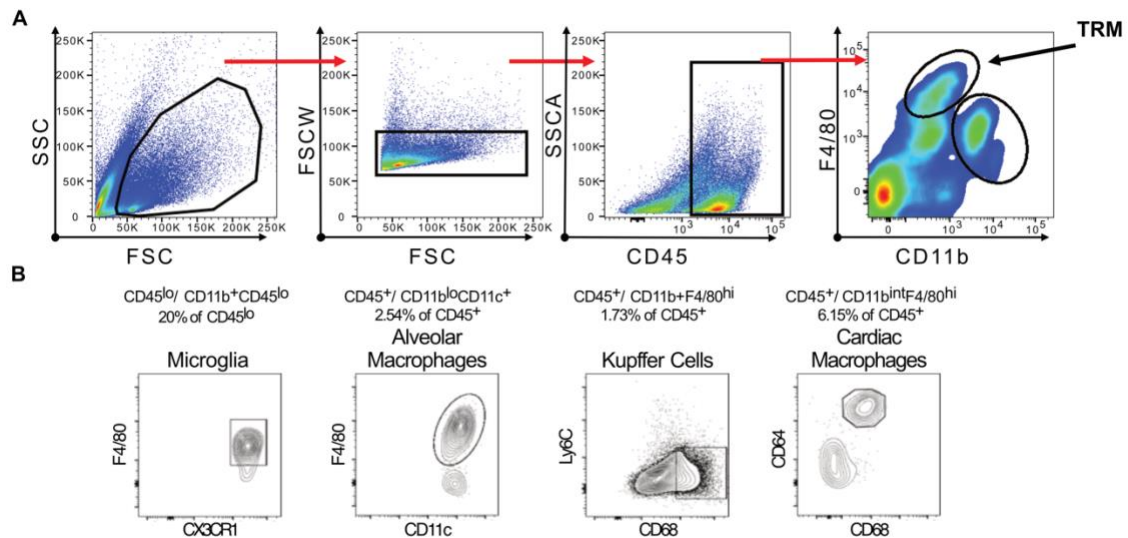


Figure 3.9 Identification of tissue resident macrophages.

(A) Flow cytometric gating strategy to identify traditional F4/80^{hi} tissue resident macrophages (CD45⁺, CD11b^{low}, F4/80^{high}). (B) Flow cytometric scatter plots highlighting secondary macrophage gating parameters for identification of specific subpopulations of TRMs. Tissue-specific TRM identification annotated as shown. Representative plots.

Using 6-month-old C57BL/6, STING^{KO}, and cGAS^{KO} mice I harvested liver, heart, lung, brain, (shown) eWAT, thymus, and epidermal tissue (data not included) to understand the role of tonic STING signaling in the maintenance of TRMs. Using this directed gating of F4/80^{hi/+} TRMs, combined with secondary markers in the liver (CD68), heart (CX3CR1/CD64), lung (CD11c/CD64), and brain (CX3CR1) to explore specific subsets, there were intriguing shifts in the total number of TRMs in the absence of cGAS/STING signaling. (Figure 3.10) While this preliminary data was generated with few mice, there were interesting expansions of TRM populations in the lung and brain in both cGAS and STING deficient mice, while only cGAS deficiency appeared to have

expanded the cardiac macrophage population. However – many of these markers, unfortunately, are co-expressed with monocyte and monocyte-derived cells such as CD68 and CX3CR1 (outside the brain) and may not adequately identify “true” tissue resident macrophages outright.

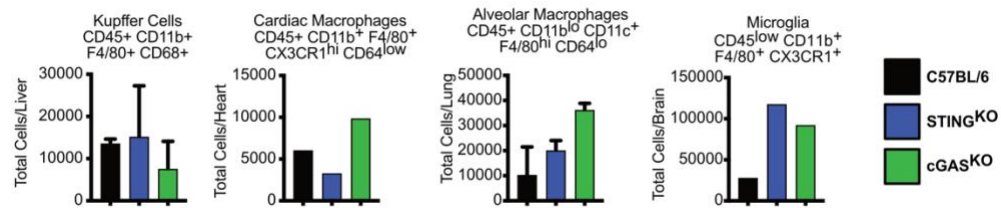


Figure 3.10 cGAS/STING signaling limits the expansion of tissue-associated macrophages in 6-month-old mice

Flow cytometric quantification of the total number of tissue-associated macrophages per organ. Cell counts acquired from the BD LSR-II and extrapolated by the total number of cells isolated during tissue homogenization. n = 2 mice per genotype, samples pooled per genotype for the heart, brain due to sample yield. Black; C57BL/6, blue; cGAS^{KO}, green; cGAS^{KO}.

With this potential heterogeneity within the tissue associated macrophage population due to STING signaling, a clearer understanding of the regulation of the tissue macrophage immune profile, as regulated by cGAS/STING signaling, became critical. As cGAS/STING is a ubiquitously expressed pathway – and interferon signaling can exert autocrine or paracrine effects – these effects on the tissue macrophage population could be intrinsic regulation by cGAS/STING or a tissue-wide effect imposed by stromal cells.

3.4 Isolation of tissue-associated macrophages

A primary goal of this body of work is to more thoroughly understand an observed immunoregulatory role of STING in innate immune cells, specifically macrophages. While whole tissue-directed qPCR of inflammatory and interferon stimulated genes is an adequate way to assess gross transcriptional changes, it is not sufficient to tease apart cell-specific changes. As such, it was imperative to isolate tissue-associated macrophages for specific analysis.

3.4.1 Physical isolation

Tissue macrophages are known to be sensitive to changes in their local environment. Previous qPCR analysis suggest that aged tissues are increasingly inflammatory when compared to younger tissues, as such it was important to perform as gentle an isolation protocol as possible to ensure maintenance of the transcriptional state. It is near impossible to bypass enzymatic tissue digestion, these steps were performed as gently, but quickly, as possible while still maintaining adequate single cell generation and remain a necessary evil in the isolation of tissue macrophages. Unlike lymphoid cells, macrophages will tightly adhere to plastic when cultured. During BMDM generation it allows efficient washing and “feeding” of macrophages with culture media and is a necessary characteristic that can be taken advantage of for selective isolation of macrophages. Performing a short culture of single cell tissue suspensions causes all single cells to settle while mature macrophages adhere, and lymphoid cells can be gently washed away.

I performed a 2-hour as well as overnight culture of tissue digestions to allow adequate time for macrophages to adhere tightly and test the potential of culture-based enrichment. However, while general macrophage concentration increased during both culture periods, I identified a significant population of macrophages within the non-

adherent supernatant suggesting that culture-based methods had a low efficiency of isolation potential (Figure 3.11A, data not shown). While it is impossible to know if these non-adherent macrophages were monocytic cells that partially matured during the overnight enrichment, macrophages that had developed an active migratory phenotype, or a subset of tissue-associated macrophages somewhat resistant to adherence, it remains a non-viable method for efficient isolation of tissue macrophages.

Interestingly, after performing qPCR analysis of baseline, 2h enrichment, and overnight enrichment I found a clear shift in the inflammatory and interferon stimulated gene expression profile of tissues and enriched macrophages (Figure 3.11B-D). Isolated or consistent alterations in the interferon profile after enriching for macrophages would be a clear indication that the tissue-associated interferon profile observed by qPCR was, rather, an average of a mosaic defined by unique cell types. This stark contrast between the young and aged enriched populations after two culture periods suggests rather that the action of adhering to a culture plate – combined with the selection of growth factors and the lack of other tissue-specific extrinsic signals – is causing rapid re-calibration of their transcriptional profile and likely their functional state. As such, culture-based enrichment methods were not a viable option to functionally test these macrophages.

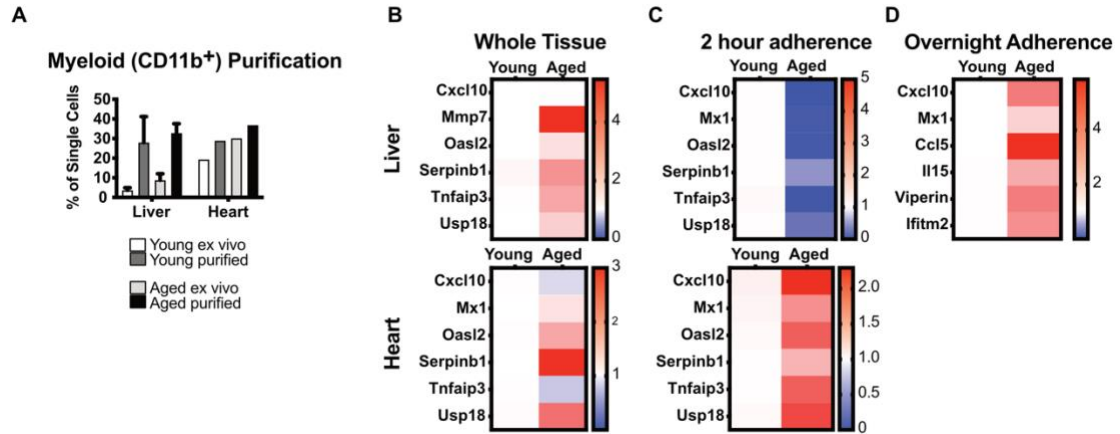


Figure 3.11 Plate-adherence enrichment protocols sufficiently alter the TRM transcriptome

(A) Bar graph of CD11b⁺ cells isolated ex vivo and after 2h enrichment in culture from young (2mo) and aged (20mo) heart and liver tissue. CD11b⁺ cells were quantified as a percentage of single events detected by flow cytometry to determine relative enrichment of myeloid cells. (B-D) qPCR analysis of select interferon stimulated genes from the liver and heart (B) as well as enriched Kupffer cells and cardiac macrophages from 2-hour enrichment (C) and 18-hour overnight enrichment. n = 3. Hearts pooled for sample size.

It has been shown that TRMs are highly responsive to signals from their microenvironment to shape their transcriptional profile and, theoretically, their functional response. Here, these data suggest that this functional integration does not solely rely on integration of danger or damage sensing but may include homeostatic signals to maintain the TRM functional state.

3.4.2 Fluorescence Activated Cell Sorting (FACS)

An alternative to plate-bound adherence is Fluorescence Activated Cell Sorting (FACS). Using an antibody-staining protocol, select populations of cells can be labelled, identified, and isolated through flow cytometry. While FACS can prevent any culture-

based adaptation of macrophage functionality, binding of antibodies to functional proteins risks synthetic activation of signaling pathways and shifts in the transcriptome. While these issues can be combated by performing the entire FACS protocol on ice or at 4°C it is not completely preventable.

I performed FACS to isolate all hematopoietic immune cells (CD45+) as well as tissue macrophages (F4/80hi CD11blo). (Figure 3.12A-B) While FACS is a very precise technique there is a very large tradeoff between specificity and efficacy due to the magnet-based sorting protocols. It can be expected that a flow cytometric-based sort will only be 70% efficient, a proportion that will decline with smaller populations – such as TRMs.⁹⁵

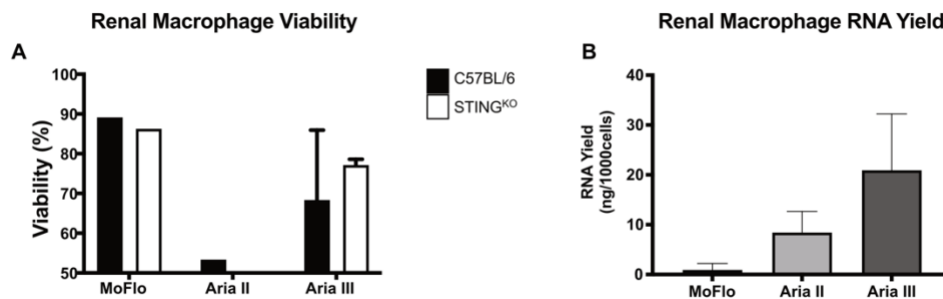


Figure 3.12 FACS sorting is not a viable enrichment procedure for tissue resident macrophage bulk transcriptomics

(A) Renal macrophage viability post FACS isolation with the MoFlow, Aria II, and Aria III cell sorters. Kidney from 5-week old C57BL/6 and STING^{KO} mice were digested and sorted for CD45⁺ CD11b^{low} F480^{hi} macrophages. Sorting system, genotypes annotated as shown. (B) RNA yield from renal macrophages isolated from the indicated FACS systems. Normalized RNA quantification indicated in ng/1000 cells. n = 2-5.

Here, I attempted to sort renal macrophages, as they routinely provided the largest and cleanest populations of the tissues assayed thus far, using the MoFlo, Aria II, and Aria III FACS systems. While the relative viability of the isolated TRMs was unchanged between C57BL/6 and STING^{KO} samples, the MoFlo sorting system retained the highest viability. (Figure 3.12A) Unfortunately, however, the total RNA yield from the

MoFlow was sufficiently low enough to prevent the specific isolation of TRMs using FACS at this time. (Figure 3.12B)

Using these two enrichment techniques incorporates massive tradeoffs between specificity and retention and were insufficient for qPCR or stimulation in culture. As such, rather than tailor the methods to the analysis, it was necessary to incorporate new analytic techniques that were capable of single-cell resolution. Next-generation sequencing techniques bypass many of the caveats discovered/discussed during qPCR and enrichment protocols.

3.5 Age-associated inflammatory profile

3.5.1 Introduction

Gross anatomical structure is a time-tested method of understanding general shifts in tissue function through architecture and immune involvement through mono- or polymorphonuclear cell infiltrates or the deposition and accumulation of tissue fibrosis. Histology and histopathology are some of the earliest forms to study the immune system in tissue, predating the understanding of lymphocytes, cancers, and antibodies. For the purpose of this project, I focused on analysis with hematoxylin and eosin (H&E). These two reagents bind to positively and negatively charged molecules and stain the tissue shades of pink and blue. This contract effectively stains the negatively charged nucleus blue and the positively charged cytoplasm shades of pink.

3.5.2 Inflammatory cytokines and chemokines are elevated in aging tissues

A major focus in aging research has been the identification of circulating inflammation to identify a biomarker of pathological aging. To best understand the physiological relevance of this project I first generated an extensive bank of serum

collected from mice across the aging spectrum, primarily targeting traditional young (2mo) middle aged (12-14mo) aged (18mo) and old (~24mo). A subset of these serum samples was sent away for multiplex ELISA analysis at Eve Biosciences. The selected multiplex targeted an array of inflammatory cyto- and chemokines to best understand the breadth and extent of age-associated circulating inflammation.

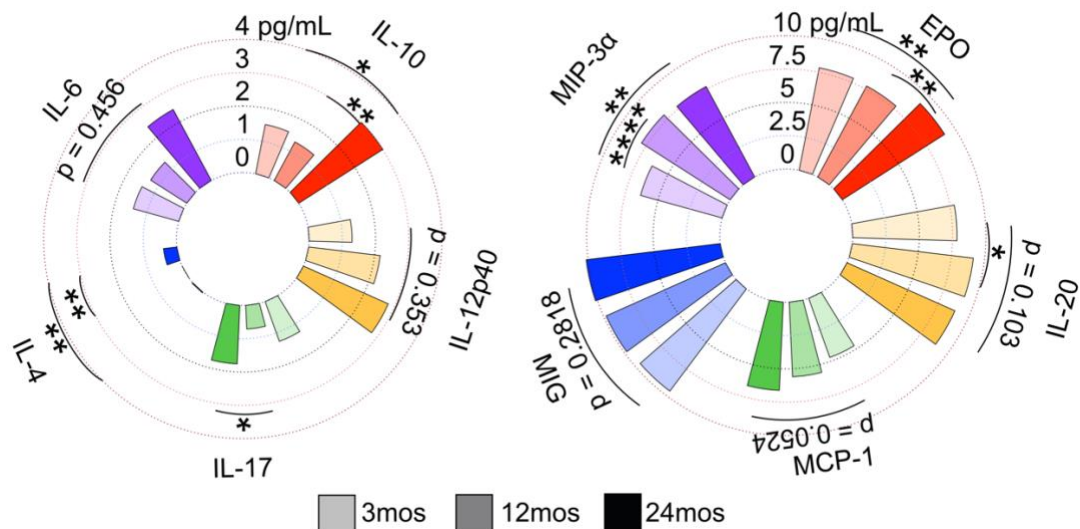


Figure 3.13 Inflammatory serum cytokines are elevated in aged mice

Radial bar charts depicting the relative concentration of serum cytokines from young (3mo), middle aged (12mo), and aged (24mo) C57BL/6 mice. Cytokines separated by concentration for visual clarity as lower than 5pg/mL (left) and greater than 5pg/mL (right). Cytokines colored and indicated peripherally to the plots. Ages marked by shading as indicated below. n = 4-5 Statistics calculated with a One Way Anova. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Here, these data demonstrated a clear elevation of inflammatory cytokines as early as 12mo in relation to the 2mo control samples. (Figure 3.13) Intriguingly, there was a clear increase in both cytokines and chemokines suggesting not only immune activation but chemotactic signaling towards an inflamed target. Combined with general

inflammatory activation of tissues based on qPCR analysis and shifts in tissue macrophage populations it became very intriguing to the local inflammatory activity of aged tissues – an aspect of aging that is woefully understudied.

3.5.3 Mononuclear cell infiltrates increase in aging tissues

As this project shifted towards a biological model of aging, the immunoregulatory role of tissue macrophages quickly highlighted the state of chronic age-associated inflammation in solid organs. While aging research is not a new field, a considerable amount of tissue-centric research has focus on how aging modulates specific innate or adaptive immune responses and rather overlooked the inflammatory status of tissues themselves rather focusing on generalized clinical inflammation. Here, we first sought to confirm the inflammatory status of these tissues through H&E staining. (Figure 3.14)

Staining of young tissues was adept at marking generalized tissue structures and highlighted consistent, organized structures throughout, while in the aged tissues there was a clear disorganization of the stromal elements as well as a prominent expansion of the mononuclear compartment. While unable to clearly determine the lineage of these cells it demonstrates a dominant expansion of local cells of a significant cellular infiltrate which could be trafficking towards the source of inflammatory chemokines noted in the serum. Intriguingly, these observations could directly account for a remarkable increase in activated, inflammatory cells within aged organs propagating a chronic inflammatory state in the tissues.

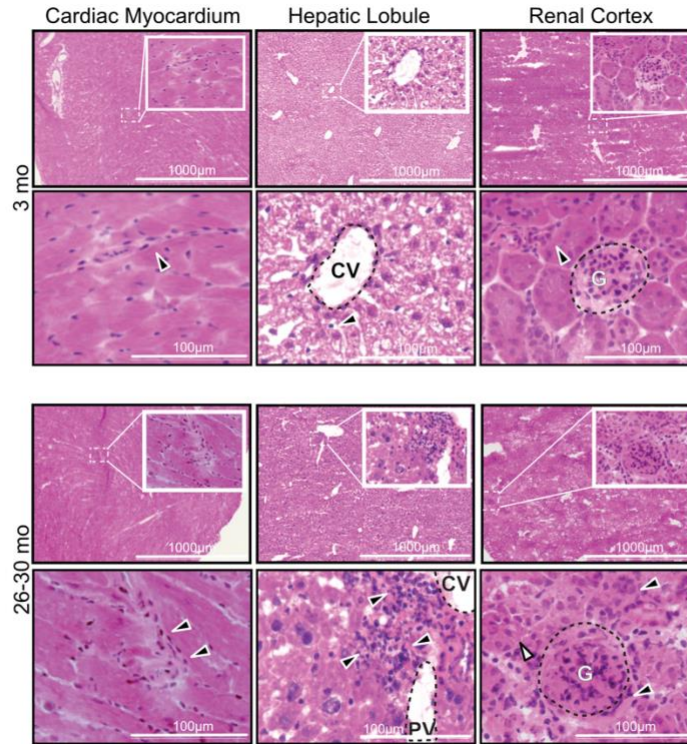


Figure 3.14 Aged tissues are marked by increased cellular infiltrates

Hematoxylin and eosin (H&E) staining of young (3mo) and aged (26-30mo) heart (left), liver (middle), and kidney (right) tissue flash frozen in OCT. Area of magnification indicated by dashed/solid box. Arrows indicate regions of local cellular infiltrate. CV; central vein, G; glomerulus. Representative images.

Combined, qPCR-identification of immune signatures in aging tissues, the shifting profile of macrophages through flow cytometric analysis, circulating inflammatory chemokines and expansion of mononuclear cells within tissues points towards significant restructuring of the tissue-centric inflammatory state with aging. While qPCR and flow cytometry are exceptional tools for highly targeted analysis, these shifts with aging are otherwise uncharacterized and require a sufficiently broad enough analysis to adequately understand the system as a whole. As such, for the purposes of this project I

shifted towards single cell RNA sequencing as a platform to investigate discrete cell types in an unbiased manner.

3.6 The effects of aging on transcriptional dysregulation

3.6.1 Background

Genetic sequencing has been a staple of biological science research for 50 years, and the speed and depth to which these experiments can be run has increased exponentially. From original experiments that sequenced individual genes, high throughput and microfluidic technologies have allowed sequencing technologies to target even single cells.

Due to the high level of transcriptional plasticity noted in TRMs, it became quickly clear that cell culture or FACS would not be sufficient enrichment techniques to adequately profile TRMs. To minimize the ex vivo processing of TRMs and still retain the ability to profile TRM heterogeneity I elected to perform single cell RNA sequencing (scRNAseq) of whole tissues. scRNAseq has been gaining incredible traction in recent years, progressing from a pinnacle technology for the most important studies to a common benchtop experiment. However, performing and analyzing scRNAseq requires the utmost control and regulation – due to the breadth and depth of the acquired dataset, a researcher can test an incredible number of hypotheses to a previously impossible number of cell types. However, not every question can be appropriately answered by any single scRNAseq experiment. Here, I utilized scRNAseq as a method of screening whole aged tissues to identify and characterize the role of STING in regulating TRMs primarily, but with the secondary ability of probing monocyte derived macrophages alongside stromal and parenchymal cells.

3.6.2 Methodology

I performed two distinct rounds of scRNAseq in an attempt to evaluate the breadth of the lifespan of mice and TRMs. Each round included young (2-3mo) and aged (12mo or 24mo) C57BL/6 and STING^{KO} mice. While the 24mo dataset was collected direct from tissues with an included Percoll gradient, while the 12mo experiment underwent mild FACS enrichment for live cells to increase the efficacy of the single cell isolation step. In part, this increased the efficiency of live cells, but also mildly enriched the population for immune cells. Aging and chronic inflammation are slow, yet progressive syndromes and it is likely that transcriptional and functional immune changes are compounding as well. As such, it is likely that any pathological cell states that are present in aged or end-of-life animals are initiated significantly earlier and even traceable from middle age. Including both of these datasets can not only provide a kinetic aspect to this dataset but can also increase the resolution of immune cells within the dataset.

With the help of a collaborating bioinformatician, I performed multiple iterations of the preprocessing protocols described above to generate distinct datasets that can each prioritize unique aspects of age-associated immune dysregulation. First, by combining all samples from each sequencing experiment inclusive of all tissues involved I was able to prioritize inter-tissue comparisons. Here, performing normalization, PCA, UMAP, and kNN procedures on all the tissues together, there is greater power to understand the magnitude of transcriptional similarity of cell types between tissues – rather than limiting the understanding to “cell type X” from tissue A or tissue B. Second, all samples from each tissue, regardless of sequencing set, were combined to, rather, prioritize intra-tissue comparisons. This iteration of the dataset normalizes and assigns clusters to cells restricted to an individual tissue. This method is increasingly adept at teasing apart transcriptional uniqueness between cell subsets, because the “background” dataset is

not muddled by unknown tissue-intrinsic differences between similar cell types that introduce excess variability in gene expression profiles. Further, as C57BL/6 and STING^{KO} samples were sequenced jointly, I elected to perform gene normalization and other preprocessing steps on both genotypes together and select solely the C57BL/6 subset of the data, when necessary, to specifically query age-associated changes.

3.6.3 UMAP generation of scSeq datasets

After sequencing data is retained as raw sequencing reads and requires significant processing and normalization before significant analysis can be performed. After raw sequencing reads are aligned to a reference genome it is necessary to begin to interpret the dataset for analysis. The most common way to visualize and begin to interpret scRNAseq datasets is with a t-Stochastic Neighbor Embedding (tSNE) or Uniform Manifold Approximation and Projection (UMAP). While generally very similar, UMAP analyses tend to run slightly faster and retain slightly better global data structure and was preferred for this project. The UMAP and cluster identification is ultimately the backbone of this, and most, single cell sequencing projects. Through the UMAP and associated cell clustering technologies individual cell types can be properly annotated, differentially expressed genes (DEGs) can be identified, and further cluster-based analyses can be performed.

After the filtering of low-quality cells or doublets (by unique genes and total gene counts) it was necessary to perform dimensionality reduction using principle component analysis (PCA; RunPCA) to use as generalized input for UMAP generation. Using principal components, rather than the entirety of the gene set, for UMAP generation created this projection much faster with considerably less processing power. After calculating sufficient PCAs it was necessary to determine the number of PCs that would be used to generate the UMAP. Including more PCs in the dataset increases the

possible resolution of the UMAP, as more information is included to differentiate different cell types. The number of PCs was determined by plotting the relative variance explained by subsequent PCs on an “Elbow Plot”. Each PC is calculated to describe the greatest amount of variation in the data (after accounting for any PCs preceding it), and it is vital to an accurate UMAP to include as much new information as possible – where the Elbow Plot begins to level off demonstrating an insignificant amount of new information per subsequent PC. It is important to compare multiple iterations of each step – PCA, UMAP generation, and cluster identification – to most appropriately process the data for proper project-specific analyses. As a major goal of this project was the classify previously unknown or poorly described cell types it was imperative that 1) the resolution of the data set was as high as possible and 2) that the data was not over processed leading to Type I errors and concluding populations are transcriptionally distinct when, in fact, they are not.

Clusters were identified using the Seurat functions `FindNeighbors()` and `FindClusters()`. `FindNeighbors()` generates a k-NearestNeighbors (kNN) graph derived from the PCs defined previously. The kNN generation provides information about the local structure of cells and provides information about relative similarity of each cell in local space. `FindClusters()` implements a Louvain algorithm to identify similar cells and group them into clusters for future analyses. `FindClusters()` contains a modifiable “resolution” variable allowing the user to increase (more clusters) or decrease (fewer clusters) the relative resolution of the dataset. This resolution variable directly tunes the capability of the dataset to profile generalized cell types or specific subclusters of cells and is one of the most important steps in processing data for scRNAseq.

Finally, reduced, clustered data was fed into the UMAP algorithm using the same PCs identified with `RunPCA()` and used for `FindNeighbors()`. The final 2-dimensional output visualized the data in such a way to determine if the data is over or under

clustered. This determination is made by comparing the cells in UMAP space to the relative clustering of the cells. If the data is over-clustered the UMAP will display smooth groups of cells that are either split by two or more clusters, or groups of cells will contain two or more clusters relatively homogeneously distributed. In contrast, under-clustered datasets will show multiple structures in 2-dimensional UMAP space that are incorporated into one k-NN “named” cluster.

3.6.4 Describing the Dataset – Population Identification

After generating, processing, and visualizing the scRNAseq datasets I began to selectively identify individual cell types within the Seurat-identified clusters. Using a combination of Top DEGs (`FindAllMarkers()`; which calculates DEGs from one cluster relative to the rest of the dataset and `FindConservedMarkers()`; which calculated DEGs expressed in each cluster from all integrated datasets) and relative DEGs (`FindMarkers()`; which compares DEGs between two select populations, when necessary) specific cell types were manually assigned to each individual cluster. Clusters were identified by specific cell types (Figure 3.15), general cell type (Figure 3.16), and general cell lineage (lymphoid, myeloid, stroma; data not shown).

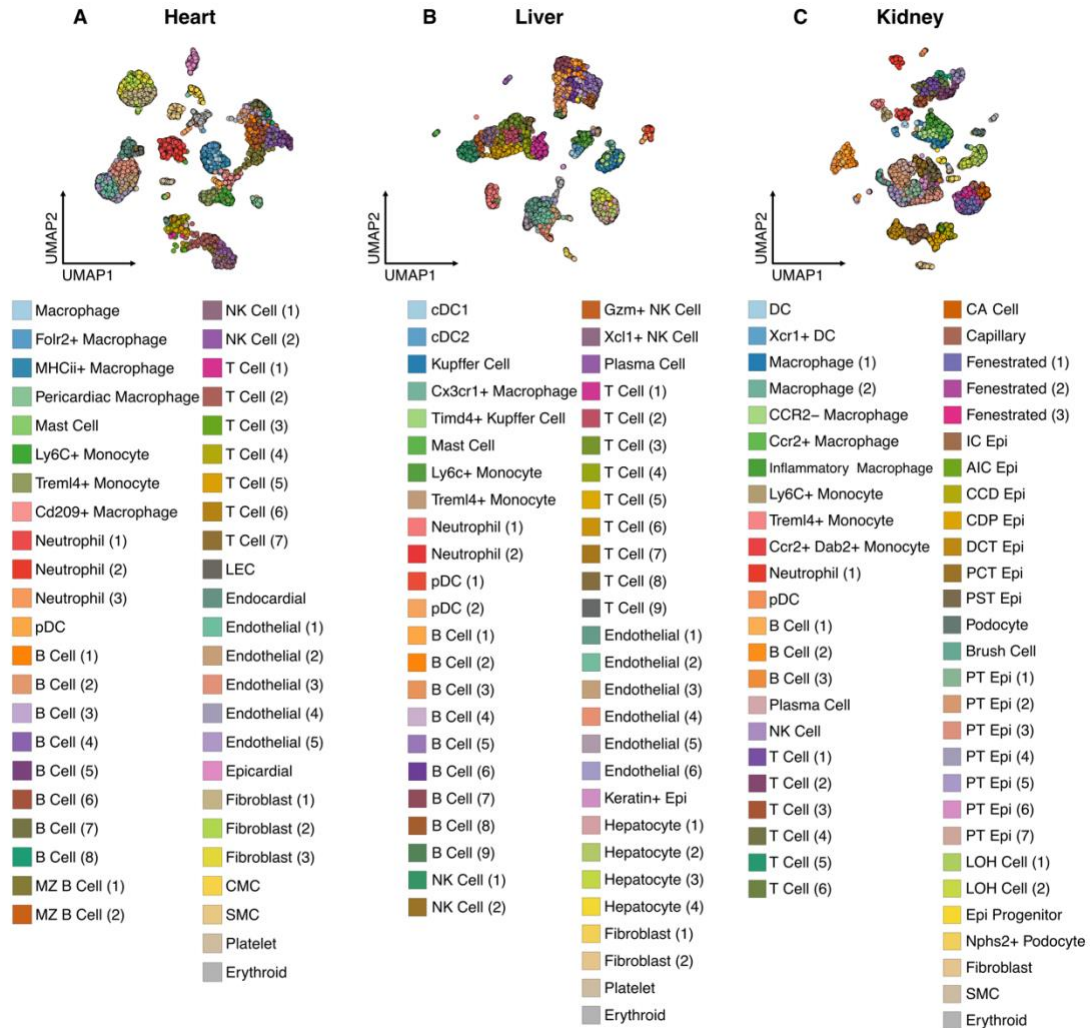


Figure 3.15 Full cell type annotation of tissue associated cells.

Using a combination of top differentially expressed genes (DEGs) and key lineage-identifying genes individual kNN-based clusters were specifically annotated for clarity of downstream analyses. Cluster-specific cell annotation for transcriptionally distinct clusters from the heart (A), liver (B), and kidney (C). Clusters color coded as shown, labeled in legend. Clusters oriented in UMAP space.

With these specific labeling strategies, it was then important to quantify the relative distribution of different cell types within the dataset, and how they were altered by age and in the absence of STING signaling.

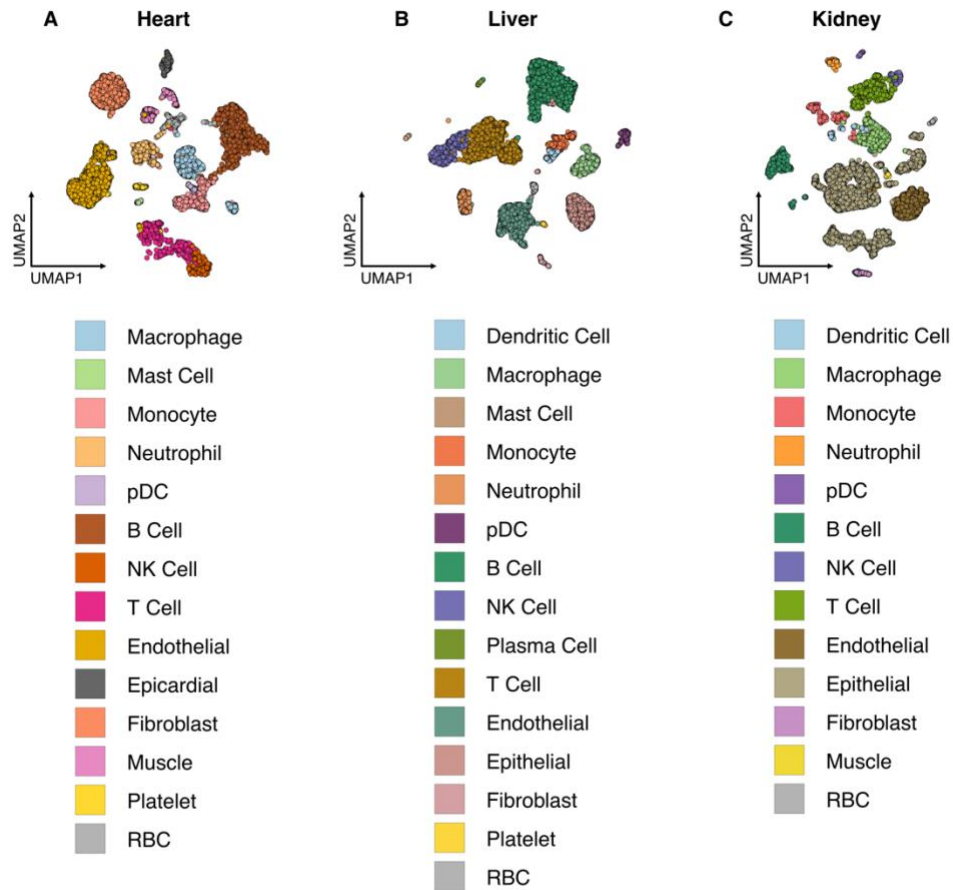


Figure 3.16 Descriptive cell type annotation of general cellular populations.

Using the methodology as in Figure 3.15, individual kNN-based clusters were grouped into generally descriptive cell populations to perform downstream analyses. Distinct clusters color-coded as depicted in the legend. Cells/clusters oriented in UMAP space.

Here, I found that, with age, there was a general restructuring of all cell types, without significant alterations due to STING signaling, suggesting that, while the global immune landscape appeared to change with age, this landscape was generally maintained in the absence of STING signaling. Unfortunately, however, it was found that between experimental scRNAseq datasets there were striking differences between population sizes suggesting some level of technical variation during sample generation or sequencing. Intriguingly, these changes do not appear consistent with complete

enrichment due to the live cell sorting applied to the 12-mo dataset. Due to these differences I decided to generally compare these datasets separately rather than perform the intended kinetics analyses (Figure 3.17).

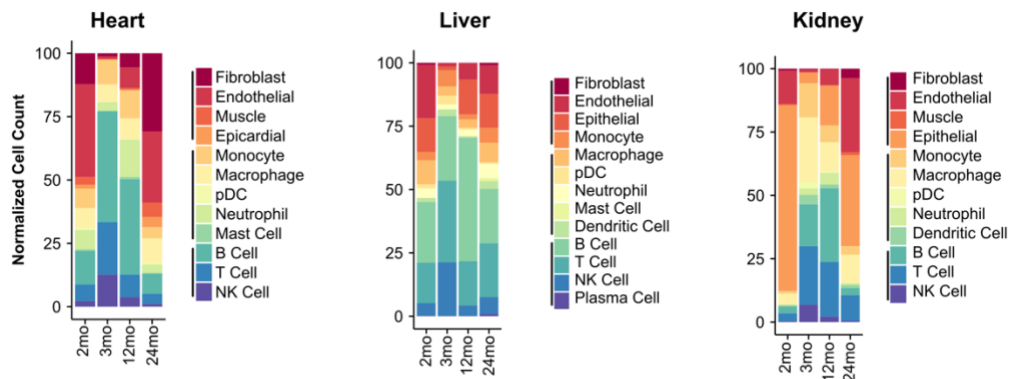


Figure 3.17 Tissue-specific cellular distribution of scRNAseq datasets.

Relative distribution of C57BL/6 sample datasets to directly compare shifts in cellularity with age. Total counts per broadly descriptive cell population were calculated and normalized per the total cells sequenced in each sample. Populations colored, annotated as shown.

3.7 Generation of Immune Signatures

3.7.1 Immune fingerprinting

Through qPCR analyses I demonstrated that STING signaling was sufficient to regulate general responses to both IFN-I and inflammatory stimuli. As such, it was first important to understand if this immune-modulating effect of STING could be recapitulated ex vivo. Primarily, I focused on the identification of age-dependent transcriptional changes to form a foundation to understand STING-dependent programs. Using DEG analysis I was able to calculate all marker genes for each tissue, sample groupings to understand if transcriptional diversity was most dependent on tissue of origin or age. (Figure 3.18A, B)

Interestingly, when plotting total DEGs it was apparent that there was an additive effect of age, and tissue of origin. While each tissue generally expressed similar genes, there was a very clear division of these tissue-identifying genes based on age.

Intriguingly, the middle-aged dataset appears to have a more striking stratification of these age-associated genes. (Figure 3.18A) While this dataset is partially enriched for immune cells, this suggests that the dominant effect of age-associated transcriptional changes may, in fact, be sourced from immune cells.

Importantly, when I examined these genes, it became apparent that a striking number of these tissue and age-defining genes were, in fact, immune-associated genes. This led to selective subsetting of these DEG maps based on inflammation (Figure 3.19) and IFN-I associated gene expression, the two readouts confirmed to be regulated by STING signaling during qPCR analyses.

Figure 3.18 Aging is marked by tissue-intrinsic gene modules.

Single cell RNA sequencing datasets were profiled for marker genes from each age/tissue designation, ordered, and sampled for the top expressing genes by average log2-fold change. The top genes segregate into unique, mostly non-overlapping gene modules with specific cassettes upregulated with age. Both datasets represented. (A) 3-12month, (B) 2-24month. Fingerprint marker genes thresholded with a Bonferroni adjusted p-value ≤ 0.1 .

Incredibly, these two immune gene sets demonstrated similar tissue/age distribution of genes compared to the total DEGs, suggesting a previously unappreciated tissue *intrinsic* regulation of immune associated genes. To better understand the transcriptional and immune “fingerprinting” of these datasets, I performed general pathway analysis on the genes identified as markers of each age/tissue combination. (Figure 3.20)

Here, using pathway analysis serves as a dimension reduction of sorts – rather than attempting to correlate tens or hundreds of genes, they can be selectively grouped based on Gene Ontology and used to describe functional potential of the dataset. Here, I performed this pathway analysis on total DEGs upregulated in the young and aged C57BL/6 samples (data not shown) as well as the *inflammatory* gene modules, specifically. Here I was able to identify a specific set of GO terms that described tissue specific functional processes in the young (regulation of defense response, endocytosis tissue homeostasis, circulatory system processes), while the aged DEGs were best described by immune associated or inflammatory terms (epithelial cell apoptotic process, monocyte chemotaxis; heart, positive regulation inflammatory response; liver, positive regulation of TNF production; kidney). These data directly suggest that a significant portion of the aged DEGs were, in fact, a result of age-associated pathological inflammation.

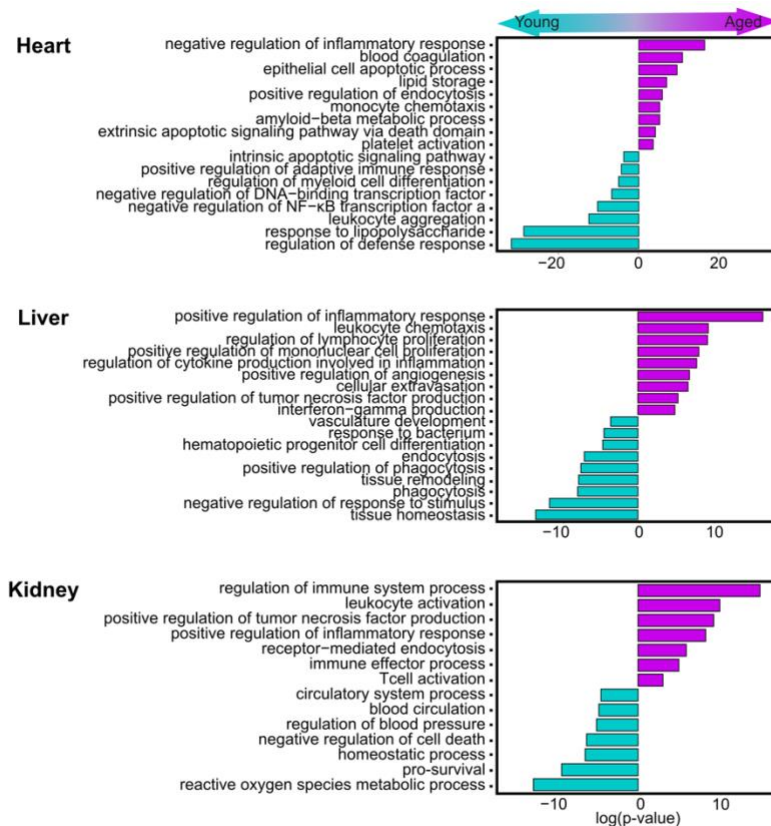


Figure 3.20 Aged tissues are enriched with reactive inflammatory gene programs

Descriptive pathway analysis of inflammatory gene modules upregulated in young (cyan) and aged (magenta) from the heart, liver, and kidney. Genes expression programs were summarized by pathway analysis and sorted by “recall” – relative enrichment of a signature within the input gene set.

This functional dichotomy of immune fingerprinting suggests severe immune dysregulation in these organs, rather than the select identification of a handful of genes that are altered in a dataset. Further, these data highlight a functional role of inflammatory-associated genes in young, otherwise healthy, mice. As such, further understanding of how immunity is altered with age was imperative. Aging is not a simple increase in inflammatory gene expression programs, but a concomitant loss of more regulatory and functional programs.

3.7.2 Relative distribution of age-associated immune fingerprinting

Profiling immune signatures by sample generated an intriguing set of immune fingerprints that was sufficient to delineate tissues by age. However, this analysis lacks an understanding of the cellular distribution of these signatures. A primary step to understanding the relative sample distribution of immune signatures was to calculate the relative activity of these genes based on the cell typing for each tissue. Each age-associated tissue fingerprint was isolated and used as an input to calculate the single-cell gene expression from each broadly descriptive cell type in each tissue. Intriguingly, while these signatures were not isolated to an individual cell, they were not equally distributed across all cell types and were selectively biased away from the lymphoid cells in each tissue – suggesting age associated chronic inflammation and immune dysregulation was primarily an *intrinsic* response rather than a chronic adaptive immune response as is so often observed in chronic immune disease. (Figure 3.21A)

Similar, but to a lesser degree, the IFN-I response pheno-copied the inflammatory signature. (Figure 3.21B) This generalized immune fingerprinting suggests that immune gene expression is fairly well represented across multiple cell types, and that individual cell types are aging *uniquely* rather than a dominant, age-associated immune signature developing temporally.

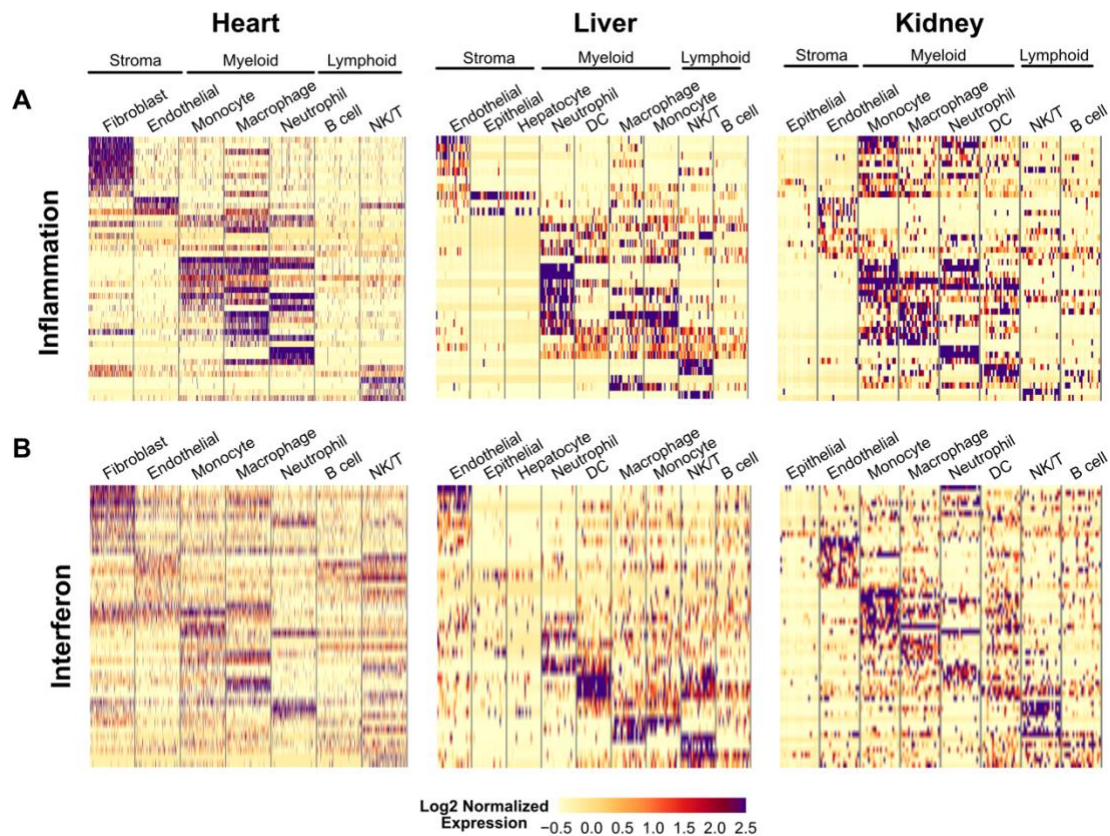


Figure 3.21 Age-associated inflammation and interferon-associated genes are distributed among aged myeloid and stromal cells

Single cell gene expression profiles from 24mo heart, liver, and kidney. Heatmaps display log2-normalized gene expression values for inflammation (A) and interferon (B) associated genes upregulated with age. Immune and non-immune clusters annotated above. Significance threshold with a Bonferroni adjusted p-value < 0.1.

3.7.3 Generation of a Network-based description of immunity in aging

After the identification of tissue-based *functional* immune fingerprints with the direct querying of inflammatory and interferon signatures, it was pertinent to understand general transcriptional dysregulation with age, and the scope of which is central to immune dysregulation. To best understand this generalized transcriptional dysfunction I

performed directed DEG analysis of individual populations for all genes upregulated with age, characterized the totality of this transcriptional signature through pathway analysis, and performed similarity scoring of all enriched GO terms to generate a network. (Figure 3.22)

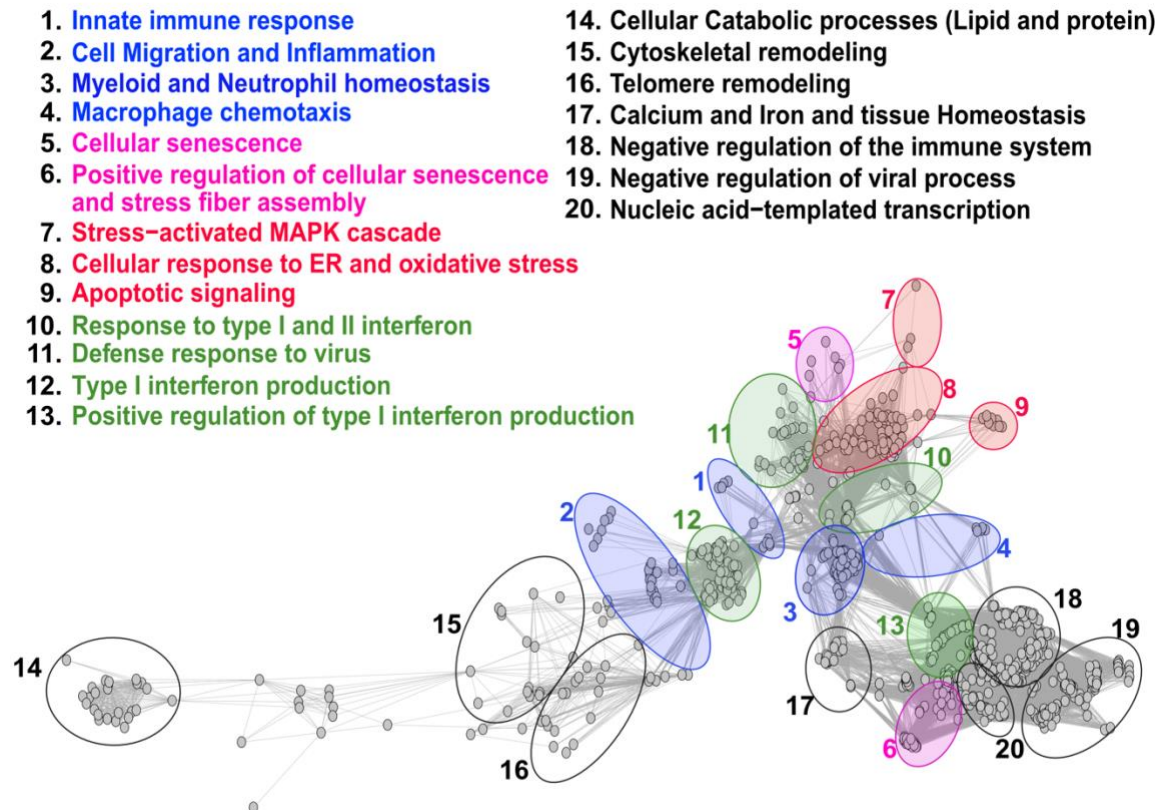


Figure 3.22 Age associated immune dysfunction is best characterized by inflammation, interferon, and senescence programs

Network-style projection of descriptive pathway analysis. Genes that were upregulated with age were summarized by gene ontology pathway analysis. A “similarity score” was generated using the online “Revigo” algorithm which was used as the basis for a clustered network of associated pathways (nodes). Clustered pathways were manually summarized and annotated as shown. Blue; immune response, pink; senescence, red; cellular stress, green; interferon response, black; cellular processes.

This “similarity network” consisted of nodes (pathways) clustered by “semantic” similarity into foci of three to dozens of nodes. Projecting the data into a network assisted in parsing higher- and lower-level GO terms into a collection that is more easily

described. All foci and associated genes were manually annotated and suggested that general, population independent transcriptional dysregulation was significantly impacted by general immune dysfunction highlighted by immune activation and interferon signaling – but a key set of terms that emerged suggested initiation of senescence was a dominant factor in age associated transcriptional dysregulation.

3.7.4 Senescence is a dominant immune program dysregulated with age

The identification of an enrichment of oxidative and replicative stress pathways was suggestive of an enrichment of senescence pathways with aging. To more specifically profile these changes, I performed similar fingerprinting analyses and extracted senescence genes from all markers genes for each sample. (Figure 3.23A) Although senescence is commonly associated with age, there was not a clear age-associated gene module that emerged with these tissues and a number of senescence-associated genes were, in fact, enriched in the young samples. Further, the core expression of key senescence markers *Lmna*, *Lmn2*, *Cdkn1a*, or *Cdkn2a* were not comprehensively elevated in aged mice, suggesting that senescence, while altered with age, was restricted to some tissue or cell-specific patterning with age. (Figure 3.23B)

3.7.5 Correlating tissue-centric immunity and peripheral cytokines

A common assumption in the field of aging is that the elevation of inflammatory mediators in the periphery bathe solid organs in an inflammatory milieu, driving chronic immune responses and tissue dysfunction. Here, we define a panel of inflammatory cytokines upregulated in the serum of aged mice in conjunction with unique inflammatory fingerprints that are altered with age in each tissue. As such, it was intriguing as to how

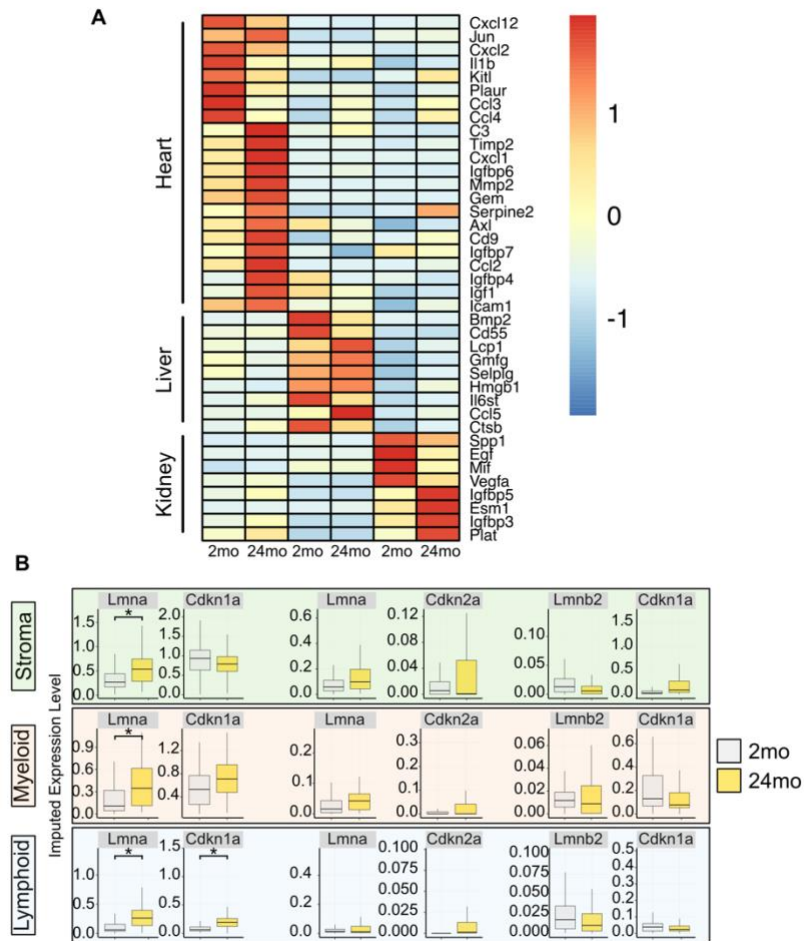


Figure 3.23 Senescence associated genes are not comprehensively regulated at the tissue level with age.

(A) Age-associated fingerprints subsetting for senescence-associated genes from young (2mo) and aged (24mo) mice. Blue; downregulated, red; upregulated. (B) Box and whisker plot for key senescence-associated genes from stroma (green), myeloid (orange), and lymphoid (blue) subsets. Plots indicate mean, first and third quartile, minimum and maximum. Grey; 2mo, yellow; 24mo. Statistics calculated as a Bonferroni adjusted p-value < 0.1. *p ≤ 0.05.

these two observations could interact – and suggests that cells are programmed to differential immune responses based on their tissue of origin. In order to more clearly parse the relationship between age-associated inflammatory serum cytokines and tissue immune fingerprinting I leveraged the NicheNet package to quantify downstream signaling potential of the queried inflammatory cytokines. (Figure 3.24)

Figure 3.24 Nichenet identifies signaling relationships between similar cell type regardless of tissue of origin.

(A) Circos plot depicting relative strength of signaling networks downstream of inflammatory cytokines (bottom) in individual cell types from the scRNAseq datasets (top). Signaling strength downstream of Il6 (orange) Tnf (brown) Il12a (teal) and Il12b (green) highlighted for clarity. Relative strength of signaling for highlighted cytokines depicted by transparency. (B) Heatmap representation of all associated signaling networks from cell types independent of tissue of origin. Values indicate mean NicheNet activity scores based on Pearson correlation coefficients. Clustering was performed on columns (inflammatory cytokines) and rows (cell types) in an unbiased manner. Tissue, and cell lineage annotated as shown on the left. Specific cell clusters labeled to the right.

NicheNet is a descriptive algorithm that integrates the relative expression of receptors for key ligands, transcriptional regulators, as well as signaling targets to understand the likelihood that a given cell has responded to the ligand in question. Here, using the multiplexed inflammatory cytokines and chemokines referenced above as inputs, I generated a communication matrix to directly visualize signaling potential of each cell in each tissue.

Remarkably, the greatest change in the signaling potential was by *cell type* and not tissue of origin. It is commonly believed that systemic, circulating factors are major drivers of age-associated tissue dysfunction and would be the source of the tissue fingerprints described here. However, the observation that signaling potential downstream of these inflammatory cytokines are similar regardless of tissue of origin suggests that any alterations in tissue immune profiles were, rather, due to tissue-intrinsic signaling. (Figure 3.24B)

3.8 Characterization of immune pathway enrichment

3.8.1 Gene set variation analysis

This description of comprehensive immune dysregulation through the use of specific gene sets spurs an intriguing question about the comprehensive nature of immune signaling in tissues and what cell types are primary contributing to this positive

signature. To best understand the contribution of specific tissue associated cells to the immune reactivity with age I leveraged the gene set variation analysis (GSVA) package. In short, GSVA performs single cell gene set enrichment analysis (GSEA) and returns a single activity score for each profiled pathway. Using this functional scoring it was possible to determine not just which cell types were enriched for specific gene sets, but to understand which cell types were primarily enriched in the signature as a whole – accounting for relative gene expression of each gene in the signature.

GSVA calculates an activity score for each cell type with a range of -1, 1. To properly visualize these terms – as the sign of the score does not indicate baseline up- or down- regulation – I performed a linear transformation to shift the datasets into the positive range (0, 2) while maintaining all relative distances of score and calculated the relative mean for each cell type in the control (young) or experimental (aged) datasets. Using these transformed values, I set out to determine if specific cell populations were selectively enriched for any of these aging-centric immune signatures. By plotting these transformed mean activity scores for each population, I was able to assess if these immune activity scores were biased towards the young or aged datasets for any specific population. (Figure 3.25)

Intriguingly, when scoring the mean transformed populations, the general trend of the data maintained a linear trajectory, with the mean expression values staying within a relative margin of error for each population. As the previous analysis suggested relative enrichment of these immune signatures with age, I applied a relative normalization to understand if these age-centric immune signatures were relatively enriched in specific populations.

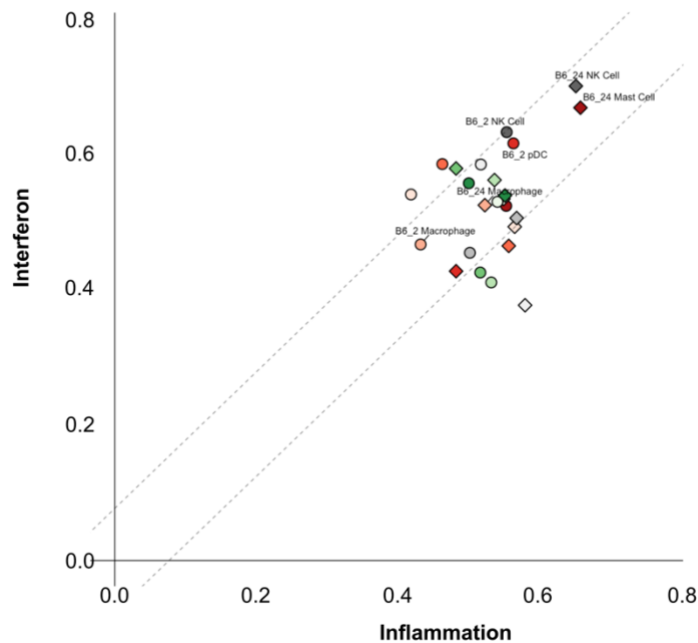


Figure 3.25 GSVA-enrichment scoring of inflammatory and interferon responsiveness is generally linear.

Scatterplot of the mean transformed GSVA-enrichment scoring to directly correlate the general enrichment of inflammatory-associated genes (GO:0006954) and interferon response genes (GO 0034340). Filled circles; 2mo C57BL/6. Filled diamonds; 24mo C57BL/6 populations. Color scale representative of individual populations from 2mo and 24mo datasets. Key clusters annotated.

3.8.2 Population specific enrichment of immune pathways

To calculate which populations were selectively enriched for these signatures I created a population-specific normalization that will calculate relative enrichment of these activity scores relative to the young C57BL/6 control sample as a relative baseline. Using the previously described linear-transformed population means, I calculated a normalized *enrichment* score for each individual cell (from the 2mo and 24mo dataset) relative to this standard baseline. Using a standard baseline for each cell type attempts to account for biologic variability between cell types and focuses the analysis on aging in its entirety. It plausible that specific cell types (e.g., macrophages or neutrophils) would score much higher for inflammatory activity than other cell types (e.g, endothelial cells or

fibroblasts) – however the relative change with aging is more likely to contribute to age-associated immune dysfunction than raw activity scoring.

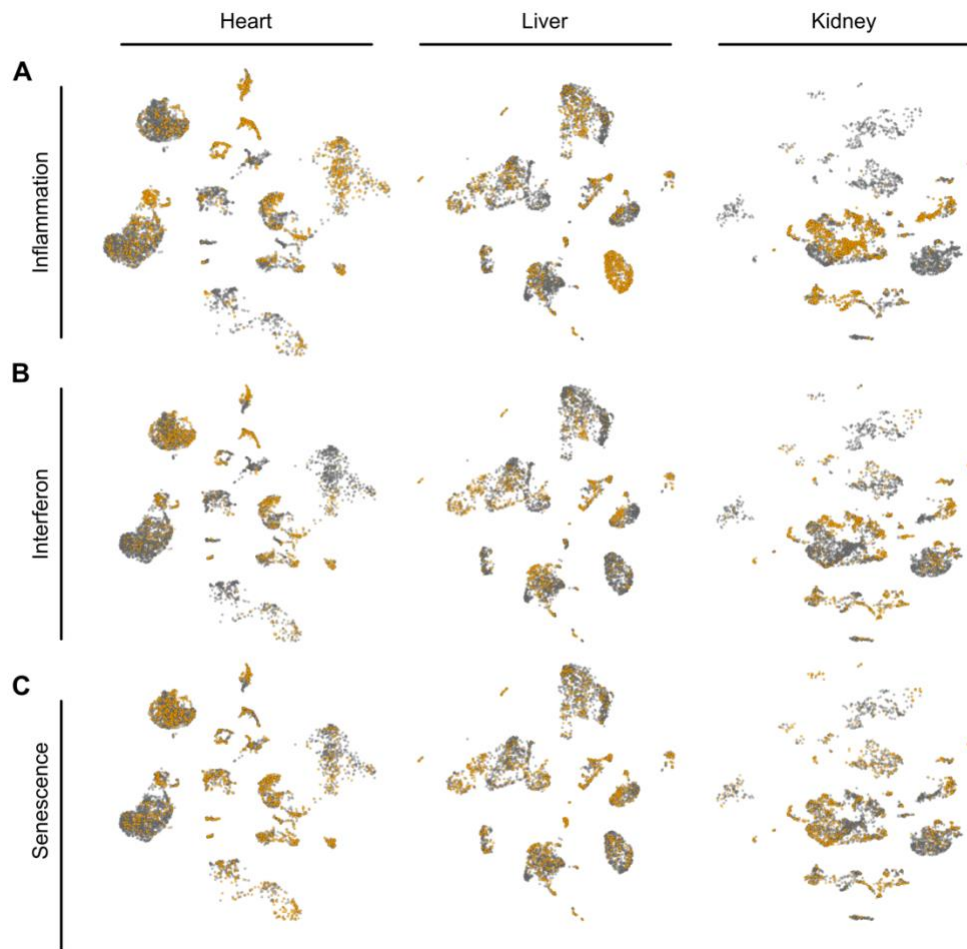


Figure 3.26 Gene Set Variation Analysis-based distribution of immune signatures

GSVA scoring of the relative enrichment of the entirety of inflammation (A), interferon (B), and senescence (C) associated genes from the heart, liver, and kidney. Cells with a normalized GSVA score above 1.2 (heart, kidney) and 1.1 (liver) highlighted in yellow. All other cells in grey. Relative strength of “enriched” GSVA scoring further annotated by transparency.

Using this normalized enrichment scoring and the UMAP described previously, I projected the inflammatory (Figure 3.26A), interferon (Figure 3.26B), and senescence (Figure 3.26C) activity scores and found that, generally, there was enrichment off these

signatures throughout each individual tissue, it appeared certain populations – specifically regions of the macrophage and stromal clusters – were consistently enriched for immune activity.

3.8.3 Comprehensive dysregulation of immune pathways

Upon the observation that specific regions of macrophages and stromal cells were consistently enriched for age-centric immune signatures I set out to determine if any cells or populations were relatively enriched for multiple immune pathways. Using the normalized enrichment scores described previously I applied a relative threshold to each immune signature and cell type based on the standard baseline of each cell type – 1.2x for the heart and kidney, 1.1x for the liver due to a lack of cells in the aged dataset. As activity scores are calculated for all cells, this threshold serves to identify which cells are sufficiently far from the mean score to consider “enriched”. With a continued focus on the three age-centric immune signatures, I could then identify cells that were enriched for individual or multiple immune signatures. (Figure 3.27A)

While the young datasets were generally enriched with cells that crossed this enrichment threshold for one of the three immune signatures, which can be easily accounted for by random distribution around a mean with standard error, the aged datasets were dominated by cells that were enriched in all combinations of two immune signatures and specific populations of cells that were enriched in all three – cells here termed as “polypositive” (Figure 3.27B, C). As observed previously, these polypositive cells are generally contained within the macrophage and stromal compartments. However, when creating an overlay of the polypositive state on the tissue-specific UMAP a very specific subpopulation of macrophage, reminiscent of the recently described TLF (Timd4+, Lyve1+, Folr2+) tissue resident macrophages, but diffuse stromal cells, are selectively polypositive.

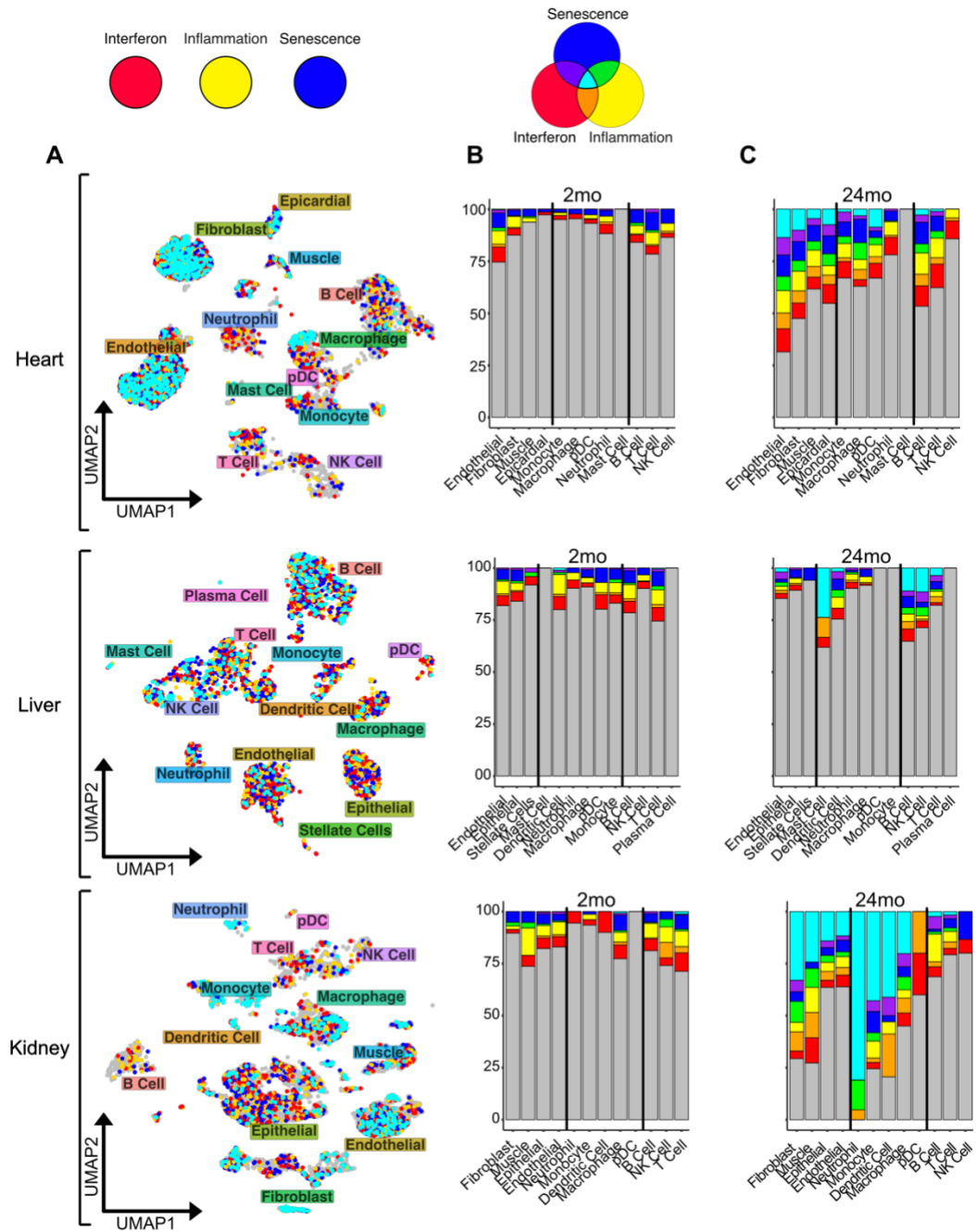


Figure 3.27 Comprehensively immune dysregulated (polypositive) cells are conserved as a function of aging.

(A) UMAP depiction of relative GSVA maximum for cells from the heart, liver, and kidney. Cells with a GSVA score greater than 1.2 (heart, kidney; 1.1 in the liver) are annotated by their maximum GSVA score. Cells with GSVA scores above threshold for all three (inflammation, interferon, senescence) pathways are highlighted in cyan. Colors for each pathway annotated above (red; interferon, yellow; inflammation, blue; senescence). (B-C) Normalized stacked bar graph to depict the relative frequency by which cells from each population are represented in young (2mo, B) and aged (24mo, C). Pathway annotated conserved from (A). Cells for which two pathways are above the GSVA threshold further annotated. (Purple; interferon+senescence, green; senescence+inflammation, orange; inflammation+interferon).

This is a particularly interesting observation, as tissue resident macrophages are considered part of the tissue's front-line defense and a focal point of the propagation of immune responses. Further, they are believed to, in part, functionally integrate with local stromal cells to orchestrate tissue immunity and regulate immune responses.

3.9 Regulation of intrinsic tissue immunity through STING

3.9.1 Aging as a model of chronic DNA sensing

Many studies modeling aging develop unique systems in which experimental animals undergo “accelerated aging” through genetic models that appear to replicate the aging process. Interestingly, most of these models induce nuclear instability or increase the level of DNA sensing within a cell. However, this not only presupposes that DNA damage/sensing is a primary cause of aging but assumes that aging can be accelerated by over activating this pathway. The immune system is incredibly complex, and it is unlikely that induction of one pathway is sufficient to accurately recapitulate all detrimental aspects of aging. Here, I took an opposing approach to the study of DNA sensing during aging by using *physiological aging* as the model. Here, I allowed mice deficient in the STING allele *Tmem173* to age, naturally, and collected tissues and serum along the same timepoints as described for the C57BL/6 mice to model aging

absent signaling through the STING pathway. Intriguingly, this model does not prevent DNA *sensing* through cGAS, only the propagation of the signaling through STING, believed to be the primary and sole adapter for the produced cGAMP.

3.9.2 Inflammatory cytokines are further upregulated in the absence of STING

For preliminary profiling of the role of STING in the regulation of age-associated inflammation I sent serum samples from STING^{KO} mice of varying ages for the same immune-directed multiplex as the aging C57BL/6 samples. (Figure 3.28) Current models of accelerated aging predict STING as a dominant promoter of age-associated inflammation, however with complete deficiency of STING, mice instead develop elevated levels of inflammatory cytokines in the sera – suggesting that STING is, in fact, acting to *regulate* the propagation of peripheral inflammation rather than a strictly dominant driver as was previously believed.

3.9.3 The absence of STING drives stromal and immune dysregulation

These data effectively recapitulate and drastically expand the concept of inflammaging from a systemic inflammatory milieu towards a model of tissue-intrinsic aging. While serum cytokines are *elevated* in the absence of STING, this does not inform the level of inflammation within the tissues – as seen in the aged C57BL/6 samples. Here, to understand the contribution of STING signaling to aged tissue-intrinsic immune profiles, I reanalyzed the tissue-specific data including the STING^{KO} samples. Importantly, the general makeup of the tissues was similar between the 24mo C57BL/6 and STING^{KO} sample sets, suggesting that these datasets are appropriately comparable and there was no significant sampling errors during processing. (Figure 3.29)

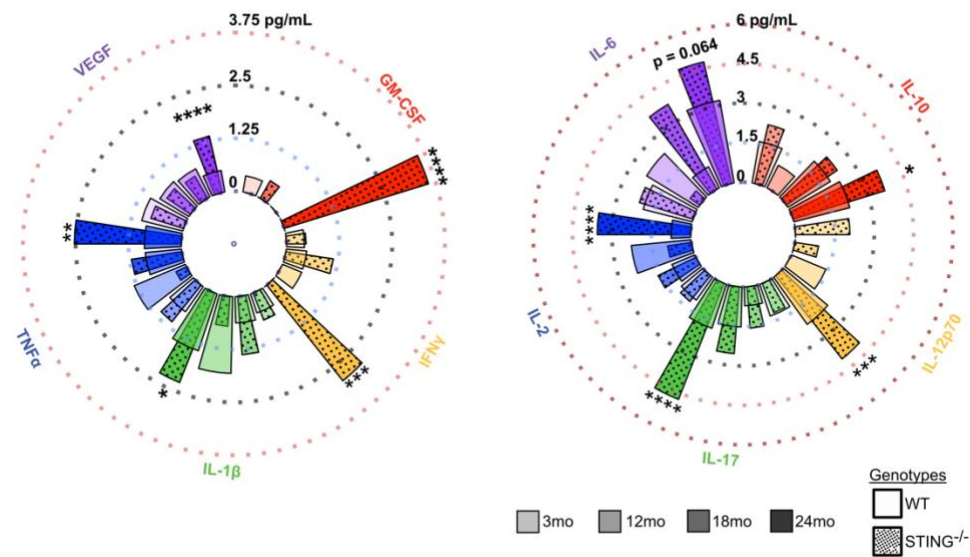


Figure 3.28 Circulating inflammatory cytokines are elevated in the global absence of STING signaling.

Radial bar chart depicting the relative concentration of inflammatory cytokines from 3mo, 12mo, 18mo, and 24mo C57BL/6 and STING^{KO} mice. C57BL/6; solid, transparent bars, STING^{KO} dotted bars. Colors highlight specific cytokines and are separated by relative concentration for clarity. n = 5-6 Statistics calculated with One way ANOVA with Bonferroni correction. * p $\leq$ 0.05; ** p $\leq$ 0.01, *** p $\leq$ 0.001.

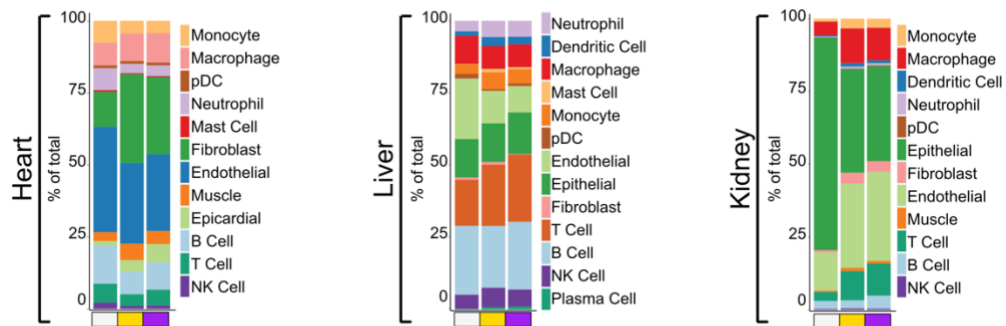


Figure 3.29 Age and STING signaling does not drastically alter general immune or stromal populations in the heart, liver, and kidney

Total cell counts from the heart, liver, and kidney were calculated, and normalized to the total number of cells from 2mo C57BL/6 (grey) 24mo C57BL/6 (yellow) or 24mo STING^{KO} (purple).

However, a gross observation of heart, liver, and kidney tissue from >24mo STING^{KO} through H&E analysis showed a series of clear stromal disruption and cellular infiltration compared to the young and aged C57BL/6. (Figure 3.30) Most notable, aged C57BL/6 liver tissues appear to contain an exceptional cellular infiltrate while the aged STING^{KO} have progressed to total stromal disruption. However, this breakdown of physical architecture remains striking across all three tissues.

A major undertaking for this body of work was the commitment to analysis of lifespan. Many models of STING-dependent pathology in the context of aging is elucidated through models of accelerated aging under genomic stress. Here, these mice age naturally and are only exposed to “normal” nucleic acid signaling within standard biological thresholds. Similar to the H&E analysis there was a stark and significant reduction in lifespan in the absence of STING signaling that was conserved in both cGAS and IFNAR deficiency suggesting a dominant function of the cGAS/STING signaling pathway that is beneficial during extended aging. (Figure 3.31) However, it remains unclear if this is a direct effect of cGAS/STING signaling or part of a more complex signaling network.

I then performed immune fingerprinting analysis as previously described, focusing on the three immune cassettes: inflammation, interferon signaling, and senescence associated genes from the 24mo dataset. (Figure 3.32)

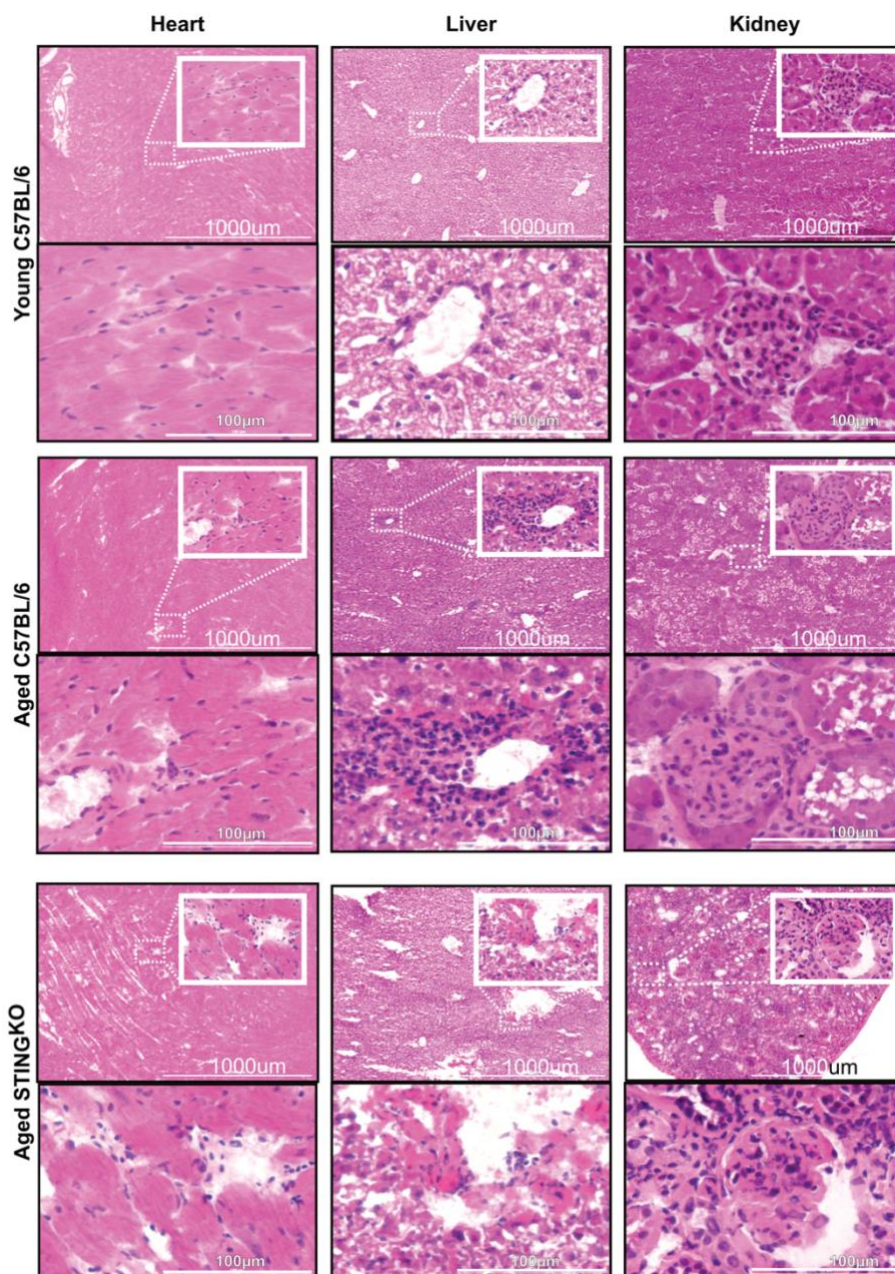


Figure 3.30 STING signaling facilitates the maintenance of physical tissue architecture with age.

Hematoxylin and Eosin (H&E) staining of young (3mo) C57BL/6, and aged (6-32mo) C57BL/6 and STING^{KO} heart, liver, and kidney. Tissues flash frozen in OCT. White boxes denote areas of magnification. Representative images.

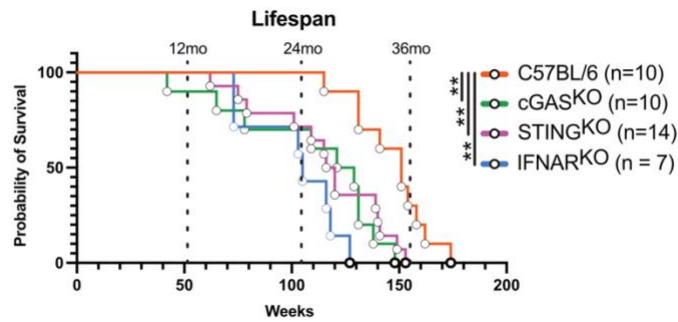


Figure 3.31 cGAS/STING signaling confers longevity

Kaplan-Meier survival probability curve of C57BL/6 (orange), cGAS^{KO} (green), STING^{KO} (purple), and IFNAR^{KO} (blue) male mice. Statistics calculated using a Mantel-Cox Logrank test * $p \leq 0.05$ ** $p \leq 0.01$.

Incredibly, while a portion of these gene sets upregulated with age were selectively reduced in the STING^{KO}, suggesting STING does positively regulate these signatures with age, there was a distinct cassette of these cassettes that were similarly upregulated in the STING^{KO}, suggesting a dominant immunoregulatory potential downstream of STING. Further, the dysregulation of these signatures was, in part, tissue dependent further supporting a previously unappreciated role of both cell- and tissue-intrinsic immunity with age.

3.9.4 Comprehensive age-associated immune dysregulation is dependent on STING signaling

While there is general restructuring of immune genes in the STING^{KO} relative to aged controls, this does not effectively address the relative concentration of immune signatures within the STING^{KO} tissues. Using the previously mentioned GSVA analysis to generate activity scores for inflammation, interferon signaling, and senescence, I generated a UMAP overlay for each immune signature's activity score. (Figure 3.33A)

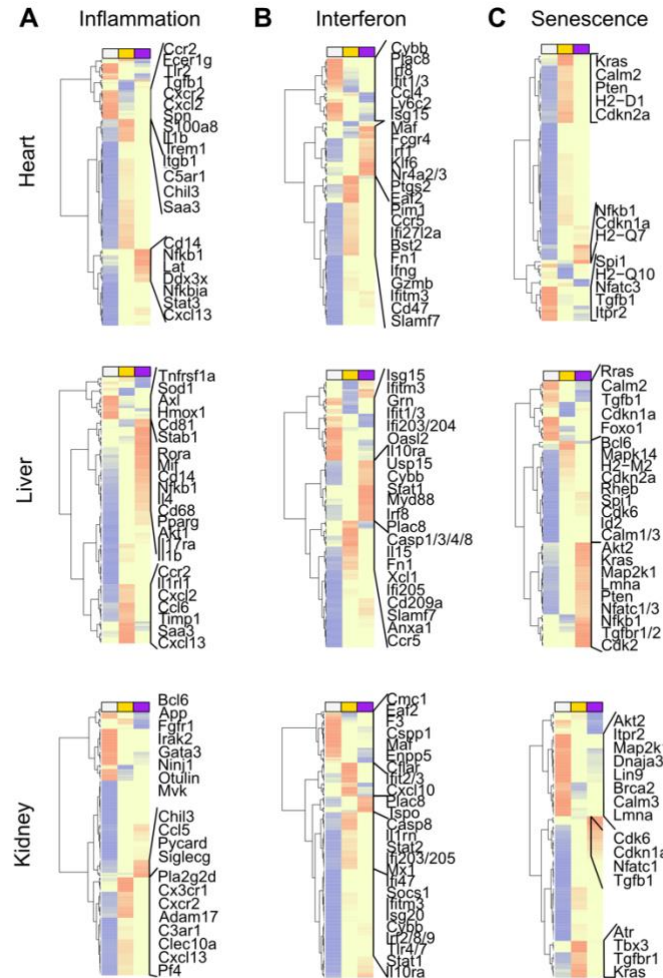


Figure 3.32 STING is a key modulator of age-associated immune signatures in the heart, liver, and kidney.

Marker genes were calculated for 2mo and 24mo C57BL/6 (grey, yellow respectively), 24mo STING^{KO} (purple) samples and inflammation (A), interferon (B), and senescence (C) associated genes were selected for fingerprinting analysis. Here, positively enriched marker genes were selected for to highlight core immune signatures associated with each dataset. Key genes within each cassette are annotated. Significance threshold at a Bonferroni adjusted p-value < 0.1.

With this analysis, it is clear to see that, akin to the C57BL/6 data, there is a general enrichment of these immune signatures within stromal and myeloid populations. In the C57BL/6 datasets, I observed general immune dysregulation within fibroblasts and specific populations of macrophages that were reminiscent of TLF+ resident macrophages. However, the STING^{KO} individual enrichments appear similar at first

glance, it was necessary to understand the relative proportion of these cells that experienced this generalize immune dysregulation in the absence of STING signaling.

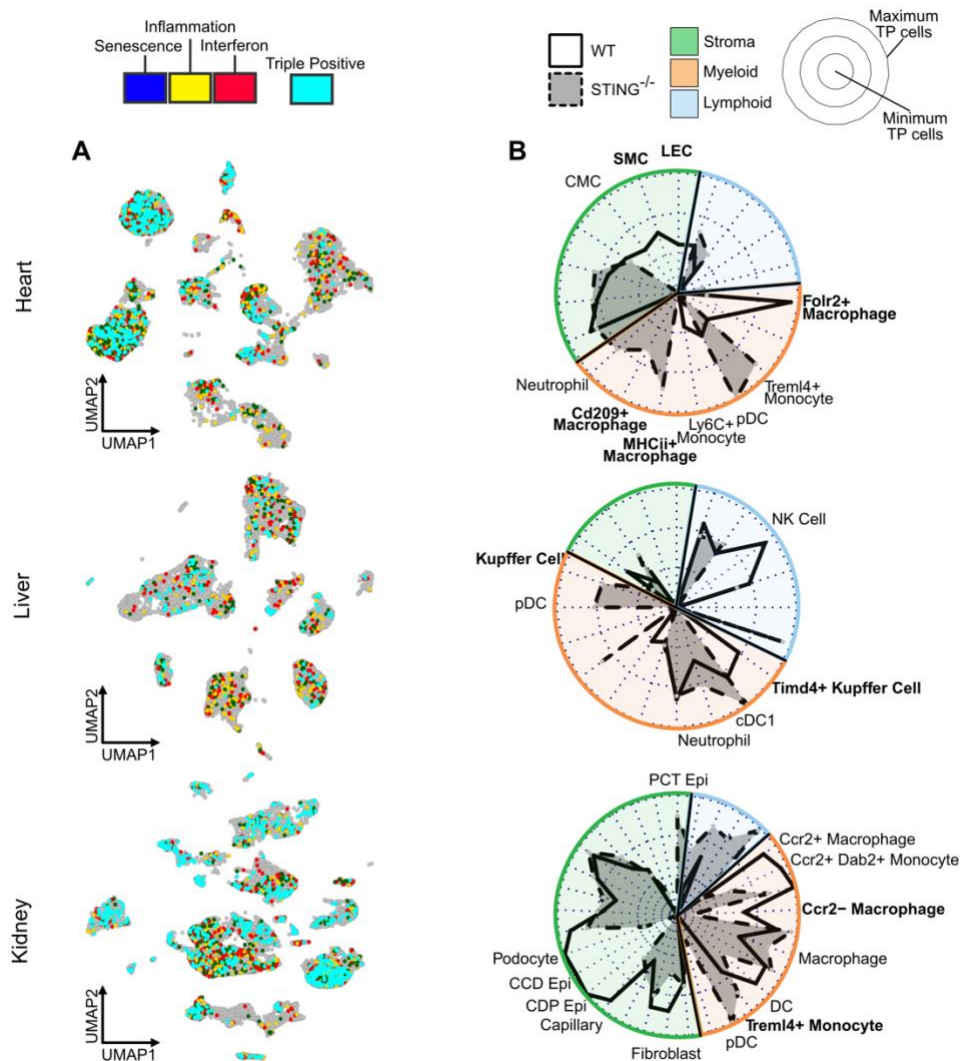


Figure 3.33 STING signaling selectively regulates immune dysregulation in stromal and myeloid cells.

Relative enrichment of GSVA activity scores were calculated as in Figure 3.26 for 24mo STING^{KO} heart, liver, and kidney datasets. (A) UMAP representation of single cell GSVA maximum enrichment scores are plotted as in Figure 3.26, with cells containing three activity scores above the tissue threshold in cyan. (B) Radar plot depiction of relative concentration of polypositive cells from 24mo C57BL/6 (solid line) and STING^{KO} (dashed line) cells. Individual cell clusters are grouped by lineage (stroma; green, myeloid; orange, lymphoid; blue) with key myeloid and stromal populations annotated as shown. Relative enrichment of polypositive cells in each cluster is normalized to the greatest percent positive for each sample. Outermost ring of the Radar plot denotes the sample maximum (not 100% enrichment), center-most point denotes sample minimum.

I analyzed the 2 and 24mo C57BL/6, and the 24mo STING^{KO}, calculating a relative ratio of *polypositive* immune dysregulated cells in each specific cluster. Here, I included specific subclusters of macrophages as they were previously observed as specifically susceptible to this immune dysregulation. A goal of this analysis is not to identify individual differences between the C57BL/6 and STING^{KO} data, but to understand which populations, as members of a whole, are becoming dysregulated immunologically. Rather than a traditional bar plot, I elected to represent these data with a “radar plot” in which the X-axis is circularized and reduced to a point allowing each dataset to extend outwards. (Figure 3.33B) Grouping the datasets by cell lineage (stromal, myeloid, lymphoid) plays into the strength of a radar plot by quickly visualizing the extent to which polypositive cells are differentially enriched in the 24mo C57BL/6 and STING^{KO} datasets. There was a remarkable shift within subpopulations of the myeloid lineage across all three tissues, while the stroma and lymphoid were, generally, representative, except the kidney which has the greatest population heterogeneity within the stromal cells.

3.9.5 STING regulated predicted physical adherence of myeloid cells

Previous analyses leveraged NicheNet to understand cell:cell communication networks through the expression of receptors, transcriptional regulators, and downstream targets. Here, we have successfully identified common *inflammatory* components of aging that are selectively enriched in stroma and myeloid cells in C57BL/6 and STING^{KO} tissues. A common aspect of inflammation and immune activation is directed chemotaxis of immune cells towards the site of stress/injury/infection. Here, given the generalized immune dysfunction and enrichment of immune signatures in stromal cells, I wanted to understand how this stromal shift in immune profiles was impacting potential chemotaxis within the tissue.

The algorithm RNAMagnet performs similarly to NicheNet, but prioritizes receptor:ligand interactions as a basis of predicted physical interactions towards populations manually selected as “anchors” that express the receptor of the receptor:ligand pairing. Using all identified stromal populations as “anchors” I performed RNAMagnet analysis on the 2, 24mo C57BL/6, and 24mo STING^{KO} samples individually from the heart, liver, and kidney datasets. Keeping these samples isolated for RNAMagnet analysis ensures transcriptional relationships are as biologically relevant as possible, as immune signatures have been shown to be altered with age and in the absence of STING. As these data have highlighted specific immune alterations within the myeloid, specifically macrophage, compartment I chose to direct my analyses towards the macrophage and monocyte population. (Figure 3.34)

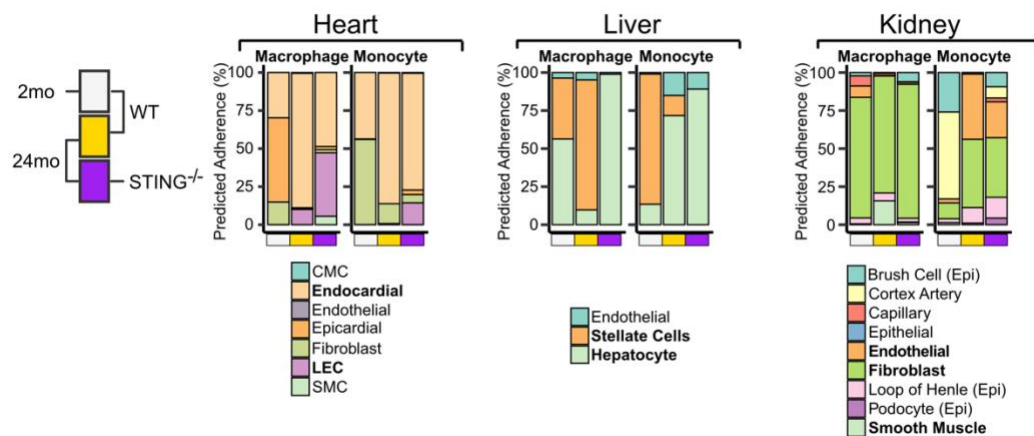


Figure 3.34 RNA Magnet predictions of cell-cell interactions

Barblots showing the predicted cell-cell interactions for macrophages and monocytes from the heart, liver, and kidney 2mo (grey) and 24mo (yellow) C57BL/6, and the 24mo STING^{KO} (purple). Relative distribution of the macrophage and monocyte “adherence” towards each stromal anchor was calculated separately for each sample. Predicted stromal anchor is annotated as shown.

When comparing 2 and 24mo C57BL/6 monocytes and macrophages there was a clear redirection of macrophages towards secondary anchors: epicardial to endocardial in the heart; hepatocytes to stellate cells in the liver, and fibroblasts to smooth muscle (vascular) cells in the kidney. Monocytes, however, increased their relative interaction with endocardial cells in the heart, but were directed towards hepatocytes and fibroblasts in the liver and kidney, respectively. Monocytes are a recirculating cell type; while certain monocytes will enter an inflamed tissue and mature into a macrophage, others will “patrol” briefly before exiting the tissue to reenter the circulatory system. What is most interesting here is that, in the aged datasets, the monocytes are uniformly being directed towards the “anchors” that the macrophages are losing their ability to interact with, suggesting that these monocytes are entering and may be attempting to replace the macrophage (physical) niche.

Further, STING deficiency has an altogether different effect on these two populations – rather than alleviating or exacerbating the effects of age. Cardiac macrophages are instead more likely to interact with lymphatic endothelial cells (LECs) while hepatic and renal macrophages restore their interaction potential with hepatocytes and fibroblasts, respectively.

3.9.6 Immune dysregulation determines macrophage responsiveness to stromal anchors

RNA-Magnet analysis was sufficient to suggest that age-associated inflammatory conditions in tissues are sufficient to support myeloid reorganization towards novel niches. While interesting, paired with comprehensive immune dysregulation in specific stroma and myeloid populations it is not able to determine if these changes are more specifically due to transcriptional shifts in the stromal anchors or the myeloid responders. Here, I selectively separated the stromal and myeloid populations by their polypositive

state and re-run the RNAMagnet algorithm. For clarity, I performed this analysis separately on polypositive anchors and polypositive responders to selectively understand how the polypositive state of each population altered its ability to interact with its cellular environment.

One of the first mechanisms of an inflammatory response is to induce the “activation” of local endothelial and other structural cells to encourage immune attachment, adherence, and migration. Using the previously described broad cell typing I subdivided each stromal and myeloid cluster into polypositive and “negative” regardless of single or double positivity. While individual immune signatures, specifically inflammatory or interferon stimulated, could play a dominant role in directing stromal interactions with myeloid cells, I specifically focused on the enrichment of all three of these pathways as it was a relatively novel state that emerged in the aged populations. Further, a primary readout of this analysis was not the selective directionality of the myeloid cells in the presence of the polypositive anchors but the relative interaction potential of the receptors and their cognate ligands. (Figure 3.35)

First, I investigated the relative contribution of stromal polypositivity to the interaction potential. Focusing on stromal polypositivity highlights the potential of these cells to promote chemotaxis or adherence and solidify the cellular makeup of their niche. Intriguingly, when I analyzed the interaction potential of stromal polypositivity there were very few receptor ligand pairings that were substantially altered between fibroblasts (the primary polypositive stromal cell) and macrophages (the primary polypositive myeloid cell), suggesting that polypositivity was not a dominant factor in the “activation” or adherence potential of myeloid cells. It is possible that aged fibroblasts were sufficiently “activated” in the aged tissues that the polypositive state was simply insufficient to increase their interaction potential.

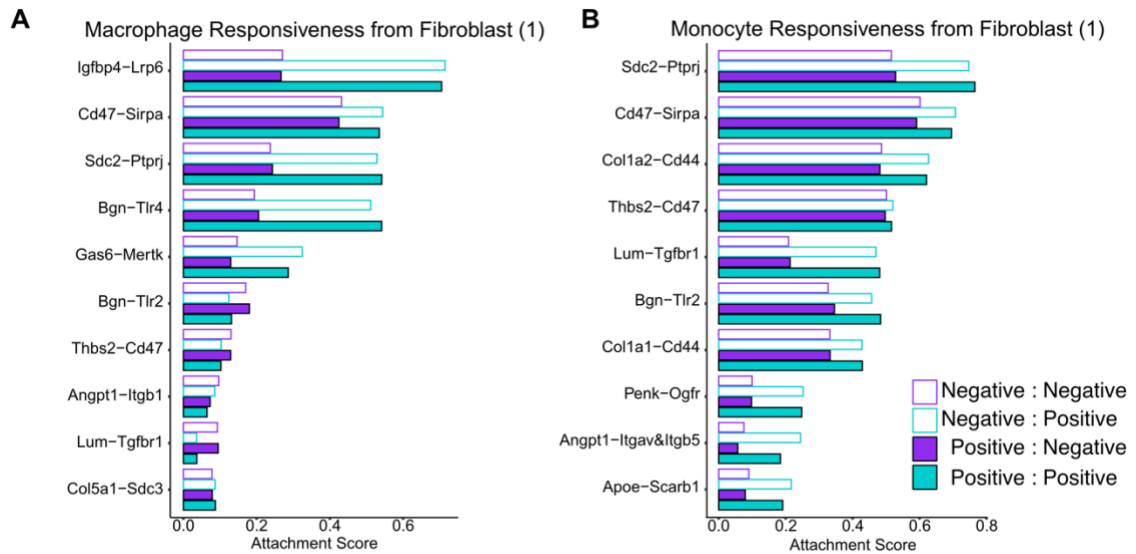


Figure 3.35 Polypositive regulation of myeloid-stromal interactions are primarily dictated by the polypositive state of the myeloid cell.

Grouped bar plot representation of the strength of receptor-ligand interactions from macrophages (A) and monocytes (B) towards fibroblasts. Ligands sourced from the myeloid population, receptors from fibroblast anchor. Open bars represent poly-negative anchors, solid bars poly-positive anchors. Cyan represents polypositive myeloid cells, purple poly-negative myeloid cells.

In order to fully understand the role of polypositivity in forming the tissue niche, it was necessary to continue this analysis for the myeloid compartment. Intriguingly, when subdividing the myeloid cells by the polypositive state there was a clear and dominant shift in the interaction potential with the fibroblasts suggesting that the polypositive state in myeloid cells, but not stromal cells, was a dominant determining factor in their adhesion potential. (Figure 3.35A). Somewhat surprising, this phenotype was conserved through non-macrophage monocytes when interacting with the same fibroblast population, suggesting that these effects are intrinsic to this polypositive dysregulated state rather than a function of the macrophage. Combined, these data are a remarkable indication of the relative effects of aging and immune dysregulation on the macrophage compartment, independent of stromal cells.

3.10 Discussion

The use of single cell RNA sequencing has exploded over the last decade, with exponential increases in the availability of data, online data management and analysis, as well as specific code pipelines for cutting edge analysis. However, as fast as the ability to use this technology has moved, the understanding and quality of controls has lagged slightly. While scRNAseq appears to be able to test any conceivable hypothesis, the power of these datasets are limited to a much greater degree than is widely appreciated. While single cell genomics can profile thousands of cells from a single organism, the necessity of true biological replicates within a single experiment is beginning to be understood. Each cell, while existing as an independent datapoint, only provides as snapshot of a dynamic population and is better understood when existing as an averaged dataset. Here, much of these analyses were performed and analyzed as a sort of “pseudo-bulk” expression set, providing a general understanding of the state of a population, rather than attempting to describe the activity of a single cell. Much of this body of work was dedicated to better understanding tissue-intrinsic regulation of age-associated chronic inflammation with a particular focus towards the relative contribution of specific cell types. Through the use of next generation sequencing technologies I was able to perform highly directed analyses to demonstrate broad immune dysregulation within aged tissues with a particular dependence of STING signaling.

Here, using single cell RNA sequencing I attempted to best describe age-associated immune dysfunction and the direct contribution of STING signaling within this pathology while maintaining an appropriate balance between high-resolution analyses and sufficient power to draw biological conclusions. Any transcriptomics analysis will run into a major caveat – the direct correlation of the enrichment of RNA transcripts to functional protein. Immune signaling itself is a highly regulated framework that, when altered, can drive significant pathology or even death of the system. As such, there are

components to regulate the transcription of DNA into RNA, the translation of RNA into protein, and the expression of protein in a functional conformation. While there are methods of more directly understanding the activity of RNA transcription, they were not available at the time of sequencing for this project. However, the relative enrichment of RNA transcripts relative to proper controls remains a viable option for understanding the state of the immune system.

3.10.1 Age-associated development of immune fingerprints

A major component of this body of work has been a clearer understanding of the temporal generation of immune dysregulation with age. While it is well accepted that the immune system, in general, is dysregulated with age it is less clear why these changes are happening and when – but most interestingly it is often overlooked the relative similarity of immune involvement in multiple tissues. Here, I attempted to understand the relative involvement of the immune system across multiple tissues with age to ascertain if individual tissues experience a specific “aging phenotype” or if there is an age-associated patterning of the immune system across multiple tissues. While other groups have attempted to identify a common aging signature, they often self-select for commonalities and overlook differences.

Tissues can be expected to have highly specific overall transcriptional profiles, as different tissues have evolved highly unique functions, but it is less clear if, or why, immune responses should be radically altered (excepting chemotactic profiles). Focusing rather on tissue uniqueness uncovers not only tissue specificity of immune profiles – inflammation, interferon, and senescence – but a unique cassette of genes altered with age, per tissue demonstrating that tissue responses to age are highly unique from an immune standpoint, and not solely a functional output. While intriguing, the importance of this fingerprinting was enhanced through the relationship identified

through serum cytokines and immune signaling. While these immune fingerprints could easily be explained through differential responses to circulating cytokines, directly testing cell responsiveness to inflammatory cytokines and chemokines, in fact, suggests that cells are equally poised to respond to inflammatory cytokines irrespective of their tissue of origin.

Tissue specificity of immune profiles, but not immune responsiveness, is one of the most important early findings from this body of work. Previously, the immune system, immune involvement, and tissue pathology have often been considered a single evolving entity – the idea that aging causes “inflammation” which induces pathology. Here, I am able to demonstrate that this age-associated inflammation is directly correlated to the tissue of interest and is not likely to be a pan-tissue occurrence. Further, rather than an outside-in model of tissue pathology these data support an intrinsic model of tissue aging that, instead, leaks into the circulation. What remains unknown, however, is what component is driving this immune involvement. While cytokine signaling is not inherently cell-specific from different tissues it does not preclude the possibility that other pathways are not being engaged that are specific to the tissue. It is entirely possible that tissues experience unique stressors that drive unique immune profiles regardless of responsiveness to inflammatory cytokines.

3.10.2 Pathway Enrichment

Often, bulk and single cell transcriptomics rely on the expression of individual genes to extrapolate towards cell functionality through curated gene profiles or pathway analysis. However, relying on the expression of individual genes can lead to missing drastic shifts in gene expression profiles because key genes of interest are missed from the dataset or do not reach the threshold of significance. Here, I employed the opposite tactic through Gene Set Variation Analysis (GSVA) to better understand the general

enrichment of the pathway within a cell. This shifts towards a more biologically relevant model of transcriptomics by profiling the significance of a pathway, rather than a set of genes. If an entire pathway is statistically enriched within a cell it is much more likely to be biologically relevant even if individual genes of interest do not cross that threshold. Using this enrichment analysis, it was quickly clear that, among each tissue, macrophages were specifically enriched for multiple immune pathways, as were select stromal populations. Noticeably absent were the lymphoid cells which may suggest simply that these cells are either recirculating cells in the absence of a significant ligand or are components of a resident memory population that exists in the absence of a ligand with age. While aging is viewed as a chronic inflammatory state, often chronic inflammation is attributed to dysregulated activation of the adaptive immune system, however these data support a model that is primarily driven by myeloid cells, specifically macrophages, and stromal cells that make up a significant portion of the tissue itself.

3.10.3 STING-dependent regulation of tissue intrinsic immunity

DNA damage, dysregulation, and mislocalization is often considered a major driver of age-associated pathology – in fact, the well regarded “hallmarks of aging” are almost entirely consisting of DNA-adjacent functions within the cell such as DNA instability, telomere attrition, and DNA damage. While errors of DNA and nuclear stability have been tested rather in-depth through disruption of DNA degradation (Trex1), nuclear stability (Progeria/progerin/Lmna1), and even STING inhibition late in life (therapeutic antagonists; H-151); the positive association of DNA sensing with aged inflammation and pathology are generally concluded through the use of overactivation of the DNA-sensing pathways – which are likely being engaged to a significantly greater extent than would be found during biological aging. Further, work that performed direct inhibition of

DNA sensing was initiated fairly late in life and does not work to directly address the establishment and progression of age-associated pathology.

Here, I utilized a progressive biological model of aging with a germline deletion of STING, allowing a full understanding of the role of STING-associated DNA sensing and signaling to the generation and progression of age-associated inflammation and pathology. Incredibly, these data demonstrate a clear negative association of STING signaling with the generation of pathology, in fact resulting in greater circulating inflammation and infiltrating cells in the absence of STING signaling with age. These data seem to be in complete opposition to the hypothesis that DNA sensing, specifically STING signaling, is a major driver of age-associated inflammation and pathology over the course of lifespan.

Intriguingly, these data suggest that STING is a critical regulator of tissue immune signatures, however suggests that the presence of STING, in the absence of otherwise known infections, rather exists as a critical negative regulator of immune activation. It remains unknown what the dominant role of STING is at baseline – whether low-level STING signaling itself is a dominant regulator of tissue homeostasis through suppression of inflammation, or if the presence of STING in a biological system rather acts as a dead end-shunt for molecular intermediates that are otherwise accessible for secondary or tertiary pathway activation. While these data are not sufficient to directly address this major unknown, what remains quite exciting is an apparent cell type-specific role of STING signaling. Here, described as poly-positive cells, I am able to identify cells that are uniquely enriched for multiple immune pathways during the aging process – however, the immune population that these cells emerge in is uniquely dependent upon STING. STING is often regarded as a general propagator of DNA sensing, however these data rather support a cell-intrinsic role for STING that is cell type or *context* dependent. This can be clearly observed through the shift of the polypositive

populations from a TRM-localized population towards monocytic cells across these three tissues, as well as the re-direction of these predicted adherence in the aged STING^{KO}.

3.11 Transcriptional profiling of tissue macrophages

3.11.1 Subsetting single cell RNA sequencing

The generation and processing of a single cell dataset is arguable the most important and impactful piece of the analysis pipeline, as it dictates the resolution and sets the background thresholding. This project utilized scRNAseq as a modality to understand and characterize broad cell:cell interactions and immune signatures in the context of aging. Partially discussed during the generation and inclusion of principle components for the UMAP, most of the variability of a tissue-wide scRNAseq dataset is comprised of the differences between unique cell *types* rather than intrinsic transcriptional states between control and experimental datasets. While this is very useful for curating the dataset and minimizing batch effects between conditions, it limits the resolution of the dataset without risking Type I errors.

With the observation of increased experimental differences specifically within the macrophage component of the tissues, it became pertinent to perform a “deeper” analysis on the macrophage compartment and attempt to selectively increase the resolution of this dataset. In brief, only cells identified as macrophages were isolated from the dataset and processed as describe above, re-normalizing the macrophage gene expression as a group. (Figure 3.36A) Further, these data were run through the principle component and UMAP generation pipeline previously described in an attempt to increase the resolution of the dataset and visualize these clusters through a UMAP for further analysis. Taking care not to over-fit the macrophage specific dataset, I was able to annotate between six and eight transcriptionally distinct macrophage clusters. (Figure 3.36B)

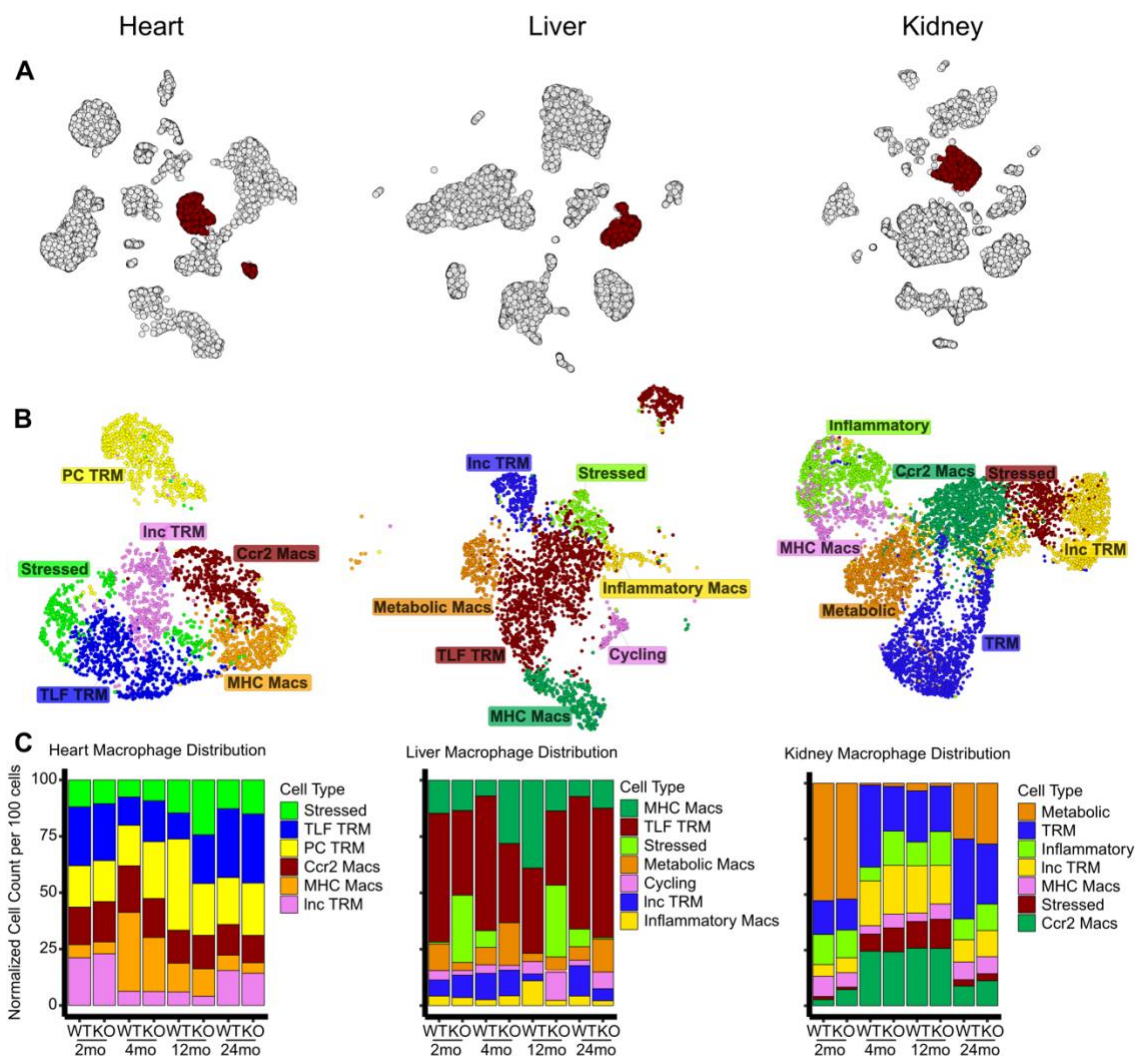


Figure 3.36 Macrophage subsets from the heart, liver, and kidney scRNAseq datasets

(A) UMAP representation of scRNAseq datasets from the heart, liver, and kidney with the macrophage population highlighted. (B) Renormalized UMAP representation of macrophages from the heart, liver, and kidney with macrophage subpopulations annotated as shown. (C) Relative distribution of macrophage subsets from the heart, liver, and kidney datasets.

3.11.2 Describing the macrophage dataset

Simply identifying more clusters does not inherently assume these clusters are transcriptionally distinct to determine them to be unique cell types. In order to first understand the transcriptional makeup of these populations, I performed cluster-identifying differential gene expression analysis and generated a heatmap of the top 25 marker genes per cluster. (Figure 3.37)

This was able to roughly identify several clusters based on the expression of key genes such as tissue residency markers (*Timd4*, *Lyve1*, *Folr2*, *Clec4f*, *Csf1r*), inflammatory marks (*Ccr2*, *Ly6c2*, *Il1b*, *Il6*), antigen presenting genes (H2-Aa), and long non-coding RNA (*lncRNA*; *Xist*, *Malat1*, *Neat1*). Interestingly, in each tissue there was a cluster of macrophages that did not retain definitive expression of any marker genes and were simply classified as “stressed” macrophages. (Figure 3.37)

While these marker genes were indicative of specific, relative populations it is difficult to adequately determine population differences with ranked expression of individual genes. As such I elected to perform pathway analysis on the macrophage clusters, using the young C57BL/6 dataset as a representative baseline for the total transcriptional profile of each UMAP-annotated cluster. As an input for the pathway analysis, I performed cluster identifying pathway analysis on each cluster focusing only on upregulated genes. These pathways were sampled specifically for GO:Biological Processes as they are more intuitively understood, and ranked by “recall” – the proportion of genes from each pathway that are represented in the input for each cluster. Using this rank order of enriched pathways for each cluster, in conjunction with the key marker genes for each cluster I was able to successfully annotate 6-7 clusters from each tissue. (Figure 3.38)

Figure 3.37 Identification of tissue macrophage subsets.

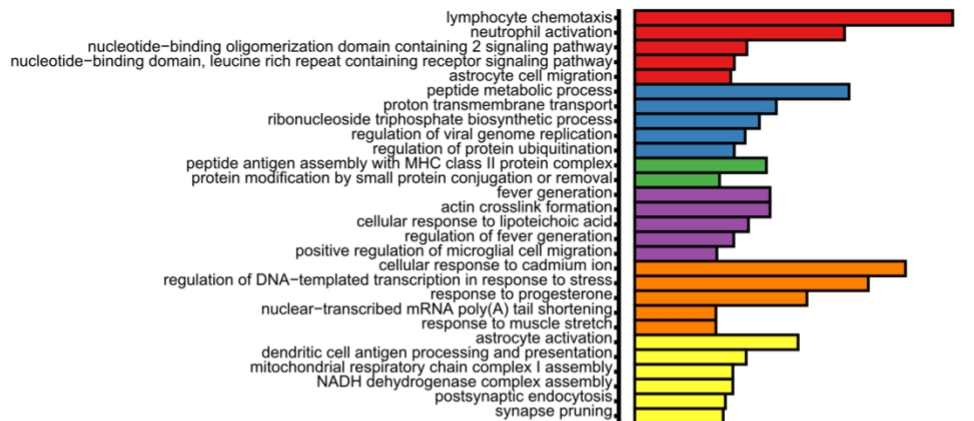
Heatmap representation of marker genes from transcriptionally distinct macrophage subsets from the heart, liver, and kidney scRNAseq datasets. Heatmaps represent top 20 (liver, kidney) or 25 genes per macrophage subset. Manually annotated macrophage typing annotated below. Significance thresholded with a Bonferroni adjusted p-value < 0.1

Here, I continued this analysis by performing the pathway analysis on the aged dataset to understand further how these clusters were changing relative to age. Using the same cluster identifying DEG and pathway analysis previously described, I was able to identify key transcriptional changes in the aged dataset suggesting a stark transcriptional change within these aged macrophage subclusters. (Figure 3.39)

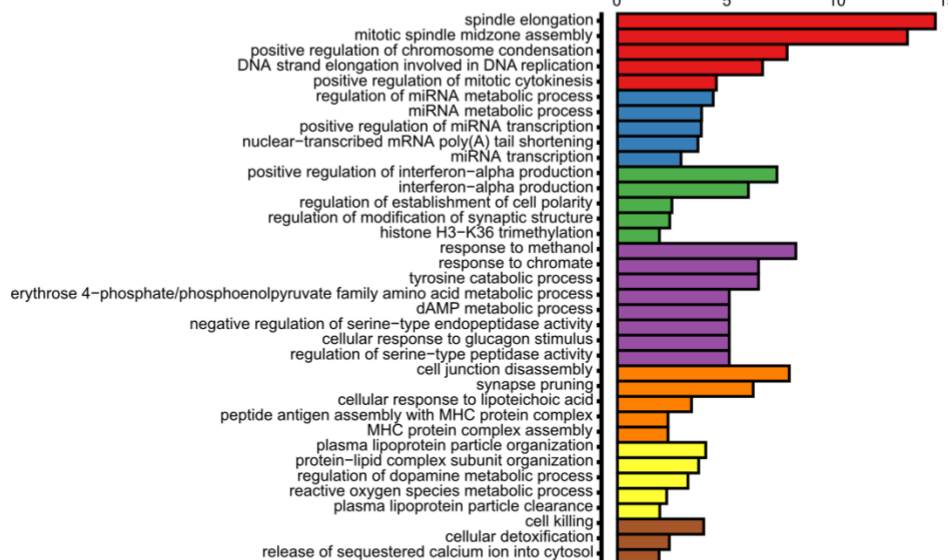
3.11.3 Expression of *Tmem173* in resident macrophages

With this macrophage specific scRNASeq dataset, it was first imperative to understand if these cells retained sufficient expression of STING (*Tmem173*) and if STING expression was altered with age. Throughout the dataset, the expression of *Tmem173* is notoriously low, and was difficult in the best populations to determine adequate expression.

Heart



Liver



Kidney

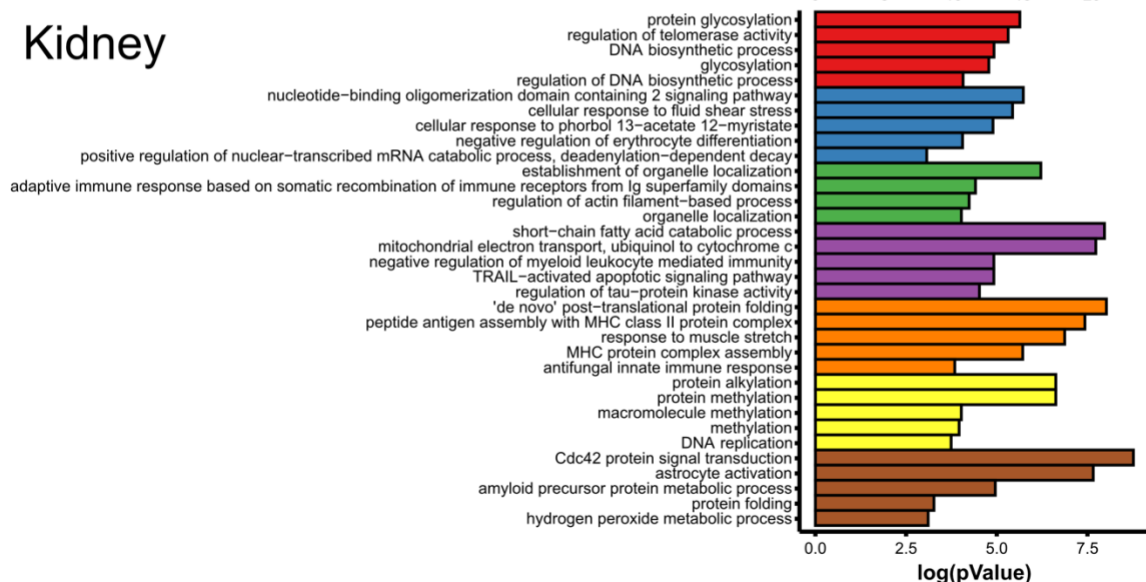


Figure 3.38 Descriptive pathway analysis of macrophage subsets

Barplot representation of GO pathways preferentially enriched in top DEGs from macrophage subsets from 2mo C57BL/6 heart, liver, and kidney. Individual GO pathways sorted by $-\log_{10}(p \text{ value})$. Marker gene significance threshold with a Bonferroni adjusted p-value < 0.1 .

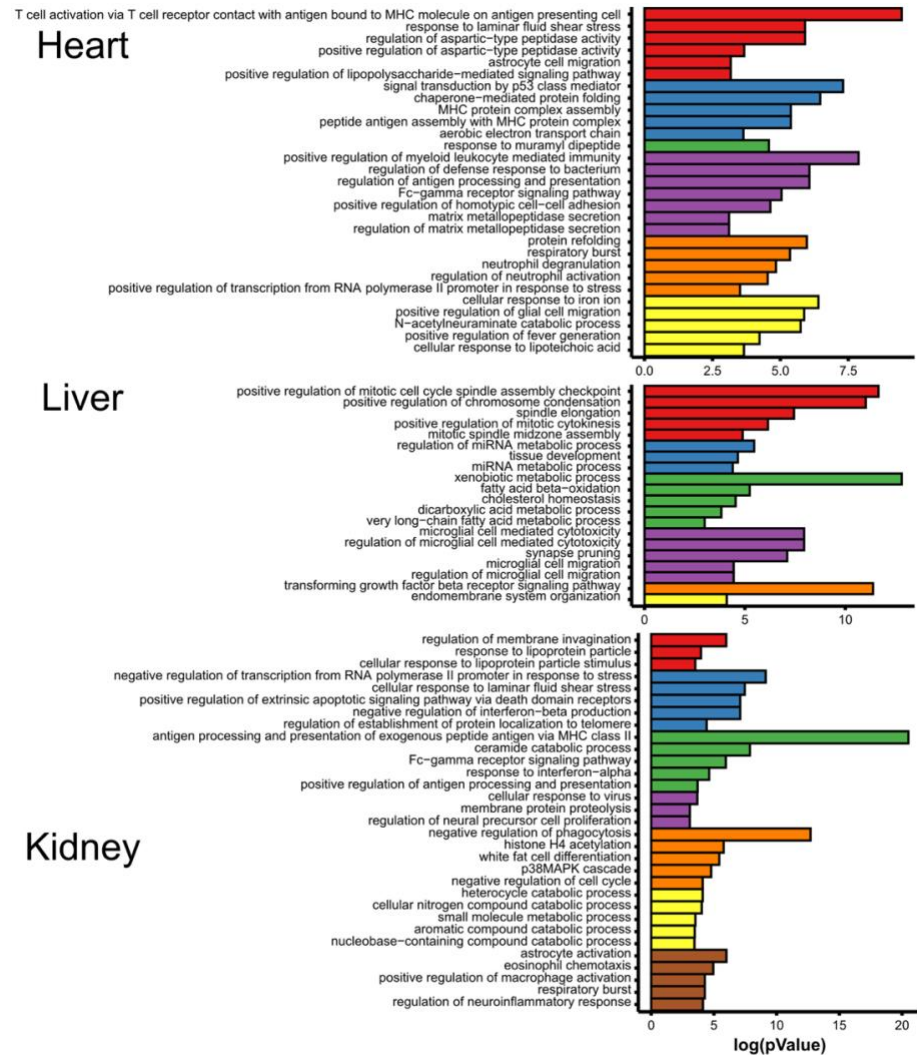


Figure 3.39 Aged macrophages are enriched in inflammatory gene pathways

Barplot representation of subset-directed differential gene expression analysis as in Figure 3.38. Marker genes were summarized by GO pathway analysis for manual annotation. Individual GO pathways sorted by $-\log_{10}(p \text{ value})$ and grouped by macrophage cluster. Marker gene significance threshold with a Bonferroni adjusted p-value < 0.1 .

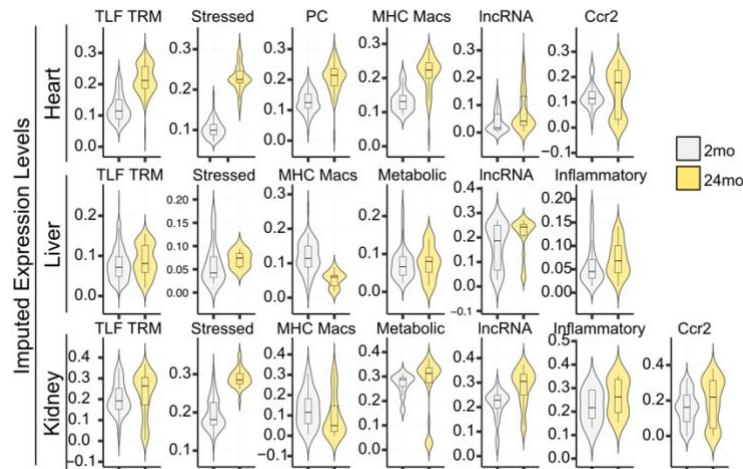


Figure 3.40 STING expression maintains elevated expression in aged macrophages

Violin plot representing MAGIC-imputed expression values of STING (*Tmem173*) in 2mo (grey) and 24mo (yellow) C57BL/6 macrophage subsets from the heart, liver, and kidney.

However, the use of the “magic” gene imputation is an effective method to rescue low-gene counts based on neighboring cell transcriptional states. Using these imputed values, I was able to visualize generally similar expression of, or slightly elevated, *Tmem173* in the aged datasets suggesting that any transcriptional differences observed is not solely due to radical changes in the capacity to signal through STING. (Figure 3.40)

3.11.4 Immune fingerprinting of resident macrophages

Preliminary investigation of the tissue-inclusive dataset identified unique fingerprints that marked aging in each tissue. Previously, TRMs have been shown to be as transcriptionally distinct from TRMs from other tissues as they are from blood monocytes, so performing fingerprinting analyses inclusive of tissue of origin was redundant. Here, I elected to perform fingerprinting analysis for each tissue-cluster

pairing in 24mo C57BL/6 and STING^{KO} TRMs. Here, I was primarily interested to know if the core immune-signature genes were significantly impacted by STING signaling. The most highly expressed inflammatory and interferon genes, while diverse among each individual cluster, were further demarcated by the ability to signal through STING. (Figure 3.41A, B) Interestingly, however, these changes were not uniform across tissues and macrophage types. For instance, the inflammatory capability of the cardiac and renal TLF-like macrophages were relatively unchanged, the TLF-like hepatic macrophages were much more inflammatory in the STING^{KO}. (Figure 3.41A) Similarly, the interferon signatures for the TLF-like macrophages in the heart and kidney were similar yet drastically altered in the liver. (Figure 3.41B)

3.11.5 Marks of senescence are not globally regulated in resident macrophages

Senescence is a core concept of age-associated dysfunction, where cells, including immune cells, lose their ability to proliferate. As senescence progresses cells can develop a secreted inflammatory phenotype (SASP; senescence associated secretory phenotype) that is believed to play a role in immune recruitment and clearance of senescent cells. However, aging is associated with the accumulation of senescent cells which was partly believed to contribute to the inflammatory state described with aging. While macrophages are a key cell type that are positioned to recognize and clear senescent cells, the state of senescence in tissue macrophages is not well understood.

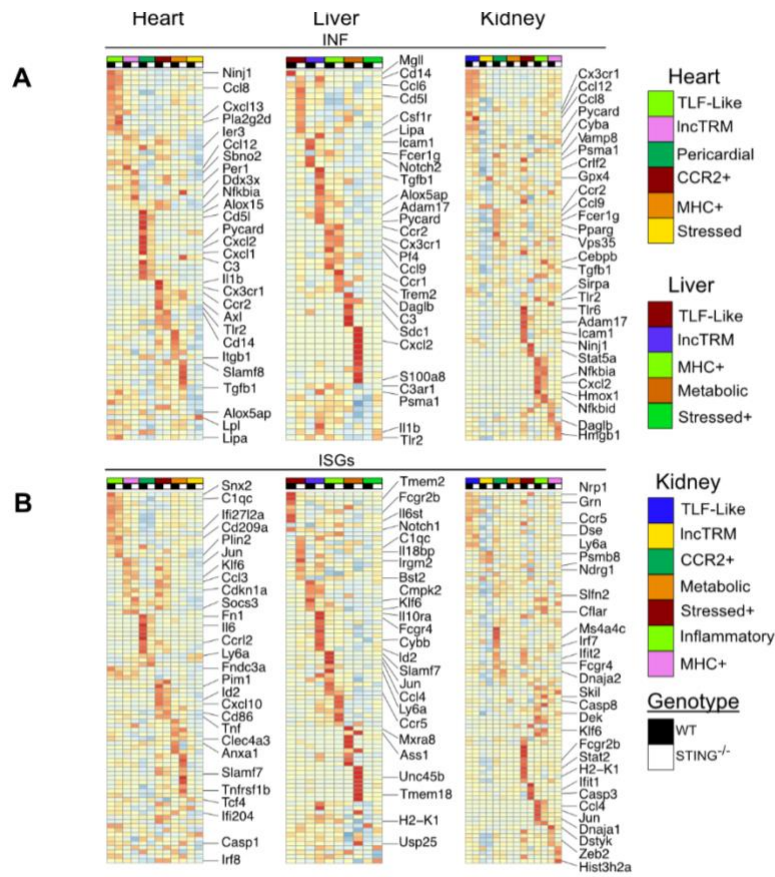


Figure 3.41 Immune fingerprints are preserved with age in tissue macrophages

Heatmap representation of top marker genes from aged (24mo) C57BL/6 (black bars) and STING^{KO} (white bars) macrophage subsets identified in the heart, liver, and kidney. Top marker genes subsetted for inflammatory associated genes (A) and interferon stimulated genes (B). Datasets scaled by row (relative gene expression among macrophage subsets). Significance threshold with a Bonferroni adjusted p-value < 0.1.

Using this aged dataset, I performed differential gene expression analysis on the gene expression imputations to understand how core senescence associated genes were altered with age. Intriguingly, it did not appear that these core, well defined senescence-associated genes were globally, or temporally, regulated with age or STING signaling. In fact, a number of senescence associated genes were upregulated in the

absence of STING signaling, suggesting, rather, an intrinsic suppressive function in the context of senescence. (Figure 3.42)

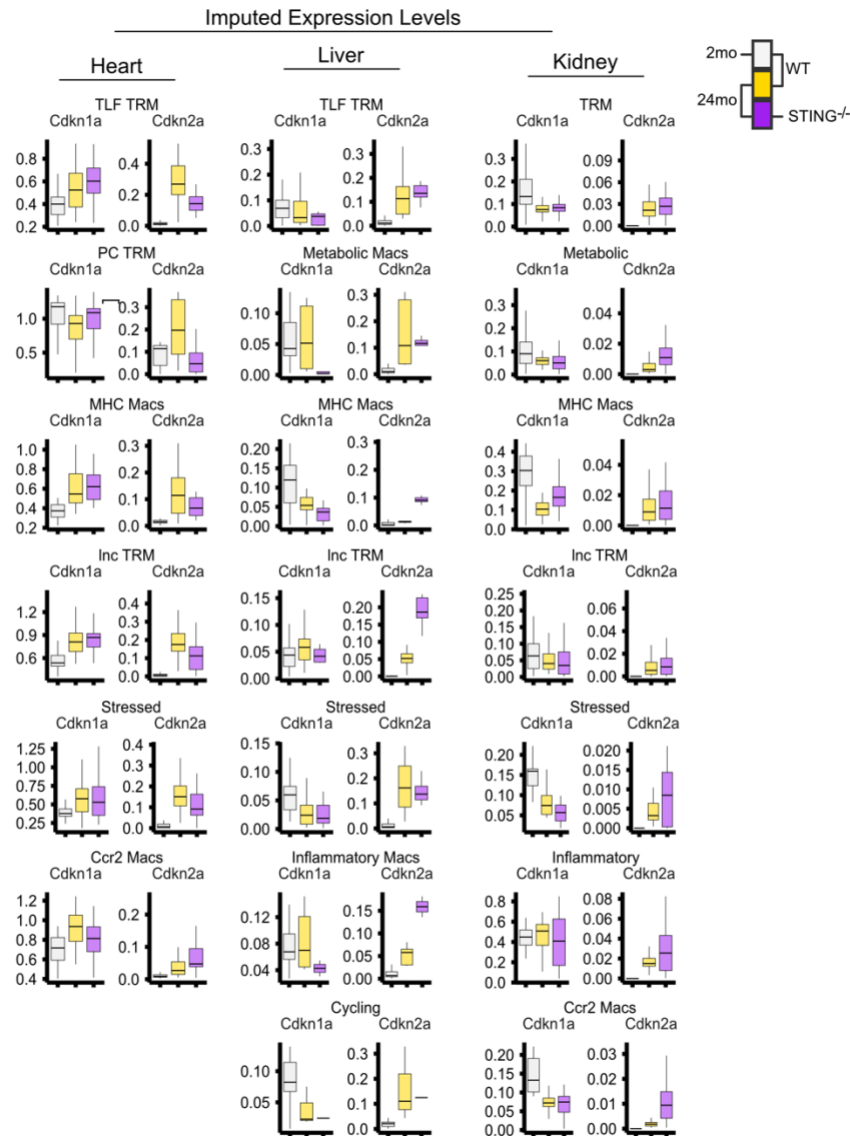


Figure 3.42 Senescence is not universally regulated by STING signaling in aged macrophages.

Box-and whisker plots of MAGIC-imputed expression of senescence associated Cdkn1a, Cdkn2a genes from specific macrophage subsets in the heart, liver, and kidney. Grey; 2mo C57BL/6, yellow; 24mo C57BL/6, purple; 24mo STING^{KO}. Macrophage clusters annotated as shown. Plots depict mean, 25th, 75th percentile, and range of the identified cells.

However, when specifically querying inflammatory and SASP-associated genes (Ccl3, Mmp9, Igfbp4, Lmna, Il1b, or Timp2), these genes were rather upregulated in the absence of STING signaling. (Figure 3.43)

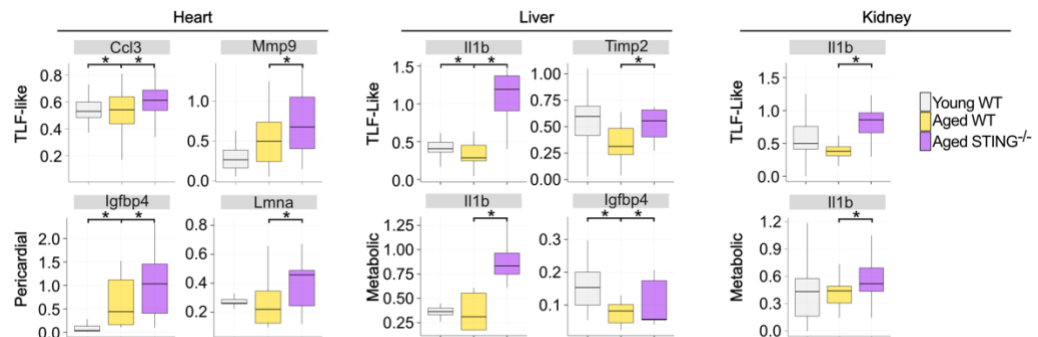


Figure 3.43 Inflammatory SASP-like cytokines are elevated in aged macrophages in the absence of STING signaling.

Box and whisker plots, as in Figure 3.42, of MAGIC-imputed expression of SASP components from 2mo (grey) and 24mo (yellow) C57BL/6 and 24mo STING^{KO} (purple) TRM-like macrophage subsets from the heart, liver, and kidney. TLF: (Timd4, Lyve1, Folr2+). Significance threshold with a Bonferroni adjusted p-value < 0.1.

STING has been suggested to be a causal driver of senescence or senescence-like phenotypes, however in biological aging there are likely a number of contributing pathways. However, given the lack of a global prevention of senescence, these data suggest that, in the absence of STING, cells that do become senescent may be much more prone to progress to an inflammatory SASP-state as if STING *maintained* senescence in tissue macrophages.

3.12 Macrophage Directed flow cytometry

Single cell RNA sequencing is a powerful tool to broadly characterize cell types and generate intricate profiles to begin to understand a biological system. A major drawback of these transcriptional based systems is the inherent disconnect between

transcriptional regulation and functional profiles. Biological systems have evolved extreme redundancy and incorporated multiple levels of regulation to protect a delicate balance, especially within the immune system where unregulated activation can lead to local inflammatory damage and a lack of response can lead to pathogen-directed damage or death of the host. Thus far, I have identified a rather significant involvement of STING signaling in the immune-pathology of aging by histological assessment of aged tissues, general identification of a loss of tissue macrophages with flow cytometry, and a rather robust immune fingerprinting of aged tissues with scRNASeq. Further, directed analysis of tissue macrophage transcriptional profiles suggest a cell-type specific involvement of STING signaling in the maintenance of TRM immune profiles during aging. To expand upon these analyses and attempt to enrich the functional understanding of these observations I elected to return to flow cytometric analyses to further characterize aged TRMs and the effect of STING signaling on the age-associated progression of the TRM inflammatory state.

Tissue resident macrophages (TRMs) are an elusive cell type and difficult to accurately characterize. Macrophages are often characterized by the presence of the F4/80 antigen, with TRMs identified as having quantifiably higher expression than mature bone marrow-derived macrophages. In this body of work, using the original TRM description of a high concentration of F4/80 paired with low expression of CD11b, I was able to identify an age-associated shift in the relative concentration of F4/80^{hi} TRMs in multiple organs. However, a major question that arose after the scRNAseq analyses was regarding previously unappreciated heterogeneity within the TRM cluster that was unable to be addressed adequately with traditional flow cytometry.

3.12.1 Identification of macrophages through spectral flow cytometry

Spectral flow cytometry increases the available protein targets two to three-fold over traditional flow cytometry by profiling the entirety of the emission spectrum. As such, an expanded fluorescent panel was necessary. I chose to include a number of common TRM-directed surface markers (Tim4, MerTK, F4/80, CX3CR1, CSF1R, CD64), inflammatory macrophage and monocytic markers (MHC-II, CCR2, Ly6C2) and select scavenger receptors (CD204, CD206, CD64, CD163) to best profile the functional and inflammatory state of TRMs among the heart, liver, and kidney. Firstly, immune cells were isolated within the analyses by flow cytometric gating on non-debris (FSC-A x SSC-A), singlets (FSC-A x FSC-H), and expression of the hematopoietic marker CD45 (FSC-A x CD45). Initial targeting of macrophages was performed by bifurcating total F4/80 expressing cells into the highest and lowest expressors. When I first started these analyses I selected all cells that retained some level of F4/80 expression to broadly capture all macrophage-lineage cells and performed subsequent gating and downstream analyses. Interestingly, many of these analyses had high variability and did not return many interpretable results.

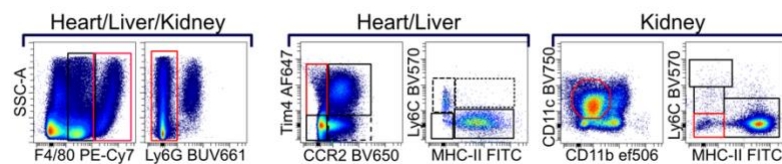


Figure 3.44 Flow cytometry gating strategy to identify tissue resident macrophages from the heart, liver, and kidney

Scatter plots with specific gating to identify and quantify F4/80hi and Tim4+ tissue resident macrophages from the heart, liver, and CD11b^{low} CD11c⁺ resident macrophages from the kidney.

However, interestingly, across all three tissues analyzed there were three distinct populations that were segregated by expression of F4/80 – F4/80 negative cells that are

non-macrophages, F4/80^{lo} cells are more akin to recirculating or monocyte-derived macrophages, and F4/80^{hi} cells which are most indicative of the long-term resident macrophages previously described and which were most likely characterized by the scRNAseq. (Figure 3.44) Interestingly, here, when the ratio of F4/80^{hi:lo} is plotted there is a very clear contraction of the F4/80^{hi} population indicative of replacement with F4/80^{lo} cells, as previously described and theorized in the literature. (Figure 3.45)

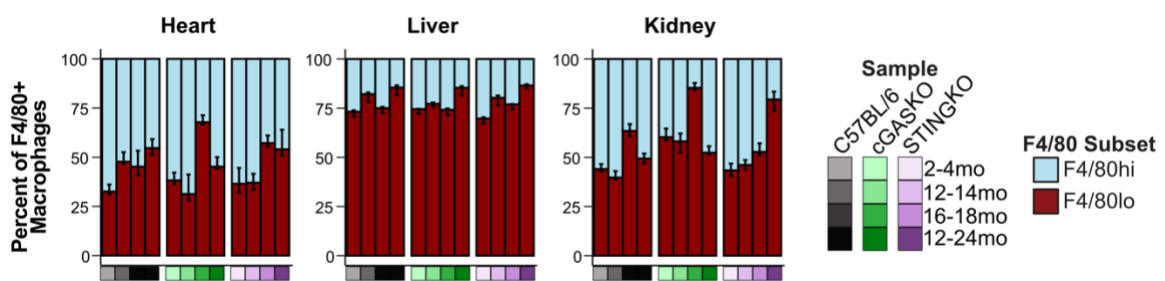


Figure 3.45 cGAS/STING signaling regulates F4/80^{hi} macrophage maintenance with age

Stacked barplot depicting the relative distribution of F4/80⁺ macrophages in the heart, liver, and kidney. F4/80⁺ cells were characterized as either F4/80^{hi} or F4/80^{lo} and normalized to the total size of the F4/80⁺ population per organ from C57BL/6 (shades of black), cGAS^{KO} (shades of green), and STING^{KO} (shades of purple) mice. Blue; F4/80^{hi}, red; F4/80^{lo} macrophages.

Here, however, these analyses have been extended through to 24mo and are able to describe a true aging trend, rather than extrapolating early-life analyses. What was remarkable here, however, was the kinetics at which this replacement occurred across tissues in the cGAS^{KO} and STING^{KO}. (Figure 3.45) In the heart the replacement of F4/80^{hi} macrophages appears to peak as early as 12-14 months in the C57BL/6 mice but as late as 18mo in the cGAS^{KO} (albeit transiently) and STING^{KO}. The liver has a slow but gradual replacement until 24mo with similar kinetics between the C57BL/6 and STING^{KO}, however the cGAS^{KO} F4/80^{hi} macrophages persist at roughly 25% of the

population through the 18month timepoint. Finally, the kidney demonstrates the most profound effects due to the cGAS/STING signaling pathway – there is a demonstrative replacement between 12 and 18mo in the C57BL/6 that contracts again by 24mo. Intriguingly, as in the heart there is a remarkable, yet transient, expansion of F4/80^{lo} macrophages around 18mo, while this expansion does not appear until the 24month STING^{KO}. While these data support previously theorized replacement of the TRM population with monocytic macrophages, these data quantify these shifts and suggest that STING plays a role in the *maintenance* of these populations with age.

3.12.2 FlowSOM to characterize and identify TRM populations

Early optimization of spectral cytometry gating strategies, given 30+ parameters, increases the potential analyses exponentially relative to traditional flow cytometry. Initially I performed subsequent gating to identify TRMs and attempted to characterize sub-populations based on different iterations of included parameters. While effective, this style of analysis retained a level of operator bias simply by including directed gating and ran the risk of both Type I and Type II analytic errors. To minimize operator bias and most efficiently characterize the immunological profile of these F4/80^{hi} macrophages I performed higher order analyses, UMAP and FlowSOM. (Figure 3.46) These analyses were partially streamlined by the analytic software Omiq (Dotmatics) and required slightly less input than described for the scRNAseq work. Here, I elected to direct these analyses towards the F4/80^{hi} population from each tissue (independently) to best characterize heterogeneity among the TRM population. These analyses are also restricted to the C57BL/6 and STING^{KO} samples for clarity, as including too many experimental samples may confound some inherent biological differences.

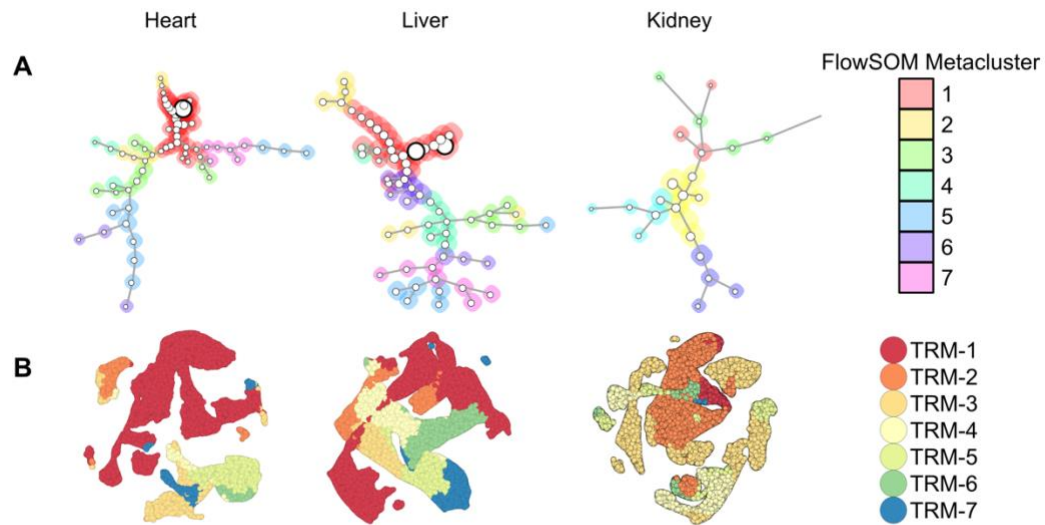


Figure 3.46 Flow-SOM identification of macrophage subtypes by spectral cytometry

(A) Flow SOM (Self Organizing Maps) projections of nodes and metaclusters based on relative fluorescence intensity of common macrophage markers. F4/80hi cells were selected and clustered based on the FlowSOM algorithm. (B) UMAP of F4/80hi cells generated from spectral flow cytometry. Color overlay is representative of the FlowSOM-identified metaclusters.

When performing the UMAP for the scRNAseq dataset, individual datasets were previously sampled during the sequencing steps and therefore had comparable numbers of cells. Initially I attempted to use all CD45+ cells as input for the UMAP and downstream analyses however quantification of UMAP clusters was ultimately driven by quality of sample collection, number of biological replicates, or the level of downsampling of each sample that was included in the UMAP. In order to normalize the input of each sample but maintain biological variability of the macrophage populations, I elected to normalize each sample to the CD45+ cells and then subset the entirety of the F4/80hi macrophage populations. This uniquely accounted for any sampling or technical variation from sample to sample as well as biological variability of the macrophage populations, specifically, across ages and genotypes.

While the UMAP was generated through the inclusion of a number of packages and functions for the scRNAseq dataset, the analysis for flow cytometry was streamlined through the Omiq software. Here, I manually optimized the number of neighbors (70), minimum distance (0.1) and left all other variables as the recommended default values. Omiq did not include a method for cluster identification on top of the UMAP, so I integrated FlowSOM analysis into the UMAP to identify phenotypically distinct clusters. FlowSOM is an additional clustering method that was specifically built for flow cytometric analysis. Here, FlowSOM is able to incorporate the fluorescent intensities of select parameters, as well as the UMAP coordinates, to generate a “self-organizing map” and assign all cells within the dataset into “metaclusters”. These metaclusters are directly associated with the UMAP and can be used to selectively identify phenotypically distinct groups of cells within the UMAP and limit the drawing of manual gates.

Considering the FlowSOM-identified meta-clusters as distinct macrophage populations within the UMAP, it is possible to quantify the relative fluorescent intensity of each target and classify each macrophage subset in a more unbiased manner. A combined UMAP and FlowSOM strategy to classify macrophage heterogeneity is a completely unbiased approach. Here, groups of cells are clustered, and populations are generated independent of the user. (Figure 3.47)

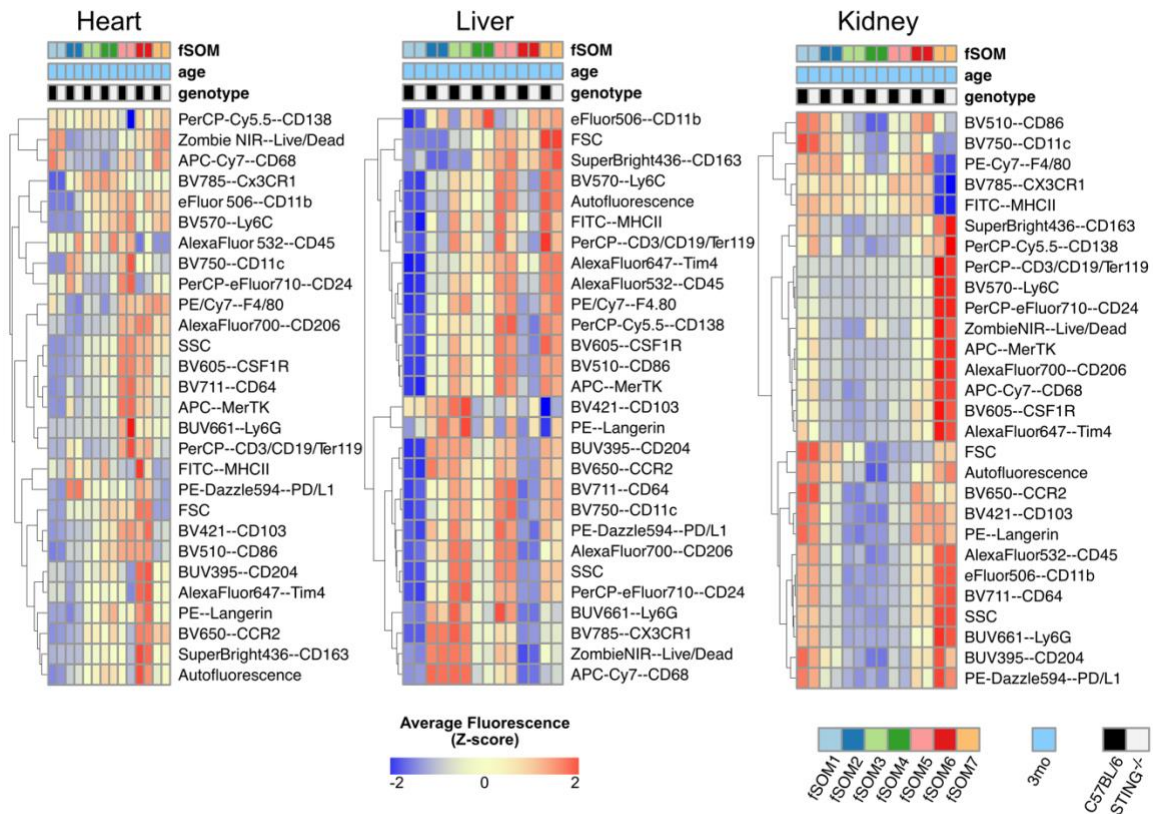


Figure 3.47 FlowSOM populations subdivide distinct macrophage subclusters

Heatmap representation of relative fluorescent intensities of common macrophage and other lineage identifying markers by spectral cytometry. Markers (rows) are clustered in an unbiased manner to identify phenotypically similar FlowSOM metaclusters from the heart, liver, and kidney.

Using hierarchical gating assigns some level of priority to each target and while a highly effective method for quantifying specific populations, it is increasingly difficult to characterize novel populations. A major portion of this thesis work has focused on identification of STING-specific regulation of immune profiles of macrophages with age. While scRNAseq was prohibitively expensive to include extensive ages and genotypes, it was feasible to more extensively test the STING signaling pathway with spectral cytometry. Here, I was able to include three additional key players of the STING signaling pathway: cGAS; the sensor of double stranded DNA, IRF3; an interferon

sensitive transcription factors, and IFNAR1; a major component of the interferon receptor to transduce cellular responses to IFN-I. Including these four components specifically allows the testing of the STING signaling pathway and provides insight into a STING-dependent mechanism of aging.

In order to streamline the analysis, I elected to focus the first round of analyses on only C57BL/6 and STING^{KO} mice from each age group before subsequent analyses including the cGAS^{KO}, IRF3^{KO}, and IFNAR^{KO} mice. Hierarchical gating strategies are relatively straightforward and can be easily applied to multiple ages, genotypes, and conditions without losing any resolution in the dataset. However, when running clustering algorithms it is important to understand the “background” of the dataset. Including extra conditions – specifically conditions that may partially recapitulate a phenotype – will drastically increase the complexity of the dataset and may cause stark changes in the observable variability.

3.12.3 FlowSOM to identify macrophage populations – C57BL/6 and STING^{KO}

Focusing solely on the C57BL/6 and STING^{KO} populations, I performed UMAP and FlowSOM analyses on the heart, liver, and kidney tissues. Here, I was able to identify up to 7 phenotypically distinct tissue macrophage populations in each tissue. Using the 3mo sample from each tissue as a relative baseline, I attempted to assign a phenotype to each population. Unfortunately, as extensive as these panels were, it was not sufficient to effectively and accurately assign unique phenotypes to each population – however each tissue was rich in a TLF-like population (heart: fSOM1, fSOM5, fSOM7; liver: fSOM5, fSOM7; kidney: fSOM2, fSOM7) that was rich in markers of residency (Tim4, MerTK, CX3CR1) or scavenger receptors (CD204, CD206, CD64, CD163). These unique FlowSOM metaclusters are unique in their ability to relatively cluster cells based on the expression of all these phenotypic marks and, in combination with

traditional hierarchical gating strategies, are an effective method to characterize STING-dependent trends in aging tissues.

First, I performed a relative quantification of each metacluster by genotype and age. Here, I found that metacluster 1 from the heart was specifically smaller in the STING^{KO} as early as 3 months of age. This was striking as the heart is an organ that has been suggested to lose the majority of it's TRM population during early life and maintain the remaining, but smaller, population for life. (Figure 3.48)

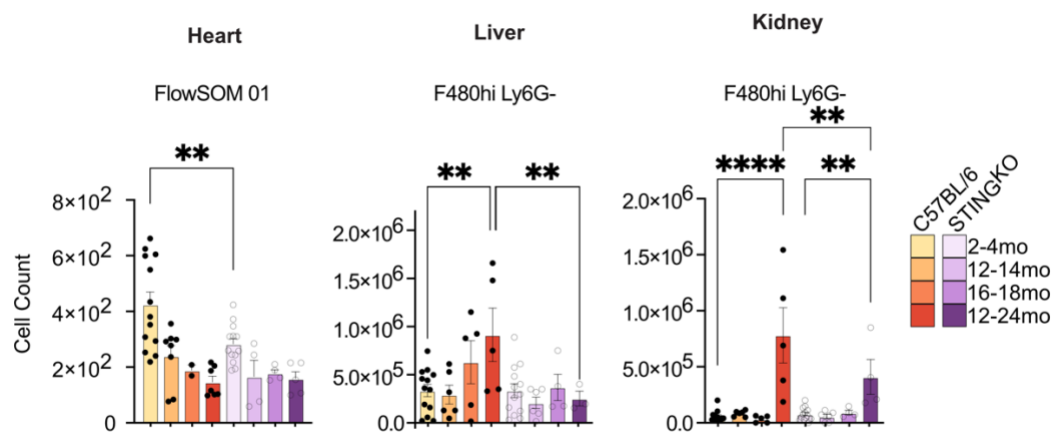


Figure 3.48 Tissue resident macrophages are decreased with age and in the absence of STING signaling.

Barplots depicting the total cell count of tissue resident macrophages from the heart (FlowSOM 01), liver, and kidney (F480hi, Ly6G⁻). Shades of red; C57BL/6, shades of purple; STING^{KO}. Samples ordered by increasing age. n = 3-13. One way ANOVA with Holm-Šidák correction.

Here, metacluster 1 is the largest of the metaclusters, suggesting it is likely the dominant homeostatic population of F4/80^{hi} macrophages. Intriguingly, when I attempted traditional gating to identify Tim4⁺ CCR2⁻ resident macrophages within the F4/80^{hi} population there were no significant differences, suggesting a relative restructuring of unidentified subpopulations.

Interestingly, the liver and kidney total F4/80^{hi} Ly6G⁻ population was significantly decreased at 24mo in the STING^{KO} tissues suggesting that there was a more drastic loss of macrophages in the STING^{KO}, with age. As such, rather than focusing directly on the FlowSOM metaclusters I was able to directly quantify STING dependent regulation of TRMs in the liver and kidney directly. (figure 3.48) Here, I was able to directly quantify a loss of resident macrophages (Tim4⁺ CCR2⁻, MHCII⁻) in the liver at 16-18months, and 22-24mo in the kidney (CD11b^{int} CD11c⁺ MHCII⁻ Ly6C⁻; as Tim4 is not reliably expressed in kidney macrophages). Interestingly, these data suggest a relative increase in F4/80^{hi} macrophage population size in C57BL/6 liver and kidney by 24mo, while the proportion of F4/80^{hi}:F4/80^{lo} macrophages decreases. These data suggest that STING is involved in the maintenance, or even expansion, of TRMs within aged tissues.

3.12.4 Tissue macrophages: traditional gating

Expanding these data to include the cGAS^{KO} samples I was able to investigate a general incorporation of the cGAS/STING signaling pathway in the restructuring of TRMs with age. While STING is capable of binding self- and bacterial-CDNs (cyclic dinucleotides) cGAS is believed to be the only endogenous producer of CDNs. This does not preclude the possibility of bacterial CDNs acting distally, cGAS/STING is yet believed to be a linear and restricted signaling pathway.

Intriguingly, there was a tissue-specific involvement of cGAS in the age-dependent expansion of total macrophages (F4/80^{hi} Ly6G⁻). While C57BL/6 cardiac macrophages did not expand, cGAS^{KO} and STING^{KO} macrophages remained fairly consistent through the aging timepoints, however in both the liver and kidney there was a remarkable expansion in the total F4/80^{hi} Ly6G⁻ macrophage population that was absent in mice deficient in either cGAS or STING. (Figure 3.49) While the hepatic F4/80^{hi} Ly6G⁻ population from the liver did not reach statistical significance these data were

comparable to the aged STING^{KO} and strongly trending ($p < 0.07$). These data were more notable when quantifying the size of specific subpopulations of TRMs – namely Tim4⁺ (CD11b^{int} CD11b⁺ in the kidney), and specific Ly6C-expressing macrophages that are generally considered reactive or inflammatory.

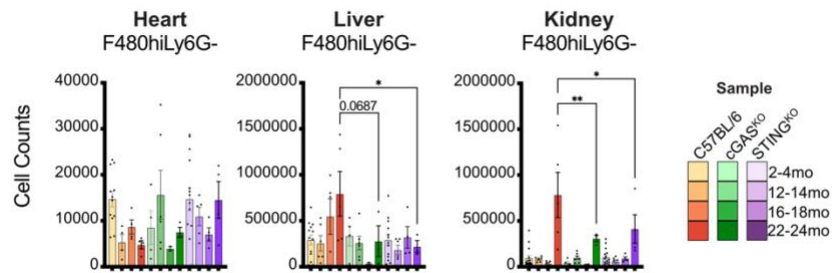


Figure 3.49 cGAS/STING signaling mediate F4/80^{hi} liver and kidney macrophage maintenance with age

Flow cytometric quantification of F4/80^{hi} Ly6G⁻ macrophages from young (2-4mo), middle aged (12-14mo), early aged (16-18mo), and aged (22-24mo) C57BL/6 (orange), cGAS^{KO} (green) and STING^{KO} (purple) heart, liver, and kidney. $n = 2-13$. One way ANOVA with Šidák correction.

In the heart, cGAS appears to play a definitive role in the age-associated loss of TRMs (CCR2⁻ Tim4⁺) during early aging, while STING restricts the expansion of a Tim4⁺ CCR2⁺ population later in life. Intriguingly, both CCR2⁺ and CCR2⁻ Tim4⁺ macrophages expand with age in the liver, which is significantly ablated in the cGAS^{KO}, and while STING^{KO} macrophages did not reach statistically significant differences from C57BL/6 controls, these macrophages maintain levels comparable to their young (2-4mo) counterparts. The contribution of cGAS and STING signaling in the kidney was most striking, which a significantly reduced expansion between 18 and 24months in both the cGAS^{KO} and STING^{KO} samples. (Figure 3.50A)

This tissue-specific heterogeneity of TRMs and the integration of cGAS/STING signaling was further exemplified with the profiling of Ly6C-expressing “inflammatory”

TRMs. In the heart there as a specific expansion of Ly6C⁺ (but MHCii⁻) Tim4⁺ CCR2⁻ TRMs that did not reach statistical significance in a similar Tim4⁺ CCR2⁺ population. The liver saw a robust ablation of an age-associated expansion of CCR2⁺ MHCii⁺ in cGAS^{KO} and STING^{KO} mice suggestive of some impaired or misaligned functionality of moTRMs with age in the absence of cGAS/STING signaling. Further, while CCR2⁻ Ly6C^{hi} TRMs from the kidney are drastically decrease in 24mo cGAS^{KO} and STING^{KO} there was a progressive expansion of a CCR2⁺ CD11b^{hi} CD11c⁺ Ly6C^{hi} population that may be indicative of a monocyte-derived macrophage population attempting to fill the CCR2⁻ void. (Figure 3.50B)

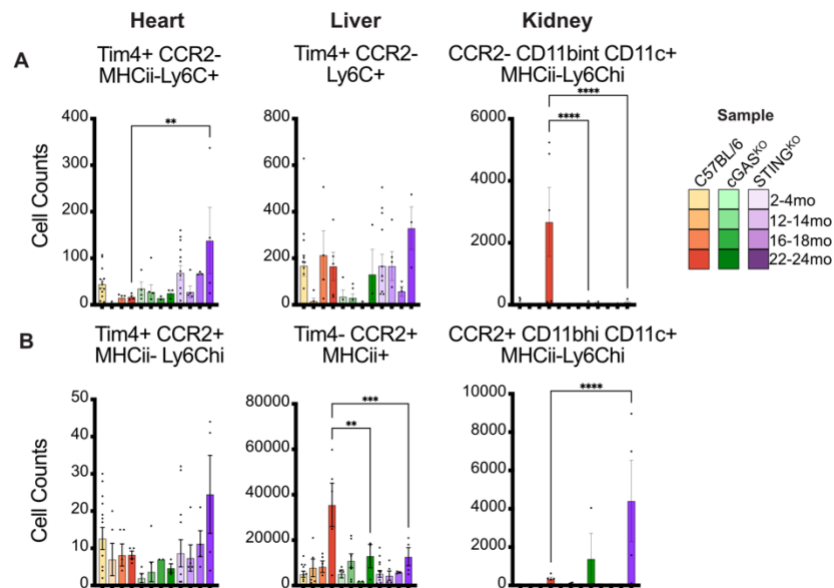


Figure 3.50 cGAS/STING signaling regulates the inflammatory composition of F4/80^{hi} TRMs

Flow cytometric quantification of Tim4⁺ CCR2⁻ Ly6C⁺ TRMs (A) and Tim4⁺ CCR2⁺ Ly6C⁺ TRMs (B) in the heart, liver, and kidney. Samples pre-gated on CD45⁺ Ly6G⁻ F4/80^{hi} macrophages from young (2-4mo), middle aged (12-14mo), early aged (16-18mo) and aged (22-24mo) C57BL/6 (orange), cGAS^{KO} (green), or STING^{KO} (purple). n = 2-12. One way ANOVA with Šidák correction.

3.12.5 Homeostatic immunophenotype of TRMs.

These data suggest, rather than a direct gain or loss of TRMs with age and STING signaling, that there is, rather, an intricate restructuring of TRMs by STING signaling. Here, in an attempt to further characterize the phenotype of TRMs I directly queried the relative expression of key homeostasis associated genes, such as scavenger receptors and inhibitory immunoreceptors within the scRNAseq dataset. TRMs have long been associated with homeostatic functions in tissues – clearance of stressed or dying cells, turnover of extracellular matrix, and general sensing of damage or pathogens. Profiling of the transcriptional signature of key macrophage populations can aid the understanding of the (theoretical) function of these cells as well as further understand if there is any functional decline of these cells in the absence of STING signaling. Previously, I have shown a contraction within the TRM population in the absence of STING signaling and it is important to understand the relative contribution of these cells to the TRM functional profile. Here, relative size of a population can quickly be offset by the functional contribution of each cell. The absence of STING signaling results in a relative contraction of the TRM population however if the only cells that remain have higher potential function, they could fully recapitulate the necessary functions of the TRM.

First, I compared the relative homeostatic potential of key TRMs from the heart (TLF-like, pericardiac macrophages), liver (TLF-like, metabolic), and kidney (TLF-like, metabolic). Using both middle-aged and aged datasets I calculated the average expression of curated homeostatic-associated genes from the young C57BL/6 and middle- or aged C57BL/6 and STING^{KO} TRMs from each dataset. (Figure 3.51)

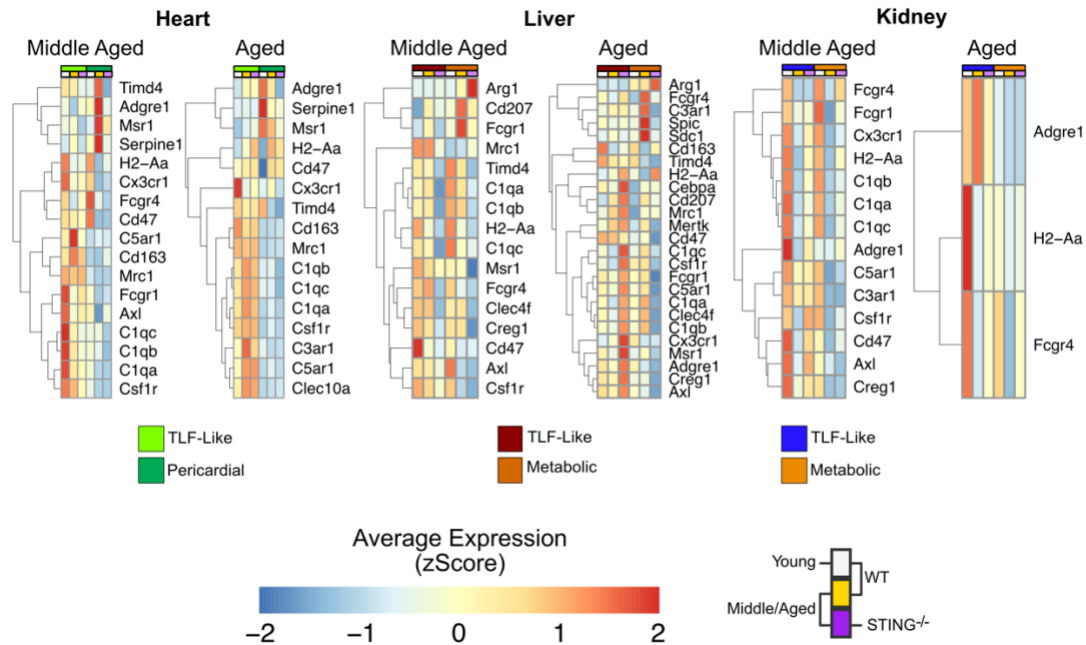


Figure 3.51 Homeostatic genes are preferentially enriched in TLF-like TRMs

scRNAseq profiling of a curated list of homeostatic genes consisting of phagocytic or scavenger receptors, complement receptors, and other key macrophage markers. Macrophage subtype, genotype indicated above the heatmap. Middle aged; 12mo dataset, aged; 24mo dataset. Grey; 2mo C57BL/6, yellow 12/24mo C57BL/6, purple; 12/24mo STING^{KO}. Average gene expression normalized across rows. Blue; downregulated, red; upregulated.

The TLF-like macrophages from each tissue tended to have a greater expression of this homeostatic signature across all age-groups, supporting the original cell-typing of these as the most canonical TRM population. Interestingly, In the middle-aged datasets for all three TLF-like macrophage populations there was an apparent loss of this gene signature in the C57BL/6 and STING^{KO} macrophages, while there was a potential increase in the aged dataset relative to the young control. This suggests a gradual loss of function within the population with age, whereas later in life the per-cell average expression of this signature starts to increase. This could be a per-cell increase in gene expression or a relative concentration of this signature in cells with a higher function. It is quite possible that there is an intrinsic or autocrine signaling mechanism within these

long-lived cells that, with retained function, supports their survival. The primary understood purpose of the TRM is to surveil the tissue niche and respond to changes, and any loss of this ability would be the quickest mechanism to begin clearance of a defective TRM.

A major difficulty of single cell -omics is understanding when to assay the dataset at a single cell resolution and when to generate population averages. Generally, scRNAseq datasets are assayed at the single cell level to generate specific cell-typing before the remainder of (basic) calculations are performed on the population as quasi-bulk sequencing. In an attempt to understand this dataset with the greatest resolution possible, I shifted towards the single-cell capability of this dataset.

Paired scRNAseq and spectral cytometry provides an exceptional opportunity to profile both the transcriptional profile as well as a protein-based immunophenotype which has much greater potential to ascribe functional potential to a TRM subset. (Figure 3.52) Interestingly, cardiac TLF-like TRMs had lower-per cell expression of a number of homeostatic genes, such as *Adgre1* (F4/80), *C3ar1* (complement receptor 3a), *Cd47* (CD47), *Csf1r* (CSF1-receptor), and *C1qa* (complement C1q subunit a) in the STING^{KO} suggesting that, combined with a contracting population these cardiac TRMs likely had less functional ability per cell. When I profiled similar protein markers from the spectral cytometry dataset (on FlowSOM metacluster 1) I saw a relative loss of the homeostatic marks CD163, CD86, CSF1R, CD206, and MerTK in the aged STING^{KO} macrophages supporting a role for STING signaling in the maintenance of TRM functional ability with age. (Figure 3.53)

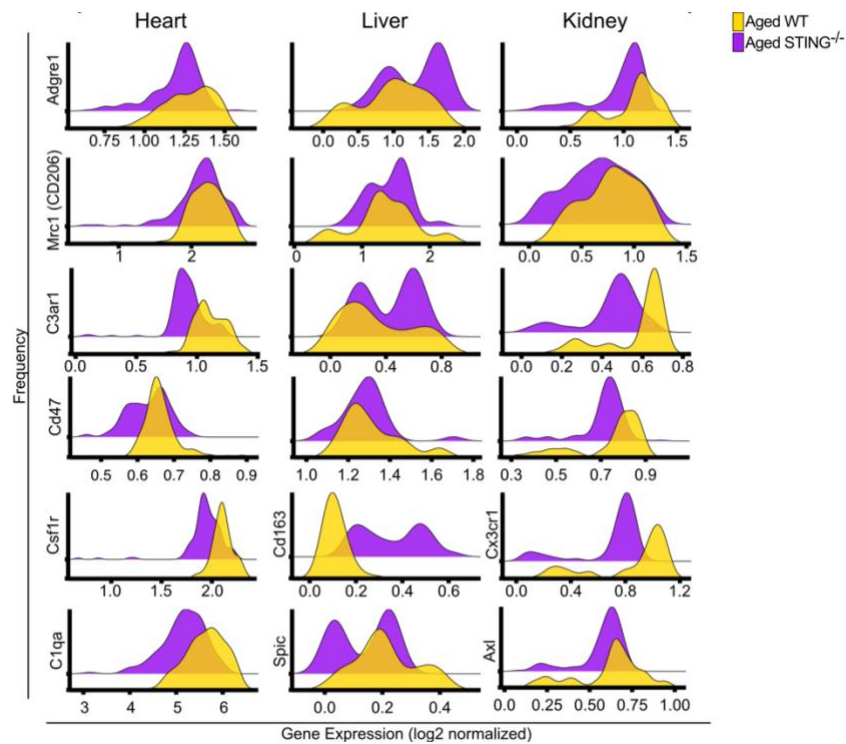


Figure 3.52 Homeostatic gene expression is decreased in the absence of STING signaling

Histogram representation of the frequency of expression of core homeostatic markers in 24mo TLF+ TRMs from the heart, liver, and kidney. Yellow; C57BL/6, purple; *STING*^{KO}. Genes annotated as shown, genes manually curated based on association with TRM identity and function as a scavenger receptor.

The liver, however, had a much different profile. These cells were more strikingly lost with age in the absence of STING signaling but retained a greater variation in the homeostatic signature, potentially suggesting an intrinsic dysregulation of transcriptional homeostasis in hepatic TLF-like macrophages. Cardiac TLF macrophages had consistent unimodal expression of homeostatic genes, while hepatic macrophages developed a consistent bi-modal profile of *Adgre1*, *Mrc1*, *C3ar1*, *Cd47*, *Cd163*, and *Spic* (a transcription factor critical in TRM ontogeny) in the absence of STING signaling. (Figure 3.52) Interestingly, these cells did not consistently lose expression of these genes, in the exception of *Spic* and potentially *C3ar1* supporting a critical role of these genes in the TRM population. Further, the protein expression of these homeostatic

marks, assayed by spectral cytometry, was fairly similar among F4/80hi hepatic macrophages suggesting these macrophages are more affected by population loss than functional dysregulation. (Figure 3.53)

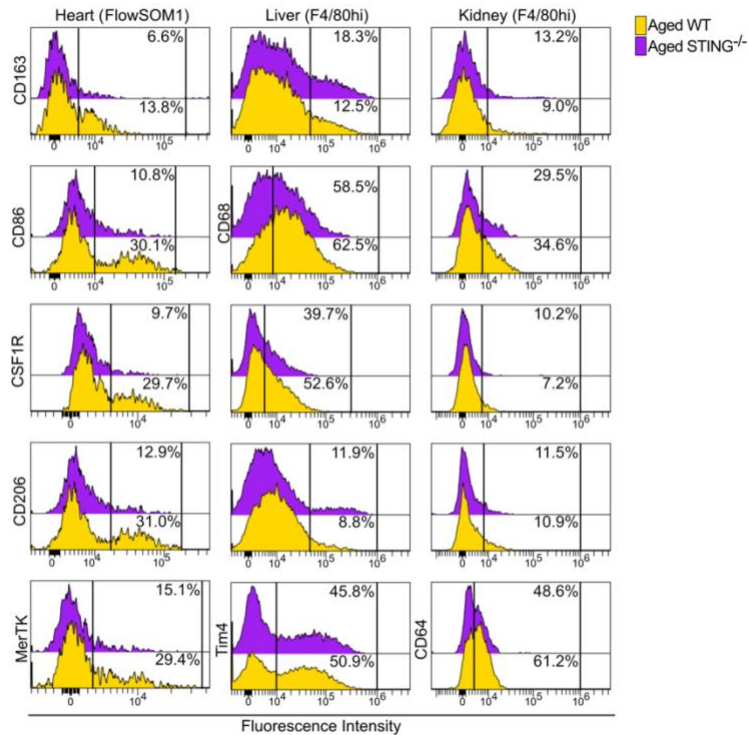


Figure 3.53 Protein expression of homeostatic receptors are reduced in the absence of STING signaling

Histograms depicting the expression of homeostatic-associated proteins from 24mo C57BL/6 (yellow) and STING^{KO} (purple) in the heart (FlowSOM1), liver, and kidney liver (F4/80hi). Protein expression was quantified as relative fluorescence intensity through spectral cytometry. All samples were concatenated to generate the representative datasets shown.

Kidney macrophages have previously been shown, supported in this dataset, to have low to no expression of the key TRM marker TIM4 (Timd4). As such, the expression of this curated homeostatic signature was intriguing, as it had potential to be quite different than the heart and liver. Here, renal macrophages experienced a reduction in homeostatic associated genes (Adgre1, Mrc1, C3ar1, Cd47, Cx3cr1, and

Axl) similar to cardiac macrophages; however, the expression of similar protein antigens (CD163, CD68, CSF1R, CD206, and CD64) were very lowly expressed in aged mice to a similar extent in both C57BL/6 and STING^{KO} F4/80hi macrophages. (Figure 3.52, 3.53)

3.13 Ex vivo analyses of TRM viability and vitality

3.13.1 Assessment of TRM intrinsic cell viability

After performing the GSVA pathway enrichment and network analyses there were several pathways that helped shape the subsequent understanding of the scRNAseq dataset. While much of the data analysis focused on the enrichment of broadly characterized immunity, there was an unmistakable enrichment of pathways regulating cell stress and cell death. After further analysis of the spectral cytometry data, and the specific contraction of TRM populations in the absence of STING signaling I revisited the enrichment of gene ontology pathways within the TRM dataset.

To best understand the effects of STING signaling within individual TRM populations as a function of aging, the change in GSVA activity score (as a function of aging) was calculated for each genotype and the magnitude of change is compared between each genotype. The relative rate of change with age is a very precise method to understand the causal role of STING signaling on different TRM populations with age. The relative magnitude of change was used to account for any baseline differences in the young C57BL/6 and young STING^{KO} TRM. This linear modeling can only compare two samples, as such it is difficult to impossible to know if something increases with age in the C57BL/6 and in the STING^{KO} if, ultimately, with aged sample reaches a “greater” activity score. As such, terms were identified as having positive or negative direction in the C57BL/6 with a positive (but smaller) direction in the STING^{KO}, no change in the STING^{KO}, or a negative direction in the STING^{KO}.

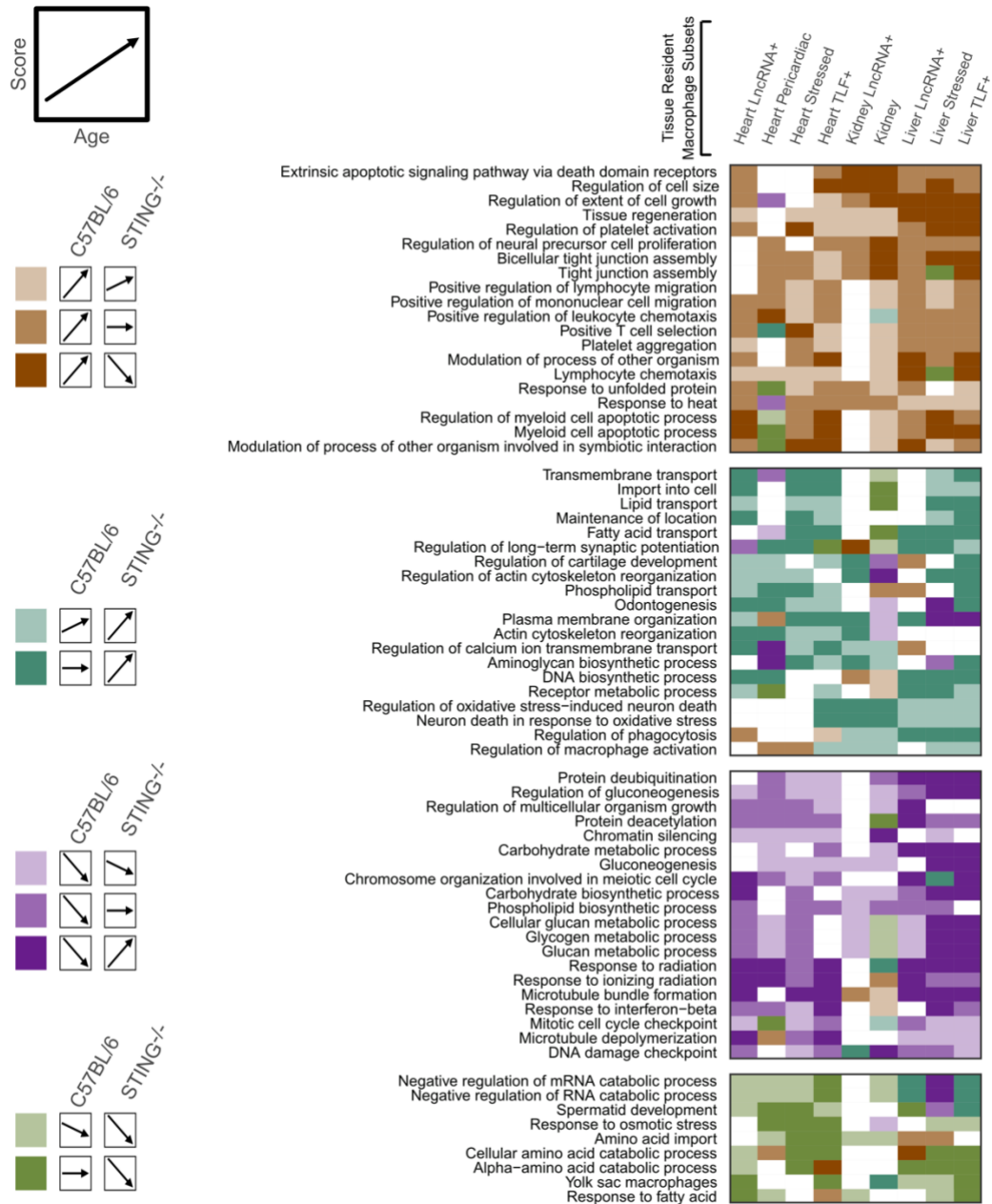


Figure 3.54 Relative rate of change of GSVA activity scores in TRM subsets

Heatmap with segmented color scales to represent the relative change in GSVA activity scores with age for TRM subsets from each tissue based on STING signaling. Pathway direction of change depicted in legend for C57BL/6, STING^{KO}: shades of brown; positive rate of change in C57BL/6 greater than the STING^{KO} (regardless of rate of change in STING^{KO}), shades of teal; positive change in STING^{KO} greater than C57BL/6, shades of purple; negative change in C57BL/6 less than STING^{KO}, shades of green; negative change in STING^{KO} less than C57BL/6. Gene ontology pathways depicted as shown. Significance threshold with an FDR adjusted p-value < 0.05.

Of the pathways preferentially enriched in the C57BL/6 TRMs there was a notable increase in apoptotic signaling gene sets with age coupled with a greater increase in biosynthetic, inflammatory responses and membrane reorganization in the STING^{KO} TRMs. (Figure 3.54) While it is possible that any enrichment of cell stress and death pathways are a direct result of tissue homogenization protocols – the C57BL/6 and STING^{KO} tissue samples were exposed to identical protocols, yet it is still a pertinent readout to the general health of the cells. Healthy, viable cells will succumb slower to extrinsic stressors than poor quality, less viable cells. While these scRNAseq datasets were not designed to statistically quantify population sized between ages or genotypes, there were intriguing shifts within the TRM populations indicative of transcriptional reorganization or loss of TRMs with age and in the absence of STING signaling.

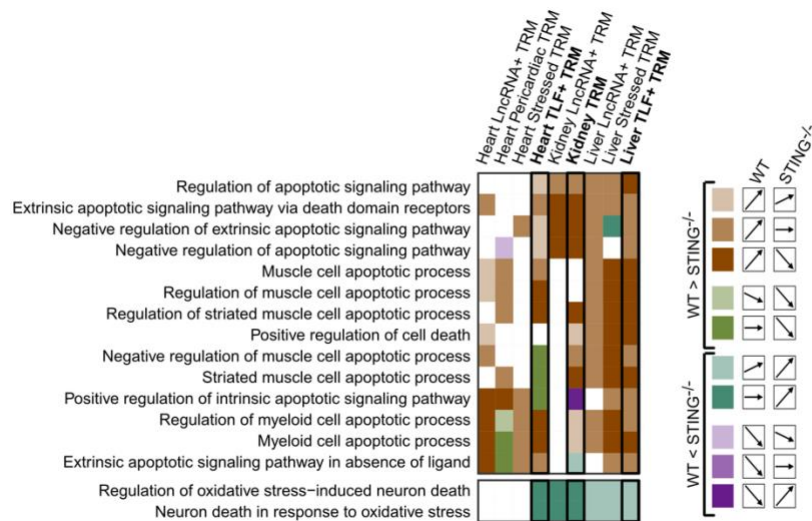


Figure 3.55 STING signaling regulates apoptotic signaling in aged TRMs

Heatmap showing the relative rate of change of death-associated signaling pathways in aged TRMs as a result of STING signaling. Magnitude of change as a component of aging was calculated for each genotype, and the relative change of this magnitude was compared to understand the role of STING signaling in the age-associated engagement of these pathways. Significance threshold with an FDR adjusted p-value < 0.05.

Here, profiling a selected list of cell death-associated gene signatures we noted a distinct enrichment of apoptotic cell death pathways in the TLF-like C57BL/6 macrophages, while oxidative stress-induced death was enriched in STING^{KO} TLF-like macrophages suggesting that STING, while a critical regulator of immune responses downstream of DNA sensing and often implicated in senescence and cell death, was in fact protecting TLF-like macrophages from oxidative stress-associated death and biasing these cells towards an immunologically silent apoptotic cell death. (Figure 3.55)

Cell death is emerging as a critical regulator of immune responses and not simply the removal of a cell. Cell death ranges from the “silent” apoptosis which relies on compartmentalization and segregation of the cell to be cleared towards highly inflammatory necro- or pyro-ptosis. TRMs are a critical component of the tissue immune landscape and act as sentinels of stress and damage. While long-lived, individual cells have to turnover in the life of a tissue. While this biological aging is theoretically absent of such direct damage, the mode of turnover of the TRM is likely a critical mediator of tissue immunity. What is unknown, however, is if TRMs are prone to immunologically silent, or inflammatory, cell death and how this mode of death regulates the maintenance of the homeostatic TRM niche.

Here, using previously collected tissues throughout the lifespan of this body of work it was necessary to understand if there was not only quantifiable cell death in the TRM pool, but if it was further regulated by STING signaling. TUNEL staining specifically detects breaks in DNA which is linked to fragmentation of DNA during cell death. While it is incredibly difficult to detect dying cells in a “snapshot” in the absence of an external stimuli, there was a quantifiable increase in TUNEL+ Kupffer cells with age (3mo, 12mo, 18mo) in C57BL/6 mice, as well as a relative increase in STING^{KO} livers at 12mo which equilibrated with C57BL/6 by 18mo. (Figure 3.56A, B)

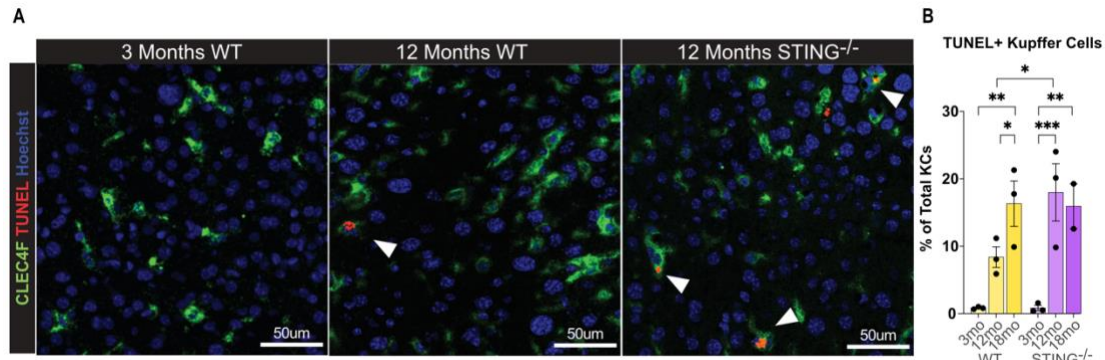


Figure 3.56 STING signaling prevents the accumulation of double strand DNA breaks in aging Kupffer cells.

(A) TUNEL staining of liver from 3mo C57BL/6 (WT), 12mo C57BL/6, and 12mo STING^{KO} highlighting the emergence of cells indicating DNA damage. Blue; nuclear staining (Hoescht), green; CLEC4F (Kupffer cells), red; TUNEL staining (DNA breakage). (B) quantification of the percentage of Kupffer cells co-staining with TUNEL to indicate Kupffer cells that acquire direct DNA damage in 3mo, 12mo, and 18mo livers. 3 fields of view per tissue, n = 3. Two Way ANOVA * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

3.13.2 In vitro induction of intrinsic TRM cell death

These two previous pieces of data, enrichment of apoptotic cell death pathways aged C57BL/6 TLF-like TRMs and increased TUNEL+ Kupffer cells in 12mo livers, at first glance appear to be at odds with each other. While TUNEL+ cells are often ascribed to be undergoing apoptosis, without specific identification of cell death pathways it is not possible to confirm that these cells are apoptotic. Further, cell and tissue processing and subsequent curation of the scRNAseq dataset likely would remove any cells that were in late stages of cell death. Transcriptional enrichment of cell death pathways are likely cells that are beginning to engage death pathways or, interestingly, are primed towards these pathways.

To better understand the engagement of cell death pathways in TRMs, it was necessary to specifically initiate cell death pathways within TRM populations and test the relative bias for TRMs to undergo specific modes of death. In order to perform these analyses most directly on TRMs and bypass tissue homogenization that might overtly

stress heart, liver, or kidney TRMs, peritoneal macrophages were selected. Peritoneal macrophages are often used for in vitro analyses after elicitation by thioglycolate – an inflammatory compound that drives substantial inflammatory monocyte and macrophage infiltration into the peritoneal cavity. However, at baseline peritoneal macrophages are dominated by resident macrophages that are embryonically derived, similar to solid organs. In a series of experiments performed by another student in support of this body of work, peritoneal macrophages were isolated and stimulated for 16h with Etoposide (DNA stress/damage associated death), Nigericin (necroptosis) and H₂O₂+3MA (oxidative stress associated death). While tracking the relative incorporation of propidium iodide through live imaging, it was shown that aged STING^{KO} TRMs were uniquely more susceptible to each mode of cell death (data not included). Most interesting, however, when these assays were performed on bone marrow-derived macrophages there was a complete phenocopy of aged C57BL/6 and STING^{KO} BMDMs. These data suggest that the role of STING regulating the specificity of cell death is intrinsically linked to the TRM lineage, and may not be a conserved immune process.

3.13.3 TRM distribution and disorganization

These data directly show a loss of TRMs with age in the STING^{KO} with a propensity and sensitivity to multiple forms of cell death. Further, through the scRNAseq dataset I was able to show that there was a potential reorganization of the TRM niche through predicted receptor-ligand interactions. After describing this in vitro susceptibility to cell death stimuli in the absence of STING it was intriguing to understand if this niche-redistribution was recapitulated in vitro. Using previously collected C57BL/6 and STING^{KO} heart, liver, and kidney the relative distribution of TRMs (heart: CD68, CD206; liver: IBA1, CLEC4F; kidney: F4/80, CD169) in relation to endothelial cells (all: CD31) and lymphatic vessels (heart: LYVE1) was quantified. (Figure 3.57)

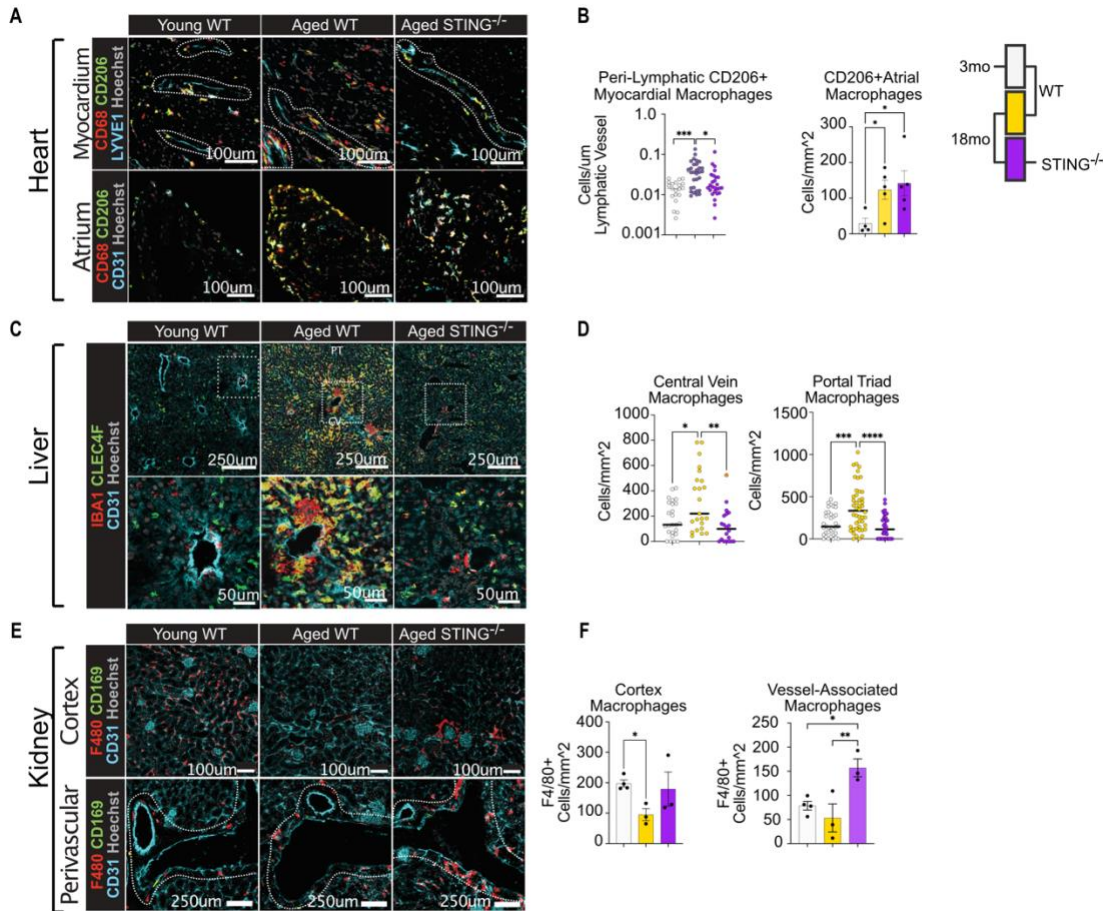


Figure 3.57 STING signaling is a critical regulator of TRM distribution in aged tissues.

(A, C, E) Immunofluorescent imaging of heart, liver, and kidney from young (2-3mo) and aged (24mo) C57BL/6 and STING^{KO} mice. (A) Myocardium and atrium. Red; CD68, green; CD206, cyan; LYVE1 (myocardium), CD31 (atrium), grey; Hoescht. Peri-lymphatic space defined as any region within 25µm of LYVE1+ staining. (B) Quantification of CD206+ myocardial macrophages within the peri-lymphatic space, normalized per 1µm lymphatic vessel, quantification of CD206+ macrophages within the atrium. (C) Liver sections with focus around the central vein (CV), portal triad (PT). Red; IBA1, green; CLEC4F, Cyan; CD31, grey; Hoescht. Macrophages associated with the CV, PT are any CD206+ cells within 50µm of a CD31+ CV, PT. (D) Quantification of total CV- or PT-associated macrophages. Normalized per unit area (mm²). (E) Renal cortex and perivascular area. Red; F4/80, green; CD169, cyan; CD31, grey; Hoescht. Vessel associated macrophages are any F4/80+ cell within 50µm of a CD31-staining region. (F) Total F4/80+ cells in the cortex, or F4/80+ cells surrounding the large vessel. At least two fields of view per tissue, n = 3-5. Statistics calculated with One Way ANOVA *p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001

In the heart, it has been shown that there is a concentration of TRMs in the atria with significant involvement in electrical conduction through the atrio-ventricular (AV) node. Here, my data supports this concentration of macrophages within the atria, although specific staining of the A/V node was not performed and highlights a clear expansion of atrial macrophages with age, although with no apparent contribution from STING signaling. (Figure 3.57A) Intriguingly, however, was the identification of clear tracts of LYVE1+ staining indicative of lymphatic endothelium. Further, there was consistent CD206 staining surrounding these lymphatic vessels. (Figure 3.57A) While these data are unable to determine the capacity in which these macrophages are interacting with the LYVE1+ vessels, there was a clear STING-dependent concentration of macrophages around LYVE1+ regions with age. (Figure 3.57B)

The liver is a highly vascularized and patterned organ with repeating lobules consisting of hepatocytes surrounding sinusoids that drain into the ventral vein. Further there is the “portal triad” consisting of branches of the hepatic artery, portal vein, and bile ducts. These structures are highly repetitious and easily identified with endothelial staining (CD31+ regions). With such highly regular structures the liver is an exceptional tissue to profile for macrophage distribution and reorganization. CLEC4F+ Kupffer cells in the young C57BL/6 mice are highly ordered and loosely organized around the central vein. (Figure 3.57C) With aging, however, there is a huge efflux of IBA1+ and IBA1+ CLEC4F+ macrophages towards the central vein. While few if any IBA1+ macrophages were identified in the young C57BL/6 livers, these data demonstrate a likely population of monocyte derived macrophages that have entered the liver and attempted to fill the Kupffer Cell niche. The presence of CLEC4F suggests that these macrophages are somewhat recapitulating the Kupffer cell phenotype, but not their highly ordered organization. Especially interesting, macrophages of the portal triad underwent a similar STING-dependent concentration with age suggesting that this (dys)regulation of

macrophage polarity was not directed towards nutrient or pathogen flow but rather to general endothelial cells. (Figure 3.57D)

The kidney is a vascular tissue and highly involved in the regulation of nutrients and waste products, while TRMs have been implicated in renal blood vessel morphogenesis. Here, as in the young livers, there was a clear homogeneity to the distribution of macrophages within the cortex of the kidney. While there was a clear loss of total renal macrophages with age, there was an even more striking loss of spatial homogeneity. (Figure 3.57E, F) Any macrophages that remained were often concentrated in specific regions. While this numerical loss of macrophages was not seen in STING^{KO} kidneys, there was an intense concentration of F4/80+ macrophages around structures resembling glomeruli, with tightly interacting regions of CD31+ staining. As with the heart and liver, renal macrophages appeared to be spatially interacting with LYVE1- or CD31-staining endothelial cells. (Figure 3.57E) However, although there was a significant loss of total macrophages within the renal cortex, the relative concentration of vessel-associated macrophages did not change with age and aged F4/80+ macrophages could easily be identified intercalated within the CD31+ renal endothelium. However, in the STING^{KO} kidneys there was as significant increase in vessel-associated F4/80+ cells relative to both young and aged C57BL/6 kidney in the absence of cortical macrophage loss.

3.14 Contributions

All experiments and analyses for figures 3.1-3.12 were performed by Jacob Hopkins. Samples for Figures 3.5-3.57 were collected by Jacob Hopkins, Katherine Sulka, or Ronald Brown. Integration of single cell datasets, as well as Figures 3.24, 3.54, 3.55 were performed or generated by Dr. Reed with input from Jacob Hopkins. Imaging and

analysis for Figures 3.56 and 3.57 was performed by Machlan Sawden and Kimberly Carroll on samples collected by Jacob Hopkins, Katherine Sulka, or Ronald Brown. Unmixing of Cytex data was performed by Katherine Sulka. All other single cell analyses, Cytex analyses (Figures 3.13, 3.15-3.23; 3.25-3.27; 3.29; 3.32-3.53) were created by Jacob Hopkins; Figure 3.28 created by Jacob Hopkins and Katherine Sulka. ELISA data was run by Eve Biosciences and analyzed by Katherine Sulka. Slides for H&E imaging (Figures 3.14, 3.30) were cut and imaged and analyzed by Katherine Sulka, stained by iHisto INC. Life span collection was a collaborative lab effort. All experiments and analyses were overseen by Dr. Shruti Sharma.

Chapter 4: Discussion

4.1 General Overview

The drive to understand the progression of aging, why we age, and the potential to slow or even reverse the aging process has been at the forefront of human curiosity forever - more recently becoming a major focus in academic research. The effects of aging are quite well characterized, but a general understanding of the underlying cause remains elusive. As a chronic, generalized inflammatory state coincident with multi organ dysfunction and failure, aging remains one of the greatest burdens on the modern healthcare system. The drive to understand the physiological cause of aging has focused primarily on alterations in the microbiome, generalized immune dysfunction, and a progressive accumulation of genetic damage and dysregulation. While often considered a disease, or a dominant risk factor for several inflammatory associated diseases, aging itself is not a disease and should not be treated as such. A further understanding of the genetic, immunological, and physical attributes coincident with aging are required to better understand how to extend the human “healthspan”.

It is intriguing to consider age associated inflammatory pathology simply as “associated” pathology and not a core function of aging, carrying the implication that this pathology can be limited if not prevented independent of the process of aging. As such, this inflammation is often considered as a driver of tissue dysfunction rather than a symptom that derives from the tissue – however the data described here directly describes heterogeneity of the immune response across multiple tissues that cannot be directly attributed to common circulating inflammatory mediators. Chronic inflammation is often generalized and attributed to chronic exposure to a stressor or activation of the adaptive immune system. Less often is the contribution of the innate immune system fully appreciated although many chronic inflammatory diseases bear specific hallmarks of innate activation with the potential to regulate all downstream responses.

Accumulating DNA damage or mislocalization of nucleic acids is a common hypothesis for the induction of age-associated inflammation as an underlying cause of general tissue and immune dysfunction; however, a direct linkage of these systems has yet to be described in biological aging. In models of DNA damage/nuclear instability, DNA sensors have been shown to play a critical role in the development of pathology – however, induced activation of these pathways may not completely replicate the effects of biological aging, which is associated with slowly progressing or accumulating dysfunction not an acute exposure to a pathological burden. Here, my body of work aimed to generate a more robust understanding of the role of key nucleic acid sensing pathways – specifically the cytosolic DNA sensing cGAS/STING pathways – to the development of age-associated inflammatory pathology, generalized morbidity, and mortality across chronological aging.

STING has emerged as a major signaling component downstream of DNA damage, inducing robust inflammatory and interferon responses, associated with the development of cellular senescence, inflammatory pathology, and even cell death – and emerging as a key activated pathway during aging to drive associated pathology. While STING has been directly linked to inflammatory pathology in aging-like diseases such as models of progeria, my work uncovers a much more intricate model of STING signaling. A model that does not limit STING in the induction of inflammatory pathology, but rather implicates it in long-term protective roles during the lifespan. Here, the absence of STING signaling exacerbates inflammation, contributes to the viability of key homeostatic populations, drives the accumulation of immune cell infiltrates in diverse organ systems, and ultimately leads to remarkably shortened lifespans. While STING exists as a favorite pathological pathway during aging and continues as a target for multiple pharmacological interventions, the work contained herein rather suggests major

inhibition of the STING signaling pathway may yield severe detrimental effects and exacerbate age-associated pathology.

STING is understood as a critical component of the antiviral defense mechanism, and likely plays an important role throughout lifespan regulating viral infections, sensing cellular stress or damage, and inducing specific cell death pathways. These data expand upon the central role of STING and describe a system by which STING acts as a general regulator of cellular and tissue immune responses, retaining an integral role shaping inflammatory and interferon responses, as well as the development of senescence. While STING was directly observed to tune the inflammatory and interferon responses within individual tissues with apparent direct inhibition of key inflammatory gene modules, its role in senescence is less clear. Here, STING was not observed to have a profound, conserved regulatory effect on key senescence associated transcripts, yet was involved in dampening key inflammatory marks often considered as components of the SASP.

STING has often been implicated as a critical component of senescence initiation, however its role in regulating the progression to SASP is quite intriguing. While the absence of STING may likely partially inhibit the progression to senescence it is unlikely the sole stimulus to induce this state of quiescence. The absence of STING promoting the induction of inflammatory SASP suggests a general regulatory aspect of STING signaling that promotes yet maintains the state of senescence, potentially as a primary checkpoint as the cell either undergoes repair processes or progresses to an irreparably damaged state. While STING is a key mediator of immune activation downstream of DNA sensing, it is important to note that STING does not participate in direct binding with either DNA or cGAS. Rather, STING is activated through the second messenger cGAMP. While immune signaling cascades are dominated by receptor dimerization, phosphorylation, or formation of multimeric protein structures to induce

signaling pathways, second messengers are often utilized to propagate larger cascades or integrate signals from multiple sources. The presence of a second messenger in the cGAS/STING signaling pathway is, therefore, suggestive of an increasingly expanding network rather than a pathway restricted to inducing an immune response towards DNA sensing through cGAS.

The redundancy of nucleic acid sensing conveys the ability for a cell to detect nucleic acids in all compartments of the cell – nuclear, cytosolic, lysosomal, and extracellular. While this is critical for the cell to respond to stress or infection, these pathways remain distinct and do not converge on a common nucleic acid response pathway. It is increasingly intriguing to consider that unique, compartment specific responses to nucleic acids confer unique pieces of information – repairable stress, controlled infection, unrestricted infection – with the express purpose of inducing a state of arrest to repair genomic stress, induce bystander activation to prepare for an infection, or engage death pathways to attempt to control unrestricted infection or irreparable genomic damage.

Further, the cell-specific integration of these signaling pathways may necessitate unique responses. Critical structural cells may predispose stress responses towards permanent arrest before engaging death pathways, while lymphocytes may directly engage death pathways long before genomic stress-induced senescence. Tissue resident macrophages, critical responders to tissue stress and important players involved in the clearance of stressed, senescent, or dying cells, would then be considered part of the tissue-integral populations engaging STING-dependent cellular arrest (senescence). Combined with a distinct decline in these macrophages with age and a transcriptional enrichment of death associated pathways in the absence of STING signaling, my data reinforce a core concept in which STING plays a critical role regulating the balance of engagement and maintenance of senescence with the

propagation of stress-induced cell death. While DNA stress and damage can be a sufficient signal to initiate senescence or death, induction of senescence likely precedes any initiation of cell death, at least within these key macrophage populations.

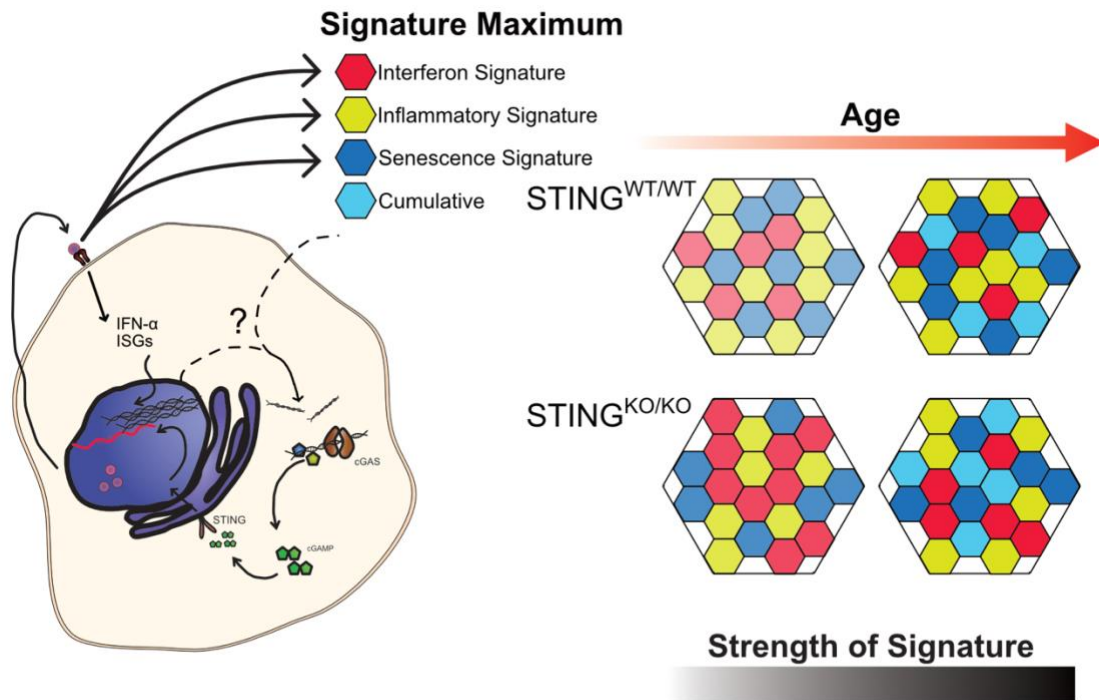


Figure 4.1 Representative model describing the regulation of multiple immune signatures by STING signaling during aging

STING was shown to directly regulate inflammatory, interferon, and senescence signatures (left) in primarily myeloid, but inclusive of stromal, cells. Intriguingly, the distribution of these signatures was shifted with age, and dependent on STING signaling as represented by the filled hexagons (right). The relative enrichment of these signatures is depicted by a graded transparency.

4.2 Significance of the work

4.2.1 Aged tissues are marked by immune fingerprints independent of circulating cytokines

A major focus of aging research has relied on human subjects to better understand *human* morbidity and mortality. As such, a traditional understanding of aging has focused on circulating inflammatory cytokines due to their abundance and ease of collection. Due to this “inflammaging” a prevailing belief in aging research has suggested that aging tissues are bathed in this pro-inflammatory soup, driving immune dysfunction, inflammatory disease, and other morbidities. The belief that a circulating factor drives age-associated pathology has led to teams of researchers performing serum transfers, heterochronic parabiosis studies, and even clinically directed human serum transfers to better understand circulating drivers of aging and attempt to alleviate these associated issues. While there are many age-associated pathologies that can be recovered through serum transfers, without a clear understanding of how tissues are aging and what inflammatory pathways are active during aging it is dangerous to assume that circulating factors are the dominant cause of age-associated pathology.

Here, I identified tissue-specific gene modules altered during aging that demonstrate a potential divergence of immune signature within tissues. While this could be easily explained as selective responsiveness to these circulating factors, specifically profiling the potential of cells to respond to circulating factors unequivocally showed cells of one lineage responded to the same cytokines to similar degrees. These data support, rather, a *tissue-intrinsic* model of aging where tissues develop inflammatory signatures independent of the serum.

This observation carries significant weight as it suggests that inflammaging at the tissue level may precede the development of an easily trackable circulating inflammatory signature. If circulating inflammation is a symptom, rather than a driver, of inflammaging,

it would ultimately only act as an indication of the severity of inflammatory disease rather than a diagnostic biomarker. Here, it becomes imperative to understand the specifics of inflammatory involvement within a tissue before any inflammatory biomarkers can be determined or a definite ligand for this inflammation can be identified.

4.2.2 Comprehensive immune dysregulation

During aging, many dysfunctional immune pathways have been identified in response to stress, pathogens, or damage and is major contributor to a decreased healthspan. However, while well described regarding microbial infection, vaccine responses, and wound healing, immune dysfunction in the absence of extrinsic stressors (inflammaging) is not clearly characterized. Although a vital and necessary component of immune responses and homeostatic maintenance, “inflammation” is only a subset of the immune response. Here, I expand the understanding of immune dysregulation to a previously poorly described immune pathway in the context of aging – the interferon associated gene signature.

Interferon signaling is a powerful regulator of every aspect of the immune response and a critical regulator of inflammatory signaling. The previous lack of understanding of interferon signaling during aging, therefore, is shocking. Further, interferon signaling does not exist solely as a response during a propagating immune response but actively participates in the activation of most cell types. This identification of an interferon signature in aged tissues is novel and suggests a greater involvement of the innate immune system during aging than was previously appreciated. While chronic inflammation is often attributed to unrestricted activation of adaptive immunity, age-associated chronic inflammation bears hallmarks of innate immune activation – particularly aging through IL1b, IL6, and TNF α .

Here, not only did these data highlight the emergence of an inflammatory and interferon signature with age, but identified specific gene modules present in each tissue that were most highly upregulated in the young suggesting that these genes were selectively lost with age. Often, the function of inflammation and other immune responses are viewed solely through the lens of a reactive mechanism to infection or damage, however the immune system is tightly integrated into the tissue at steady state, playing key roles in homeostatic processes, growth, and regeneration. As such, there are certainly key aspects of the immune system that are prominently active during adulthood that are progressively lost during age-associated immune dysfunction. Here, I not only identify age-dependent inflammatory and interferon gene cassettes but comprehensively categorize these genes into functional biological fingerprints.

Homeostasis is often considered as a “baseline” absence of inflammation. Here, I describe a general enrichment of inflammatory genes in young mice best described by pathways that suggest functional integration with the tissues of interest, while age-associated genes appeared to drive reactive immunity, likely contributing to age-associated immunopathology. While it is vital to understand the inflammation that is gained with age and how that contributes to tissue dysfunction, it is just as critical to understand these immune-directed pathways that are progressively lost with age. My data, instead, suggest a core immune and inflammatory component that is quite active in the absence of an infection, creating a homeostatic “zone” with immune activation a state of flux.

Further, the emergence of cell and oxidative stress pathways in these analyses was indicative of senescence pathways which are a key topic in the context of aging with the administration of senolytics to help revitalize the aging system. However, with these data there was not a clear temporal regulation of senescence initiation in this biological setting. Often, senescence is studied in the context of cell culture or with

pharmacological initiation – senescent cells in a biological system are very difficult to detect. These data rather suggest a more complicated mechanism by which cells enter and remain within a senescent state. If cells remained in a senescent state, unable to be cleared, there would be a progressive accumulation of cells expressing these key marks. It is possible that in vivo senescence is much more complex or that cells enter a more temporary state of senescence – either through directed clearance or a progressive shunt into an inflammatory death.

One interesting aspect of this project has been the incorporation of gene set variation analysis (GSVA), allowing a directed analysis of inflammatory and interferon stimulated genes alongside the enrichment of senescence. Here, understanding relative enrichment of these pathways was critical to better understand immune dysregulation with age. However, the finding that certain cell types – specifically subsets of macrophages or stromal cells, were consecutively enriched with inflammatory, interferon, and senescent pathways was highly intriguing. It remains to be fully understood if the senescence profile in these cells is reactive to immune dysregulation to silence the cell, or if the inflammatory component of these cells is rather part of an inflammatory component to mark these senescent cells for clearance. While inflammation is a well-regarded pathological adaptation during aging, many instances of chronic inflammation stem from otherwise proper immune responses. It is quite plausible that the key factors that are attributed to senescent cells are only temporarily expressed, while others are involved in the maintenance and progression of senescence – which is identified through the general enrichment of the senescence gene program.

4.2.3 STING signaling is a critical component in healthy aging

STING is understood to be a critical component of the innate immune system; integrating and propagating second messenger signals from cGAS to respond to viral

infection and induce proper immune responses. As such, with the assumption that DNA damage and mislocalization are integral drivers of age-associated inflammation, it has been suggested that STING is an integral signaling component in age-associated pathology. However, many of these STING-directed studies depend on overly-active DNA sensing pathways through nuclear disruption – rather showing that DNA sensing has the capacity to disrupt homeostatic processes with some dependence on STING. Genetic models that induce direct disruption of the nucleus, DNA damage, or nucleic acid mislocalization likely activate DNA sensing pathway to a much greater extent than is experienced in vivo, in the absence of severe cell damage or infection that leads to death of the cell. Here, a biological model of aging maintains any activation of DNA sensing pathways within “normal” levels and can directly address the ability of STING to drive age-associated inflammatory pathology.

Interestingly, in the absence of STING signaling there was a measurable elevation of inflammatory serum cytokines with age, significant destruction of tissue order, and a complete restructuring of immune gene cassettes. While there are a number of immune genes positively regulated by STING signaling, there are a surprising amount suppressed by the presence and activity of STING. This dominant phenotype culminates in novel myeloid cells progressing towards “polypositivity” – trending towards monocyte-like populations rather than the TRMs described in the C57BL/6. Further, this dominant immune dysregulation results in a significantly decreased lifespan in the STING^{KO}, highlighting the extent to which immune regulation by STING is critical during aging.

4.2.4 Potential ligands

A gap in these studies is the identity of the ligand that engages constitutive STING signaling in health, disease, and aging. STING is well-regarded as an interferon and inflammatory-generating pathway as is likely involved in multiple pathogen-sensing

pathways during normal aging. However, it remains unknown how STING, in the context of aging, is providing the clear protective or even homeostatic signal described herein. While intriguing to consider STING as a critical integrator of tissue health, it remains possible that STING signaling is a primary response to low-level activation from “homeostatic” microbes to reinforce barrier tissues during mild dysbiosis.

A common metric of cellular health focuses around metabolic and mitochondrial health. While STING is presented both as an integral sensor of mislocalized or damaged self and non-self-DNA through cGAS, cells are rather full of non-self-DNA through the mitochondria. Stressed, damaged, or dying mitochondria can be very potent sources of non-self-DNA into the cytoplasm and serve as a direct ligand for cGAS to activate STING. However, in the context of aging, reports suggest that mitochondrial DNA may not be as likely a driver of aging and age-associated pathology.⁹⁶

Alternatively, STING has more recently been implicated in the sensing of endogenous retrovirus elements (ERVs) as a source of interferon production.^{97,98} It is plausible that STING could be a primary sensor of ERVs as an indicator of generalized cellular stress or, rather than DNA damage, transcriptional dysregulation. While the cGAS/STING pathway has long been understood to be a sensor of cytosolic double stranded DNA, recent work has indicated that cGAS may, in fact, be restricted to the nucleus.^{99,100} Although the role of cGAS in the nucleus has yet to be elucidated, its ability to bind double stranded DNA has not been disputed. When packaged in the nucleus, DNA is tightly wound into dense structures around histones. To be transcribed, there are a number of DNA binding proteins required to bind specific promotor regions of DNA to allow sufficient room for different polymerases to bind and begin transcription. However, not only are there proteins that bind to the promoter elements, but also to proximal and distal enhancer elements to ensure transcriptional fidelity. It is possible that cGAS, and in effect STING signaling, could localize to the nucleus to sense and prevent

erroneous DNA transcription by binding regions of accessible DNA that lacks proper DNA binding proteins within these promoter and enhancer elements.

Infectious viruses require host machinery to transcribe and translate their genetic material, and many ultimately fully integrate into the host genome – in fact a substantial portion of the mammalian genome is viral in origin. While viruses can integrate into the mammalian genome and hijack transcriptional machinery, it is unlikely that all the required axillary binding proteins are involved, allowing cGAS to bind accessible DNA to engage the cGAS/STING signaling pathway. In the context of aging, cGAS and STING signaling may be a critical component to recognize and prevent erroneous transcription. For many years, proteins were key actors for cellular functions, but the engagement of RNA, as long non-coding RNA or short hairpin RNA, have more recently become appreciated as key regulators of the immune system – through direct inhibition of transcription, translation, or protein function itself. In the absence of STING, there cell may lose it's primary early sensing pathway for transcriptional dysregulation, allowing the cell to progress to an increasingly dysfunctional state, or for secondary, highly reactive “failsafe” pathways to activate damaging the cell or its surroundings to signal a severe damage response.

4.2.5 Tissue resident macrophages are distinct immune elements of aging tissues

Here, I identified a significant portion of age-associated immune dysregulation that was focused within the macrophage component of tissue resident immune cells and was further perturbed by the absence of STING signaling, as specifically emphasized with the comprehensive immune dysregulation described previously. To characterize macrophage-specific perturbations with age that are dependent on STING signaling

more specifically, I performed secondary processing and cluster identification on the macrophage population of these scRNAseq datasets. Intriguingly, the macrophages were not uniformly associated in a single UMAP cluster, but I was able to identify up to 7 transcriptionally distinct tissue resident macrophages from each tissue. While multiple, critical functions have been ascribed to tissue resident macrophages they are generally referred to as a single entity – whereas these data demonstrate transcriptional, and potentially functional, heterogeneity within the TRM pool.

Across all three tissues analyzed here, I was able to identify TRMs with distinct enrichment of canonical TRM markers such as *Timd4*, *Lyve1*, *Mrc1*, *Cx3cr1*, or *Csf1r*. While this group remained one of the larger transcriptional clusters across all tissues, they did not represent the majority of the cells. While the general interpretation of scRNAseq clusters indicates transcriptionally distinct – and stable – clusters it is intriguing to consider the concept of macrophages in a state of transcriptional *flux*. All immune cells exist through periods of resting, activation, quiescence, exhaustion, and death; but most immune cells are quite short lived and are either activated and die or circulate in a state of inactivation until they are replaced and thus have distinct, and well characterized, activation profiles. TRMs are quite long-lived and, as such, are not believed to expand as robustly as a T cell when activated or to apoptose post activation as a neutrophil. TRMs are understood to be plastic and robustly adaptable to a shifting microenvironment, which is likely reversible. It is intriguing to consider the concept of a transcriptionally flexible TRM that exists within a basal state and conforms to different functions as necessary to respond to damage, infection, stress, or repair.

Here, these TRM clusters are more tightly grouped than would be expected from completely distinct cell types suggesting a core set of genes are conserved, but they continue to cluster within distinct nodes indicative of specific gene modules. Here, rather than describing conserved uniqueness among these TRM clusters these data may only

capture a snapshot of the TRMs highlighting distinct reactive subsets of TRMs to a variety of stimulants. Distinct or in flux, these data highlight an unappreciated diversity of the resident macrophage phenotype. Conserved across at least two tissues I identified TRMs more adept at antigen presentation, propagation of inflammatory responses, enrichment of metabolic responses highlighting a potential to ingest and process lipids, distinct enrichment of long non-coding RNA, and a conserved population with an indistinct transcriptional phenotype.

This transcriptional heterogeneity clearly emphasizes the potential of the TRM within the tissue to respond to stressors (inflammatory macrophage or MHC-II+ macrophage), act as a distinct functional component of the tissue (TLF+ or metabolic macrophage), or a previously uncharacterized macrophage (stressed, lncRNA+ macrophage). Using pathway analysis to more broadly characterize TRMs with age replicated a similar phenomenon observed in the pseudo-bulk tissue samples in which young TRMs were preferentially enriched with pathways more suggestive of a physiologic role while aged were indicative of a reactive inflammatory phenotype suggestive of a clear pathological shift in the TRM transcriptome.

While whole tissues generated immune fingerprints with age, tissue macrophages remained more transcriptionally distinct between cell types than in the absence of STING signaling – however many macrophage subsets retained unique STING-dependent immune signatures, suggesting some cell-intrinsic (or state of flux) role of STING signaling within aged macrophages. While the transcriptional phenotype will not definitively determine the functionality of the macrophage, it is likely that any phenotypic dependence on STING signaling could be dominated by another functional response, such as a necessary phagocytic and antigen presentation program or the propagation of a STING-independent inflammatory response. While it remains unclear what functional output is regulated by STING signaling in the aged macrophage, there is

a distinct transcriptional program that is activated in aged macrophages. Intriguingly, STING did not appear to significantly drive a classical senescence transcriptional profile in TRMs, demonstrating a clear positive and negative regulation of a number of key senescence associated genes. The inflammatory component of senescence, the senescence associated secretory phenotype (SASP), however, was elevated in several TRM-subpopulations in the absence of STING signaling. While STING was expected to play a dominant role in the age-associated generation of senescence and the inflammatory SASP, these data suggest an uncertain role for STING in the generation of senescence, and rather seems to be involved in preventing the progression of senescence towards a SASP phenotype.

Senescence is generally considered a state of quiescence and generally believed to be irreversible; however, there is an increasing appreciation for multiple stages of senescence with varying degrees of permanence. Senescence, or senescence-like, phenotypes are observed as critical components of homeostatic or resolving immune responses such as wound healing. Functionally, it is not likely that a large percentage of cells would enter senescence at the same time, as it could be detrimental to the host, as it would be difficult to clear and replace all these cells. During aging, an accumulation of senescent cells suggests either increased rate of senescence initiation, or the removal of these cells is inhibited. Macrophages are key cells that engage in the phagocytic removal of senescent cells and may become overwhelmed by this steady accumulation of senescent cells. Further, it is unknown what the consequences are of a tissue resident macrophage entering a state of senescence. Tissue resident macrophages are often homogeneously distributed within a cell, and the loss of one would require replacement through the hematopoietic system or a neighboring resident macrophage. While the context by which these cells engage

senescence and other immune pathways remains unclear, it is a vital path for future study.

4.2.6 Tissue Macrophages are lost with age and functionally regulated by STING signaling

Tissue resident macrophages and their importance have been well characterized during development and during acute stressors such as tissue damage and infection. While there is an overarching understanding that their relative composition within tissues decreases due, in part, to monocytic infiltration and turnover of the niche, these experiments have generally been focused on the first few months of murine life, extrapolation post solid organ transplants, or during directed stress in the tissue. It is quite unclear, however, if this monocytic bias within the TRM niche progresses to total replacement of the niche or is limited to an expanding organ. Further, the extent to which replacement monocyte-derived TRMs functionally mimic canonical fetal-derived TRMs is entirely unknown.

While these data show a clear shift towards F4/80^{lo} macrophages within these tissues with age, the overall abundance is surprisingly stable compared to the replacement identified in early life, suggesting that, in the absence of stressors, both fetal and hematopoietic macrophages may fill more clearly defined niches than previously expected after an initial surge in F4/80^{lo} macrophages in the first 3 months of life. It was expected that a general enrichment of F4/80^{lo} macrophages would be indicative of a general loss of functionality within the TRM populations with age. While there was no clear correlation of F4/80^{lo} macrophages and tissue function, it remains difficult in the absence of true lineage tracing experiments to confirm which macrophages are canonical fetal-derived and which are sourced from the adult bone marrow. While initially this was a major goal of this project, the feasibility quickly became

obvious and was abandoned. Here, using the expression of F4/80 to demarcate mature, long-lived TRMs (high expression) from immature, likely recently established tissue macrophages (low expression) can provide some information regarding the “health” of the tissue-macrophage pool where a greater concentration of F4/80hi macrophages is indicative of a healthy, mature, and functional tissue macrophage population.

Through single cell RNA sequencing it was clear there was a dominant heterogeneity within the tissue macrophage population, however transcriptomics can provide exceptional depth and granularity to a biological system with less information regarding the functional state. As previously discussed, the transcriptional heterogeneity of these tissue macrophages may only provide a snapshot of a population in a state of flux that does not truly differentiate into unique populations. The flow cytometry experiments described here provide a greater functional description of these macrophages, but with much reduced specificity – and while unbiased clustering identifies upwards of 7 macrophage populations within each tissue, many of these clusters appear to exist across some phenotypic spectrum with 2-3 dominant groups. While less striking than the transcriptomic data, these data appear to support that these macrophages do exist through some state of flux and are not functionally restricted. TRMs are functionally integrated within each tissue to varying degrees. In the heart they are critical for regulating electrical conduction through the atrio-ventricular node, and in the kidney, they drive “branching morphogenesis”. While these cells are understood to be transcriptionally plastic, it is highly likely that this state of flux specifically integrates multiple sensors of the tissue environment to tailor their functional state to the physiological needs of the tissue.

What remains a major curiosity of these data is the dependence on STING signaling that appears to maintain the population of macrophages within these tissues. While prevailing thought has assumed that STING was driving pathology within tissues,

it appears that STING is critical to the maintenance of the declining TRM population – rather than causing their decline directly as may be attributable to direct STING pathology through activation of inflammatory or cell death pathways. Further, STING signaling appears also to regulate the functional state of the remaining macrophages. While this phenotype appears to be dominant within the transcriptional state of TRMs, the loss of homeostatic receptors was identifiable through protein expression as well. Together, the loss of TRMs and the disruption of their homeostatic potential suggests that STING contributes to the maintenance and functionality of TRMs with age.

While a novel homeostatic role for STING signaling during aging is intriguing, it is not the sole possibility. Many immune cells require activating input to support their growth, expansion, and maintenance. I propose that the correct dosing of STING signaling provides sufficient TRM activation to support their growth and expansion but not enough of an intrinsic pathological signal to induce inflammatory death. As such, the loss of STING would remove a gently activating stimuli that, while a pathological stimulus, provides necessary survival factors to maintain this critical population. What remains unknown, and is unanswerable through the study discussed here, is if aged TRMs are functionally equivalent in the absence of STING signaling. While STING^{KO} TRMs appear to have dysregulated homeostatic receptors, these cells theoretically are not experiencing any additional activating factors and would require specific stressors to determine if they could functionally activate to non-STING-mediated pathology.

4.2.7 STING calibrates TRM vitality and sensitivity to cell stress

These data have suggested, that rather than simply driving a dominant pathological phenotype, STING itself plays a critical role tuning and shaping the age-associated inflammatory response. Furthermore, TRM populations are lost faster and homeostatically dysregulated in the absence of STING signaling suggesting that

activation through STING facilitates population maintenance or even expansion in aged organs. Curiously, STING activation has previously been linked to the engagement of senescence and inflammatory death pathways which, rather, would promote stagnation and loss of the TRM population if STING was activated.

Here we describe a model where, STING signaling is regulating and preventing the engagement of cell death pathways and functionally regulates DNA damage responses and cell death in TRMs but not BMDMs.

Intriguingly, this may be a result of STING directly engaging and inducing early senescence-type phenotypes or maintaining a senescent phenotype in TRMs. While senescence is often considered an irreversible state, the classification and identification of senescence is still very difficult. The point at which a cell engages permanent arrest is likely long after the cell has transiently arrested in an attempt to manage and repair any intrinsic stresses. Specifically in long-lived cells, the ability to repair genomic stress would be critical rather than committing to death or a permanent state of arrest. However, none of these options prevent the possibility that STING activation directly provides some survival stimuli directly to the TRM or other long-lived cells. STING engagement has been shown to induce major signaling cascades: interferon response factor 3 (IRF3) which drives interferon production and nuclear factor kappa B (NFkB) to support inflammatory responses. However, NFkB has been linked to a plethora of cellular responses including many that support cell survival.

As TRMs are long-lived, their effective maintenance is as likely critical to the general health of the tissue, and by extension the host. Many immune cells, such as lymphocytes, exist in a state of bioenergetic loss: generated, matured, and removed without ever binding their antigen. TRMs, on the other hand, have a constant and consistent function and may not be adequately replaced with aging and damage. It is very likely that these cells, in the context of intrinsic stress, need to be prone to engage

these protective responses to prevent unnecessary death. Here, STING is shown to be a critical component of TRM survival in the context of cellular stress (aging) and the engagement of cell death pathways in a way that hematopoietic bone marrow derived macrophages are not. While it is possible that STING is a critical response factor to engage senescence during early death engagement, the data contained here suggest that any protective shielding by STING signaling is limited to resident macrophage populations and not a standard survival tactic of the immune system.

Although the macrophage was first identified almost 150 years ago (1882-2023) it wasn't until the last 10 or so years that the unique lineage of TRMs was discovered. the extent to which the TRMs are functionally integrated to their niche has been astounding, and recent discoveries suggest that TRMs even develop a heightened ability to clear cellular debris without promoting an immune response. In fact, given some of these findings and the uniqueness of the fetal and adult immune systems, it is difficult to impress just how little we may understand about these persisting macrophages. Recent discoveries of additional fetal-derived and persisting immune cells underscores how critical it will be to functionally segregate immune cells by ontogeny for all future studies.

4.3 Consequences of Senescence

Aging as a biological process is incompletely understood but is highlighted by accumulating pathologies that are increasingly burdensome on the population as a whole. A major issue coincident with aging is DNA mutagenesis that leads to the activation of oncogenes and tissue destruction through tumor metastasis. While oncogenic activation is most associated with aging – because of the potential for slow accumulating mutations – this is not exclusive to aging and has been met with selective pressure over the course of evolution. The emergence of senescence is similar to cancerous oncogenesis, is most often attributed to aging and its associated pathology,

however senescence is believed to exist in direct opposition to oncogenesis by limiting continuous replication in the presence of genomic stress. Senescence, in the context of aging, has been shown to be induced in response to DNA damage in order to halt all cellular growth in an attempt to repair any genetic abnormalities and prevent oncogenesis.

While senescence is often considered to be a permanent state of growth arrest, the further understanding of senescence has uncovered multiple stages of senescence that may not be entirely irreversible. The senescence of aging appears to become problematic when it progresses towards an inflammatory state of senescence. While the effect of this inflammatory senescence (SASP) is not entirely understood, its primary purpose may exist to induce clearance of any cells that have progressed to a permanent state of senescence. The use of senolytics, senomorphics, and senostatics have seen an incredible rise in geriatric research and even clinical trials. The outward effects of removing senescent cells may have more complicated effects than can be fully understood. A major issue remains in the effective identification of senescent cells. While there are certain markers that have been associated, and are generally accepted indicators of induced senescence, these markers are very difficult to identify within tissues, raising questions as to how useful many of these markers truly are. As such, senolytics and senomorphics tend to be increasingly broad spectrum and may have more off target effects than are intended – especially due to the relatively unknown function of senescence in vivo.

It is often assumed that senescent cells are entering a terminal state, but if these cells have engaged senescence rather than some other death pathway – often the default response to excessive damage – it is possible that these cells are required for critical functions within the tissue or preserve some other unknown element required for ongoing processes. While senescent cells may not grow and function appropriately, they

may be incredibly more beneficial to the tissue than, say, a stiff fibrotic response specifically in the heart, skin, or vasculature that require extreme pliability and flexibility. A sudden removal of senescence cells may require extreme collagen deposits to maintain the immediate physical properties of the tissue in question that are ultimately debilitating to the proper functionality of the tissue. Further, as these drugs are broadly effective, extended treatment may have more detrimental effects outside of inhibition of permanent senescence, as many cells likely engage early senescence mechanisms but never progress to late or permanent senescence phenotypes.

4.4 Incomplete TRM replacement

Over the course of this body of work, a major focus has been on the further understanding of the homeostatic immune function of the tissue resident macrophage (TRM) during the course of aging and how STING signaling shapes this age-associated inflammatory state. While TRMs are well characterized during development and early life, there has been considerably less work defining their state and function throughout life – only that this population is believed to persist during steady replacement from bone marrow-derived macrophages. Thus far, effective identification of fetal-derived and bone marrow-derived tissue macrophages has required specific lineage tracing experiments – however these models were primarily developed to isolate and understand these macrophages during fetal development and early life. While accurate models for this usage they do not remain infallible throughout the lifespan of the mouse as these specific markers (Cx3cr1 and Csfr1) become slightly promiscuous and can reliably identify other myeloid and lymphoid cells.

The persistence of the fetal-derived TRM is likely incomplete, as these cells experiences a lifetime of stress, growth, and death; however there is a well-accepted mechanism by which monocytes can mature into macrophages and develop into TRMs

(moTRMs) in their own right. However, in the absence of temporal lineage tracing experiments it remains unknown if these moTRMs are long-lived, or constantly replaced, nor is it known the extent to which these moTRMs functionally replicate the canonical TRM. TRMs have been described to regulate productive immune responses, retaining an ability to silently clear small lesions and prevent mass infiltration of neutrophils and monocytes. As monocytes appear to be programmed to respond en masse to tissue damage, the full understanding of the potential of moTRMs to limit tissue immune responses and inflammation remains highly important. The lack of immuno-restrictive TRMs to regulate inflammatory responses and the presence of moTRMs that may propagate inflammation unnecessarily could be a dominant driving factor in the generation of age-associated inflammatory pathology.

4.5 Future DirectionsProposed model

The work here contains a model of innate immune contributions to age-associated inflammatory dysregulation and proposes the idea that not only are innate immune pathways critical to health and longevity but that the absence of these pathways are detrimental. Chronic DNA sensing through the cGAS/STING signaling pathways is believed to be a major driver of age-associated inflammation, immunosenescence, and overall morbidity and mortality.

While activation of these pathways are acutely and quantifiably detrimental, the majority of these model systems rely on systems with likely a much greater load than would be expected in a biological context. Here, in a biological model of aging it can be seen that the absence of cGAS/STING signaling is, in fact, more detrimental than any steady-state activation of this pathway. While this does not inherently suggest that cGAS/STING signaling is antagonistic towards age-associated inflammatory pathology, it does reveal that the current understanding of inflammation as it pertains to chronic

inflammation and inflammation-associated pathology is not complete. It is simple to suggest, with a directed stimulus or genetic model, that key immune pathways are detrimental to the system and blocking or removing them alleviates pathology. However, in vivo, biological models of disease are considerably less clear cut. Here STING, a primary candidate for driving age-associated inflammation and inflammatory pathology, retains a dominant regulatory or even protective effect to the aging host – the absence of which is clearly detrimental leading to a significantly shortened lifespan.

These data dominantly highlight the critical fact that, while immune pathways can clearly have chronic, unresolving, pathological consequences they remain critical defensive responses to secondary or tertiary insults that are likely overlooked in models of excessive insults.

4.5.2 Overview

A majority of this body of work was dedicated to the further understanding of the role of STING signaling in the regulation of chronic inflammation and progressed specifically towards aging as a model of (otherwise) sterile inflammation. Aging is an intriguing model to study for the regulation of chronic inflammation as a definite ligand that drives this inflammation is still unclear. A strong belief that the dysregulation of nucleic acid stasis drives these responses through ligation of the cGAS/STING signaling pathway has been generally supported through models of accelerated aging that specifically drive extreme levels of nucleic acid damage, dysregulation, or leakage and may not act within a biological context. As such, these data uncovered an intricate regulation of age-associated immune networks which spans inflammation, interferon signaling, and the progression of senescence across multiple cell types. Furthermore, these data suggest a previously unappreciated balance of protective and pathological responses downstream of STING signaling that requires considerable further

understanding before these pathways are pharmacologically blocked in an attempt to alleviate age-associated inflammatory disease.

4.5.3 STING signaling in human health and disease

A major goal of this study remains to directly correlate the connection of STING signaling in the regulation of chronic inflammation to human health and disease.

Although there are biologics in clinical trials to activate STING for cancer therapy, and hopes for STING inhibitors for treatment of inflammatory disease, they are not progressing as quickly as anticipated. Importantly, the data contained herein suggests that, while these therapies may have beneficial targeted effects there is a likelihood of significant detriment through long-term application. As such, the role of STING signaling in human health and not only disease must therefore be more thoroughly studied.

Animal research has the benefit of specifically querying the function of specific genes and proteins through specific genetic knockouts, while human studies must rely on generally rare gain- or loss-of-function mutations. While volunteers with these mutations can be incredibly valuable to better understanding of their various conditions, there are not inherently mutations for every symptom or disease. Within the human population, however, there exist multiple single nucleotide polymorphisms (SNPs) that impart a loss-of-function mutation for STING signaling, gain of function mutations that result in a severe interferonopathy known as SAVI (STING-associated vasculopathy with onset in infancy). While SAVI mutations remain quite rare, the “HAQ” loss-of-function mutations are highly predominant in the human population, though concentrated in certain ethnic groups.

This prevailing loss-of-function STING mutation is incredibly intriguing as it suggests that there is a significant population with some reduction in their ability to activate STING appropriately during everyday life, infection, and aging. As such this

suggests that STING signaling is a prime candidate for direct human translational research. As a preliminary step, publicly available datasets could be directly leveraged to perform a retrospective meta-analysis of the prevalence of STING loss-of-function mutations in specific diseases or even aging datasets provided sufficient sequencing information was available. While not all publicly available human datasets provide sequencing information, and fewer of those are sequenced sufficiently to characterize STING SNPs, these mutations exist at a sufficient level within the human populations that relatively small studies could yield sufficient patients.

Unfortunately, these retrospective meta-analyses would be entirely correlative in nature, and while unable to determine a direct role of STING in health and disease it could certainly stratify STING as a risk factor for any number of diseases or the severity thereof. Further, a clearer understanding of the functional role of STING signaling in human health could more directly inform the use of biologics in the amelioration of inflammatory disease. Given datasets with sufficient associated patient data, it could quickly be determined if these STING loss-of-function mutations were able to impart significant protective effects against the initiation of inflammatory disease (specifically age-associated), or if these mutations predispose the population to cross-tissue age-associated inflammatory disease as suggested by these data.

Given the large proportion of potential patients with STING loss-of-function mutations it is plausible, although expensive, that patients could be recruited and selected based on their STING polymorphisms for directed analysis and longitudinal studies. Even small, matched cohorts could be generated from these patients for numerous analyses. Using circulating immune cells and cytokines as a direct readout of immune responses, the effects of these loss-of-function mutations could be extrapolated into effective immune responses to vaccines or even specific bacterial or viral infections through a longitudinal analysis.

While the full extent of the role of STING regulating health and disease remains to be fully understood, these data describe a critical involvement in the shaping of chronic immune responses, specifically during aging. Fortunately, STING appears to be a prime candidate for human research given the extreme prevalence of loss-of-function mutations, coincident with severe (but rare) gain-of-function mutations. Forming a more complete understanding of these signaling cascades would be difficult but appears to be highly critical. The most direct step would likely be retrospective analyses in GWAS datasets but could quickly culminate in including STING status as a variable for cohort stratification or even directly profiling immune responses based on functional STING signaling.

4.5.4 In vitro assays – STING and IFN responsiveness

Early investigation of the regulatory aspect of STING signaling for this body of work focused primarily on direct stimulation of bone marrow-derived macrophages with DNA analogues, Type I and Type III interferon. While this work was set aside to focus on STING in the context of chronic, not acute, inflammatory stress these experiments left a considerable number of unanswered questions. DNA sensing through STING is adequately induced by the DNA analogue poly(dA:dT) and is known to induce a robust interferon response. While there was a notable delay in the induction of interferon stimulated genes between poly(dA:dT) when compared to direct stimulation with Type I Interferon, when these macrophages were treated overnight with IFN-I, but not poly(dA:dT), there was a distinct reduction in the interferon signature 4 hours after a secondary stimulation.

There is an intriguing possibility that while IFN-I drives stable expression of inhibitors of IFN-I in order to self-regulate the production of IFN itself, stimulation with poly(dA:dT) functionally limits this “refractory” period to IFN-I stimulation allowed a

macrophage to be continuously receptive to IFN stimulation. Further focus on the induction of IFN repressors and interactions of various signaling intermediates is crucial to understand if this lack of a refractory response to IFN is persistent.

4.5.5 Flow cytometric characterization of TRMs

Although tissue resident macrophages are one of the most critical immune cells in a tissue they remain poorly described. Initially described primarily by an enrichment of F4/80 expression, these cells (during development) are frequently described and characterized by their dependence on transcription factors. While lineage tracing experiments using *Cx3cr1* and *Csf1r* were critical to the initial characterization of TRMs, these antigens are insufficient to distinguish TRMs from other myeloid cells in adulthood. Recent work has attempted to delineate TRMs from other myeloid cells through the expression of three markers – Tim4, Lyve1, and Fcrl2 – with the understanding that these markers are not collectively identifying any singular population but expressing any of these markers is sufficient.

While this method may be more effective than, say, an unending unified marker of resident macrophages, it rather suggests that these macrophages are highly specific to their tissue of origin and yet consist of multiple subsets within each tissue. While the work described here began an attempt to functionally characterize tissue macrophages, the primary goal was to identify conserved changes as a result of age and STING signaling rather than direct characterization of specific populations of tissue macrophages. Highly specific characterization of these macrophages for immunophenotyping will require specific usage of lineage tracing combined with transcriptomics or proteomics to identify novel, rather than expected, antigenic indicators of tissue resident macrophages and their respective subsets within different tissues.

4.5.6 Integration of immune reactivity

Activation of the immune system involves a highly integrated balance of stimulation and inhibition to maintain adequate, yet regulated, responses. An intriguing finding from this body of work is the combined dysregulation of immune signatures among myeloid and stromal cells across multiple tissues. These data accentuate the core concept that inflammation and immune reactivity is not restricted solely to the production of more canonical inflammatory cytokines and signaling intermediates but, in the context of aging, involves multiple broad immune systems. However, preliminary investigation into the relative association of the enrichment of these immune pathways identified an inverse relationship between the relative enrichment of the interferon and inflammatory gene signatures. These cells were sparse, and while this analysis was not continued as part of this thesis suggested the existence of a robust interferon signature that would exist in the absence of an inflammatory program. It would be essential to understand more fully if the interferon signature expressed by these cells was identifiable as a dominant repressive signature, especially if these genes could be corroborated in alternate datasets.

4.5.7 STING regulation of senescence progression/maintenance

STING is well-described as a critical initiator of senescence in multiple models of STING initiation. What remains to be understood is the temporal engagement of STING that is critical for a cell to undergo senescence, repair, or death. A progressive state of senescence has become more greatly appreciated in recent years and is no longer believed to be a fully committal step. While STING engagement is required for certain aspects of senescence, it is unlikely the sole initiator and regulatory checkpoint. Transcriptionally, these data demonstrate a non-specific role of STING signaling in the development of key senescence associated transcripts and a negative regulation of

inflammatory components linked to the SASP. This is suggestive of STING acting not as a checkpoint for senescence initiation, but a regulator of the development and progression of senescence and SASP. While a much clearer understanding of the progression of early- to late-phase senescence is critical, a temporal understanding of the role of STING throughout this process is lacking. While STING may regulate the initiation of early- or late-phase senescence, the data contained herein highlight the possibility that STING is rather involved in maintaining the senescent phenotype to prevent the inflammatory SASP.

4.5.8 STING regulation of cell death

STING has previously been linked to the initiation of cell death, however these data demonstrate accelerated engagement of cell death pathways with multiple extrinsic stressors in the absence of STING signaling. These stressors similarly engage DNA damage (etoposide) and oxidative stress (H₂O₂) and the accelerated engagement of death is reminiscent of elevated inflammation observed in the STING-deficient aged mice. While there is little contention that STING activation is involved in cell death, what is not clearly understood is the balance of inflammatory and silent cell death pathways. STING may be involved in the early detection of cellular stress to drive an early senescence-like phenotype prior to the engagement of regulated cell death pathways while the absence of this early sensor precludes the cells to an accelerated inflammatory death as sufficient damage has accumulated.

4.5.9 Alternate activation of STING signaling

STING is a critical component of the cytosolic DNA sensing cascade, yet STING does not directly bind nucleic acids, rather to a second messenger generated by cGAS –

2'3'cyclicGMP-AMP (cGAMP). Intriguingly, unlike other second messenger systems cGAMP is strictly linked to cGAS/STING signaling with no other source or receptor. Biochemically, second messenger systems are generally described as cascades, propagating a response with significant downstream effects. cGAMP, uniquely, is only believed to bind STING. Further, while cGAS uniquely generates cGAMP and alternative isoform of cGAMP (with a 3'5' linkage) is generated by bacteria and is also sufficient to activate STING signaling.

As STING is critical to the induction of immune responses downstream of DNA sensing and is believed to be a critical component of the age-associated inflammatory response, it is incredibly intriguing that a more clear understanding of the responses to 2'3'cGAMP and 3'5'cGAMP are not more clearly understood. Human polymorphisms have been suggested to allow differential responses to mammalian and bacterial-derived cGAMP, but it is unclear if this is a conserved function of STING. Further, while STING is ubiquitously expressed and shown here to be a critical mediator of inflammatory health, it is unclear if the second messenger cGAMP served to integrate additional signaling cascades alongside – or in opposition to – STING. Certainly, a more comprehensive understanding of the activation of STING is required.

4.5.10 Independent signaling through cGAS, STING

STING is most often considered to be directly activated by cGAMP as produced by cGAS, however there are a number of cyclic di-nucleotides (CDNs) that are capable of activating the STING pathway¹⁰¹⁻¹⁰⁴. Further, many of these CDNs, and other nucleic acid second messengers, have been shown to regulate more intricate signaling networks rather than a direct linkage between to proteins¹⁰⁵⁻¹⁰⁷. STING is emerging, rather, as a complex integration point for multiple CDN-based signals. As a focal point for multiple signals, it is critical that STING is not solely investigated as a pathway

downstream of damaged or mislocalized DNA, as STING may integrate CDN-based signals from multiple sources, a general sensor of cellular health and disease.

Further, if cGAMP (or other CDNs) are acting as true second messengers, it is possible that there are multiple sources downstream of DNA activation of cGAS *independent* of STING activation and signaling. In the absence of STING there would, therefore, be an “excess” of cGAMP to stimulate secondary (or even alternative) pathways. While there would likely be further crosstalk between these cGAMP-stimulated pathways, the absence of STING ligation would result in the overstimulation of these, albeit unproven, secondary pathways. While STING is a potent inducer of interferon, alternate signaling through cGAMP could help explain the restructuring of the interferon and inflammatory signatures in the absence of STING signaling.

4.5.11 Balance of nucleic acid sensing

The immune system exists as a critical sensor of stress, damage, and infection and is the most critical defense system that has ever evolved – as such there are multiple levels of redundancies and fail-safes to ensure that survival of the host. Redundancies are viewed as the simplest system to ensure a complex response to an infectious organism, but this understanding overshadows the idea of a balance of multiple pathways to ensure different responses. While STING signaling has been shown to drive an inflammatory and interferon response in the context of DNA damage it remains unclear if these systems are dependent upon a secondary additive stimulus. While STING is believed to be a dominant propagator of DNA sensing in the cytosol through cGAS, there are additional sensors of nucleic acids in the cytosol such as AIM2 (DNA) and RIG-I/MAVS (RNA). While STING is clearly critical for the propagation of nucleic acid sensing, the absence of STING does not abrogate the cell’s potential to respond to cytosolic DNA entirely and it is entirely possible that the adequate balance of

STING and AIM2 signaling is critical for the appropriate balance of this nucleic acid response.

Here, these data describe a clear inflammatory response in the absence of STING signaling with age. However, it is unclear if these inflammatory responses are directly inhibited by STING signaling through TBK1/IRF3/NFkB or if, in fact, it is more directly attributed to an otherwise unrestricted AIM2 signal. Redundant immune signaling may not solely be present to provide a signaling cascade in the presence of specific pathogenic inhibitors but to gauge the level of this blockage. DNA sensing through the combined responses of cGAS/STING and AIM2 may signal for a completely different response than either pathway alone which could be indicative of an infection directly inhibiting the immune response, requiring a greater inflammatory response.

4.5.12 Localization of interferon production

The bulk of this body of work focuses on the role of STING signaling in tissue resident macrophages, while attempting to describe significant and intricate regulation of immune responses by STING. It is likely that much of this observed alteration of immune function is not directly induced (or repressed) by STING but are observed due to crosstalk between numerous signaling networks acting to regulate homeostasis – which are imbalanced through the lack of STING signaling. Type I interferon is a dominant product of STING signaling that is well-regarded as a potent immunoregulatory cytokine yet is composed of a number of subtypes with overlapping gene signatures. Importantly, in the absence of direct stimulation, specific interferon subtypes are produced below the limit of detection = albeit with a quantifiable gene signature. While it is quite difficult to ascribe specific interferon stimulated genes (ISGs) to specific interferon subtypes, this family is often lumped together simply as “interferon”.

In this model we removed a dominant regulator of age-associated interferon signaling, as demonstrated through a remarkable shift in relative levels of ISGs. However, this was not noted as a general *loss* of interferon response, but rather a restructuring and shift of the interferon signature. This shift could be a result of two distinct possibilities – repressive signaling downstream of STING actively limits production of certain interferon subtypes, the presence of which induces unique cohorts of ISGs; or, alternatively, the lack of interferon signaling from STING results in a compensatory shift (or overproduction in the lack of feedback inhibition) from a secondary source.

While this work focused on STING in tissue resident macrophages, a second myeloid population – plasmacytoid dendritic cells (pDCs) – are generally considered dominant producers of Type I interferon. These cells form incredible small populations within tissues, however, have been shown to play exceptionally important roles in the propagation of the immune response specifically due to their production of Type I interferon^{108,109}. While pDCs were not a major focus of this work, STING has been shown to play a regulatory role during the activation of pDCs and are an important cell type for the future consideration of age-associated Type I interferon production.

4.5.13 Senescence, health, and disease

Senescence is commonly believed to be a protective response to balance against the threat of oncogenesis, but more recently has been appreciated to be involved transiently in multiple aspects of immunity and not simply a terminal state of a depleted cell. Initiating signals for senescence and oncogenesis both appear to be downstream of DNA damage. It remains unclear, however, what differentiates between life, death, and senescence for any single cell. In vitro techniques can induce senescence chemically through the induction of persistent DNA damage, however in

vivo senescent cells are very rare – even in an aged animal which is most commonly believed to be plagued by senescent and immunosenescent cells.

Accumulation and propagation of senescent cells has been shown to be pathological, as chemically removing these senescent cells extends lifespan in a model of accelerated aging. However, there remains a physiological reason why certain cells engage senescence as opposed to committing to death. While STING has been shown to be critical for the engagement of senescence, it remains unclear, temporally, when STING is active. It is vital to understand if STING is critical in early, reversible senescence as a cell attempts to respond to stressors or if STING, rather, is engaged late during senescence to drive a chemotactic SASP phenotype to encourage removal of a damaged cell.

The use of senolytics to pharmacologically remove senescent cells has gained significant traction, harnessing the idea that removing senescent cells, specifically those that develop the SASP, would repair tissue function and re-engage the immune system in the aging population. What remains to be understood, critically, is the alternative to the engagement of senescence. Prevention of oncogenesis appears to be a vital role of senescence, but a commitment to cellular death would be equally as effective. It is likely that these cells persist, without a function, for a distinct purpose. While senescence is best linked to aging, there is a clear role for temporal senescence during wound healing. It is intriguing to consider the fact that cells undergo an early senescence-like phenotype to support the controlled expansion of structural tissues.

STING has been implicated in the progression to senescence after DNA damage, but further linked to the progression to SASP. While it is possible that STING is a ubiquitous regulator of senescence and senescence-like phenotypes, it is highly intriguing to consider STING as a regulator of progression through senescence. Senescence is believed, in part, to regulate cellular health and prevent damaged cells

from propagating. In the data presented here, STING was shown to play a distinct but not comprehensive role in the generation of senescence – however was a critical repressor of SASP-associated inflammatory responses. It is possible that STING serves a functional role in the generation or *maintenance* of an early senescence-like phenotype that, with sufficient stress, would progress through later stages of senescence and towards the inflammatory SASP phenotype. While STING may be important for the generation of SASP in highly stressed cells, it may not be as clearly defined in a standard biological system.

In the context of aging, if surrounding cells are unable to sufficiently divide to replace damaged cells (that would otherwise undergo death processes), engagement of senescence is likely the most viable alternative. The structural cell remains with adequate integration with the extracellular matrix and prevents the deposition of completely non-functional fibrosis.

The integral role of senescence is necessary to understand. While pharmacological removal of senescent cells is clearly beneficial, this is often demonstrated during chemical, not biological, engagement of senescent pathways. Prolonged application of senolytics may have broad, unexpected, effects during a protective immune response or wound healing, and an acute bolus of senolytics in an already aged animal could rapidly and aggressively clear more cells than can adequately be replaced, resulting in excessive fibrosis, tissue damage, or mortality.

4.5.14 Lineage-specific deletion of STING

This project was designed around a germ-line deletion of STING signaling to best understand the general activity of STING signaling, as may be influenced by pharmacological interventions. However, while these assays can clearly describe STING-dependent immune regulation of multiple cell types it remains impossible to

determine which cells, if any, are primarily responsible for the observed effects. This project primarily highlights immune dysfunction within tissue macrophages, but more briefly suggests a significant effect of STING signaling within stromal cells. Further, in the absence of STING signaling there are lineage-defining differences between specific myeloid cells suggesting some cell-specific role of STING signaling with unknown regulation.

It remains critical to further understand the role of STING signaling within specific cell types in a biological context to more clearly determine where STING signaling is primarily active during aging, and other chronic inflammatory diseases. As STING is attributed to the engagement of senescence, it is possible that the loss of this senescence engagement is able to be overcome in immune cells if they rather commit to death, as even fetal-derived TRMs can be replaced partially by monocytes; while stromal or parenchymal cells committing to a death pathway results in severe tissue damage and dysfunction if surrounding cells are unable to adequately regenerate their surroundings. Through the specific profiling of cell-specific deletions of STING it would be possible to dissect these lineage-specific defects and better understand the role of STING signaling in health and disease.

4.5.15 Maintenance and function of fetal TRMs

Classically, the immune system is a function of hematopoietic stem cells that give rise to a robust set of highly specialized cells. While primarily described in the context of infections, these cells have been described in response to cellular stress as well as providing growth factors for tissues. More recently has been the discovery of a novel lineage of the immune system that arises during fetal development, completely independent of classical hematopoiesis. This fetal immune system is not simply a bridge until bone marrow hematopoiesis begins but generates cells that can be identified

throughout the life of an organism. Unfortunately, little is known about what roles these fetal-derived cells play throughout the life of the organism or if hematopoietic cells are truly their cellular equivalents.

Tissue resident macrophages are likely the best-described cells of the fetal immune system, and through lineage tracing experiments they have been identified in every organ system as key regulators of tissue homeostasis. However, these fetal-derived cells have been shown to be lost with age and progressively replaced with adult hematopoietic macrophages – yet it is unknown to what extent these cells can functionally replace fetal-derived cells. Currently, the models that exist to track fetal TRMs are best able to identify these cells during early life. While these cells are identifiable through adulthood it remains unclear the extent to which continuous tracing experiments become promiscuous and label hematopoietic cells.

4.5.16 STING in chronic injury models

The experiments described for this body of work detail the regulation of chronic inflammation during aging by STING signaling. It remains to be understood if this inflammatory regulation is an intrinsic response to aging or if STING is a master regulator of chronic inflammatory responses. Age-associated inflammation is believed to exist downstream of the cGAS/STING signaling cascade, however these data suggest a more intricate role for STING that tunes the immune response and prevents secondary pathology that is inherently more detrimental to the system. Most intriguing is the concept that STING is not simply vital for the engagement of permanent growth arrest through senescence, but the tuning of a senescence like phenotype to regulate repair, inflammation, and death.

In the context of aging, these pathways are engaged at a much higher rate than in young healthy adults, yet senescent and dying cells within a tissue remain quite rare.

The initiation of chronic inflammation associated with cell stress, damage, and death, is an increasingly rapid method to study the role of STING in the engagement of these regulatory pathways. Preliminary evidence of a transient ischemic injury of the kidney (renal artery ligation) demonstrated an elevated inflammatory response in the absence of STING signaling (data not included). While a model of chronic inflammation, this response is considerably more acute than the engagement of stress pathways during aging.

A primary readout of this experiment was the relative infiltration of inflammatory (Ly6C⁺) cells to understand the severity of the inflammatory response regulated by STING signaling. Remarkably, the infiltration of Ly6C⁺ cells was heightened in the STING^{KO} kidney demonstrating some conserved role for STING-mediated regulation of inflammation across multiple models. It is possible that in the absence of STING signaling secondary DNA-sensing pathways are more responsive – however since STING is not a direct *sensor* of damaged or mislocalized DNA, competitive inhibition for DNA binding sites may not be greatly affected. In fact, if multiple DNA sensors have the potential to be engaged, this further supports a role of STING directly antagonizing secondary pathways to limit signal propagation.

4.5.17 Regulation of cell autonomous immunity by STING signaling

One aspect of STING that was, and remains, highly intriguing is the consistent expression across all cell types – lymphoid, myeloid, stromal, and parenchymal. As STING is believed to be a robust sensor of DNA in the context of cell damage and infection, evolutionarily it should be a conserved sensor. However, as STING here has been shown to be a key modulator of immune responses with age – and ultimately provides a clear benefit throughout life – the specific role of STING within stromal or parenchymal cells is highly intriguing. The discussion of immunity predominately focuses

on the role of classical cells of the immune system (lymphocytes, neutrophils, macrophages, dendritic cells), considerably less focus on more non-canonical or alternative immune cells (innate lymphocytes, NK cells, other granulocytes), but very little work focuses on canonically “non-immune” cells such as fibroblasts, endothelial, or epithelial cells. Incredibly, in the context of damage or infection, these stromal and parenchymal cells are more likely to be damaged and contact a pathogen before local immune sentinels. As such, robust sensors of pathogens and damage are as essential in these cells as canonical immune cells – albeit with less required sensitivity for the propagation of the response.

This “cell-autonomous immunity” is an intriguing avenue to explore further, especially in the context of aging while a definitive inflammatory ligand remains elusive. Stromal and parenchymal cells retain robust expression of cell-autonomous immune pathways – specifically STING – suggesting that these cells may play a large, if not dominant, role in the regulation of immune responses with age. Fibroblast-specific STING activity is commonly assayed for in the context of interferon responsiveness and proliferative senescence – and through STING signaling these cells likely play a significant role in shaping the tissue-specific immune profile with age.

4.6 Final Conclusion

STING signaling is a powerful mediator of interferon and inflammatory responses downstream of DNA stress, damage, and mislocalization. Here, these data detail an increasingly intricate role of STING signaling that, directly or indirectly, modulates the immune profile of aging across multiple tissues and cell types. Further, I highlight a direct role of STING in the progression of senescence and engagement of cell death in tissue resident macrophages. While STING is highly regarded as a core component of inflammatory engagement with aging, and a prominent target for pharmacologic

inhibition in the course of aging, this project provides significant evidence of its greater role in modulating innate and cell autonomous immune pathways in aging and, more broadly, chronic inflammation.

This immune-modulatory role of STING signaling is an incredibly intriguing aspect of immunity that deserves a considerable amount of consideration in the field of immunology moving forward. The concept that a one pathway drives a considerable amount of pathology is clear, simple, and attractive, however the entirety of the immune response in aging is considerably more complex. Commonly considered simply an antiviral sensor, the data presented here appear to suggest STING should be appreciated almost as a sensor of cellular health. Absent an acute pathological stressor, STING is demonstrated to dampen and restrict pathological inflammatory reactions. Canonically, STING responds to the cyclic-di-nucleotide cGAMP generated by cGAS which seems to recognize mislocalized DNA. Recent work, however, has uncovered a mechanism by which cGAMP can be actively transported and internalized to activate STING. This intercellular communication potential could be a valuable method to initiate antiviral responses in neighboring cells – but the interferon system is just as capable of driving these responses.

Rather, it is intriguing to consider STING as a regulator of cellular health. Many cellular signaling cascades exist and function in direct opposition with each other, requiring sufficiently consistent activation in order to outcompete each other. Understood to be a component of the cytosolic DNA sensing pathway, cGAS/STING functions parallel to the AIM2 cascade that promotes the highly inflammatory inflammasome. These two pathways may be redundant. But they may act, rather, in opposition. STING may provide direct opposition to the generation of the inflammasome to promote cell survival (or non-inflammatory cell death). Here, the absence of STING generates robust inflammation and predisposes TRMs to inflammatory death, curiously reminiscent of

AIM2-dependent responses. At steady-state, STING may serve not to actively support homeostasis but to passively dampen secondary pathology.

Although the general understanding of STING signaling is rapidly progressing, there is still a severe lack of understanding in the breadth of stimuli that may, in fact, activate this pathway. Further, STING appears to have unique functional roles in specific cell types. Tissue resident macrophages specifically are central to the health and function of multiple organ systems but are incompletely understood and vastly underappreciated. Critically, these data highlight a vital intersection of two incompletely understood biological systems with rapidly emerging importance in the regulation of health and disease; the dysfunction of which is shown to be excessively pathological through multiple inflammatory stressors. There are few pathways that deserve a more comprehensive understanding than the full integration of STING and tissue resident macrophages in cellular and organismal health.

Chapter 5: Bibliography

1. Mark Mather, L.A.J., and Kelvin M. Pollard (2015). Aging in the United States. *Population Bulletin* 70.
2. Woodcox, A. (2019). Aristotle's Theory of Aging. *Cahiers des études anciennes*, 65-78.
3. Seguchi, M. (2007). Soul and Ageing : The Views of Plato and Aristotle on Old Age. *Journal of Classical Studies* 55, 63-75. 10.20578/jclst.55.0_63.
4. Aristotle On Longevity and Shortness of Life. *Parva Naturalia*.
5. Franceschi, C., Bonafe, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., and De Benedictis, G. (2000). Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci* 908, 244-254. 10.1111/j.1749-6632.2000.tb06651.x.
6. Lopez-Otin, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2023). Hallmarks of aging: An expanding universe. *Cell* 186, 243-278. 10.1016/j.cell.2022.11.001.
7. Singh, T., and Newman, A.B. (2011). Inflammatory markers in population studies of aging. *Ageing Res Rev* 10, 319-329. 10.1016/j.arr.2010.11.002.
8. Ershler, W.B. (1993). Interleukin-6: a cytokine for gerontologists. *J Am Geriatr Soc* 41, 176-181. 10.1111/j.1532-5415.1993.tb02054.x.
9. Ahadi, S., Zhou, W., Schussler-Fiorenza Rose, S.M., Sailani, M.R., Contrepois, K., Avina, M., Ashland, M., Brunet, A., and Snyder, M. (2020). Personal aging markers and ageotypes revealed by deep longitudinal profiling. *Nat Med* 26, 83-90. 10.1038/s41591-019-0719-5.
10. Divo, M.J., Martinez, C.H., and Mannino, D.M. (2014). Ageing and the epidemiology of multimorbidity. *Eur Respir J* 44, 1055-1068. 10.1183/09031936.00059814.
11. Garmany, A., Yamada, S., and Terzic, A. (2021). Longevity leap: mind the healthspan gap. *NPJ Regen Med* 6, 57. 10.1038/s41536-021-00169-5.
12. Finch, C.E., and Pike, M.C. (1996). Maximum life span predictions from the Gompertz mortality model. *J Gerontol A Biol Sci Med Sci* 51, B183-194. 10.1093/gerona/51a.3.b183.
13. Lopez-Otin, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. *Cell* 153, 1194-1217. 10.1016/j.cell.2013.05.039.
14. Krausgruber, T., Fortelny, N., Fife-Gernedl, V., Senekowitsch, M., Schuster, L.C., Lercher, A., Nemc, A., Schmidl, C., Rendeiro, A.F., Bergthaler, A., and Bock, C. (2020). Structural cells are key regulators of organ-specific immune responses. *Nature* 583, 296-302. 10.1038/s41586-020-2424-4.
15. Aging and Immune Function: Molecular Mechanisms to Interventions. (2011). *Antioxidants & Redox Signaling* 14, 1551-1585. 10.1089/ars.2010.3228.
16. Blasimme, A., Boniolo, G., and Nathan, M.J. (2021). Rethinking ageing: introduction. *Hist Philos Life Sci* 43, 95. 10.1007/s40656-021-00446-y.
17. Boraschi, D., Aguado, M.T., Dutel, C., Goronzy, J., Louis, J., Grubeck-Loebenstien, B., Rappuoli, R., and Del Giudice, G. (2013). The gracefully aging immune system. *Sci Transl Med* 5, 185ps188. 10.1126/scitranslmed.3005624.
18. Campisi, J., Kapahi, P., Lithgow, G.J., Melov, S., Newman, J.C., and Verdin, E. (2019). From discoveries in ageing research to therapeutics for healthy ageing. *Nature* 571, 183-192. 10.1038/s41586-019-1365-2.
19. Shaw, A.C., Goldstein, D.R., and Montgomery, R.R. (2013). Age-dependent dysregulation of innate immunity. *Nature Reviews Immunology* 13, 875-887. 10.1038/nri3547.

20. Gould, L., Abadir, P., Brem, H., Carter, M., Conner-Kerr, T., Davidson, J., DiPietro, L., Falanga, V., Fife, C., Gardner, S., et al. (2015). Chronic wound repair and healing in older adults: current status and future research. *Wound Repair Regen* 23, 1-13. 10.1111/wrr.12245.
21. Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., Abraham, J., Adair, T., Aggarwal, R., Ahn, S.Y., et al. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380, 2095-2128. 10.1016/S0140-6736(12)61728-0.
22. Medzhitov, R., and Janeway, C. (2000). Innate Immunity. *New England Journal of Medicine* 343, 338-344. 10.1056/nejm200008033430506.
23. Paludan, S.R., Pradeu, T., Masters, S.L., and Mogensen, T.H. (2021). Constitutive immune mechanisms: mediators of host defence and immune regulation. *Nature Reviews Immunology* 21, 137-150. 10.1038/s41577-020-0391-5.
24. Mostafavi, S., Yoshida, H., Moodley, D., LeBoité, H., Rothamel, K., Raj, T., Ye, C.J., Chevrier, N., Zhang, S.Y., Feng, T., et al. (2016). Parsing the Interferon Transcriptional Network and Its Disease Associations. *Cell* 164, 564-578. 10.1016/j.cell.2015.12.032.
25. Uderhardt, S., Martins, A.J., Tsang, J.S., Lammermann, T., and Germain, R.N. (2019). Resident Macrophages Cloak Tissue Microlesions to Prevent Neutrophil-Driven Inflammatory Damage. *Cell* 177, 541-555 e517. 10.1016/j.cell.2019.02.028.
26. Bonilla, F.A., and Oettgen, H.C. (2010). Adaptive immunity. *J Allergy Clin Immunol* 125, S33-40. 10.1016/j.jaci.2009.09.017.
27. Adams, N.M., Grassmann, S., and Sun, J.C. (2020). Clonal expansion of innate and adaptive lymphocytes. *Nature Reviews Immunology* 20, 694-707. 10.1038/s41577-020-0307-4.
28. Ivashkiv, L.B., and Donlin, L.T. (2014). Regulation of type I interferon responses. *Nat Rev Immunol* 14, 36-49. 10.1038/nri3581.
29. Galani, I.E., Koltsida, O., and Andreakos, E. (2015). Type III interferons (IFNs): Emerging Master Regulators of Immunity. *Adv Exp Med Biol* 850, 1-15. 10.1007/978-3-319-15774-0_1.
30. Chen, K., Liu, J., and Cao, X. (2017). Regulation of type I interferon signaling in immunity and inflammation: A comprehensive review. *J Autoimmun* 83, 1-11. 10.1016/j.jaut.2017.03.008.
31. Wells, A.I., and Coyne, C.B. (2018). Type III Interferons in Antiviral Defenses at Barrier Surfaces. *Trends Immunol* 39, 848-858. 10.1016/j.it.2018.08.008.
32. Klinkhammer, J., Schnepf, D., Ye, L., Schwaderlapp, M., Gad, H.H., Hartmann, R., Garcin, D., Mahlaköiv, T., and Staeheli, P. (2018). IFN- λ prevents influenza virus spread from the upper airways to the lungs and limits virus transmission. *Elife* 7. 10.7554/eLife.33354.
33. Wack, A., Terczyńska-Dyla, E., and Hartmann, R. (2015). Guarding the frontiers: the biology of type III interferons. *Nature Immunology* 16, 802-809. 10.1038/ni.3212.
34. Gough, D.J., Messina, N.L., Clarke, C.J., Johnstone, R.W., and Levy, D.E. (2012). Constitutive type I interferon modulates homeostatic balance through tonic signaling. *Immunity* 36, 166-174. 10.1016/j.immuni.2012.01.011.
35. Okude, H., Ori, D., and Kawai, T. (2021). Signaling Through Nucleic Acid Sensors and Their Roles in Inflammatory Diseases. *Frontiers in Immunology* 11. 10.3389/fimmu.2020.625833.

36. Zahid, A., Ismail, H., Li, B., and Jin, T. (2020). Molecular and Structural Basis of DNA Sensors in Antiviral Innate Immunity. *Frontiers in Immunology* 11. 10.3389/fimmu.2020.613039.
 37. Al Khatib, I., Deng, J., Lei, Y., Torres-Odio, S., Rojas, G.R., Newman, L.E., Chung, B.K., Symes, A., Zhang, H., Huang, S.-y.N., et al. (2023). Activation of the cGAS-STING innate immune response in cells with deficient mitochondrial topoisomerase TOP1MT. *Human Molecular Genetics* 32, 2422-2440. 10.1093/hmg/ddad062.
 38. Sladitschek-Martens, H.L., Guarnieri, A., Brumana, G., Zanconato, F., Battilana, G., Xiccato, R.L., Panciera, T., Forcato, M., Bicciato, S., Guzzardo, V., et al. (2022). YAP/TAZ activity in stromal cells prevents ageing by controlling cGAS-STING. *Nature* 607, 790-798. 10.1038/s41586-022-04924-6.
 39. Cohen, B.H., Chinnery, P.F., and Copeland, W.C. (1993). POLG-Related Disorders. In *GeneReviews*(®), M.P. Adam, J. Feldman, G.M. Mirzaa, R.A. Pagon, S.E. Wallace, L.J.H. Bean, K.W. Gripp, and A. Amemiya, eds. (University of Washington, Seattle)
- Copyright © 1993-2023, University of Washington, Seattle. GeneReviews is a registered trademark of the University of Washington, Seattle. All rights reserved.).
40. Gulen, M.F., Samson, N., Keller, A., Schwabenland, M., Liu, C., Gluck, S., Thacker, V.V., Favre, L., Mangeat, B., Kroese, L.J., et al. (2023). cGAS-STING drives ageing-related inflammation and neurodegeneration. *Nature*. 10.1038/s41586-023-06373-1.
 41. Patel, S., and Jin, L. (2019). TMEM173 variants and potential importance to human biology and disease. *Genes & Immunity* 20, 82-89. 10.1038/s41435-018-0029-9.
 42. Yi, G., Brendel, V.P., Shu, C., Li, P., Palanathan, S., and Cheng Kao, C. (2013). Single Nucleotide Polymorphisms of Human STING Can Affect Innate Immune Response to Cyclic Dinucleotides. *PLOS ONE* 8, e77846. 10.1371/journal.pone.0077846.
 43. Kennedy, R.B., Haralambieva, I.H., Ovsyannikova, I.G., Voigt, E.A., Larrabee, B.R., Schaid, D.J., Zimmermann, M.T., Oberg, A.L., and Poland, G.A. (2020). Polymorphisms in STING Affect Human Innate Immune Responses to Poxviruses. *Frontiers in Immunology* 11. 10.3389/fimmu.2020.567348.
 44. Froelich, G., Finizio, A., Napolano, A., Amiranda, S., De Chiara, A., Pagano, P., Mallardo, M., Leoni, G., Zambrano, N., and Sasso, E. (2023). The common H232 STING allele shows impaired activities in DNA sensing, susceptibility to viral infection, and in monocyte cell function, while the HAQ variant possesses wild-type properties. *Scientific Reports* 13, 19541. 10.1038/s41598-023-46830-5.
 45. Jin, L., Xu, L.g., Yang, I.V., Davidson, E.J., Schwartz, D.A., Wurfel, M.M., and Cambier, J.C. (2011). Identification and characterization of a loss-of-function human MPYS variant. *Genes & Immunity* 12, 263-269. 10.1038/gene.2010.75.
 46. Regulski, M.J. (2017). Cellular Senescence: What, Why, and How. *Wounds* 29, 168-174.
 47. Huang, W., Hickson, L.J., Eirin, A., Kirkland, J.L., and Lerman, L.O. (2022). Cellular senescence: the good, the bad and the unknown. *Nat Rev Nephrol* 18, 611-627. 10.1038/s41581-022-00601-z.
 48. Gasek, N.S., Kuchel, G.A., Kirkland, J.L., and Xu, M. (2021). Strategies for Targeting Senescent Cells in Human Disease. *Nat Aging* 1, 870-879. 10.1038/s43587-021-00121-8.

49. Andrade, A.M., Sun, M., Gasek, N.S., Hargis, G.R., Sharafieh, R., and Xu, M. (2022). Role of Senescent Cells in Cutaneous Wound Healing. *Biology (Basel)* **11**. 10.3390/biology11121731.
50. Wang, B., Kohli, J., and Demaria, M. (2020). Senescent Cells in Cancer Therapy: Friends or Foes? *Trends Cancer* **6**, 838-857. 10.1016/j.trecan.2020.05.004.
51. van Deursen, J.M. (2014). The role of senescent cells in ageing. *Nature* **509**, 439-446. 10.1038/nature13193.
52. Gordon, S. (2008). Elie Metchnikoff: father of natural immunity. *Eur J Immunol* **38**, 3257-3264. 10.1002/eji.200838855.
53. Nahrendorf, M., and Swirski, F.K. (2016). Abandoning M1/M2 for a Network Model of Macrophage Function. *Circ Res* **119**, 414-417. 10.1161/CIRCRESAHA.116.309194.
54. Martinez, F.O., and Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* **6**, 13. 10.12703/P6-13.
55. Orecchioni, M., Ghosheh, Y., Pramod, A.B., and Ley, K. (2019). Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS-) vs. Alternatively Activated Macrophages. *Front Immunol* **10**, 1084. 10.3389/fimmu.2019.01084.
56. Bain, C.C., Hawley, C.A., Garner, H., Scott, C.L., Schridde, A., Steers, N.J., Mack, M., Joshi, A., Williams, M., Mowat, A.M., et al. (2016). Long-lived self-renewing bone marrow-derived macrophages displace embryo-derived cells to inhabit adult serous cavities. *Nat Commun* **7**, ncomms11852. 10.1038/ncomms11852.
57. Hoeffel, G., Chen, J., Lavin, Y., Low, D., Almeida, F.F., See, P., Beaudin, A.E., Lum, J., Low, I., Forsberg, E.C., et al. (2015). C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity* **42**, 665-678. 10.1016/j.immuni.2015.03.011.
58. Hoeffel, G., and Ginhoux, F. (2015). Ontogeny of Tissue-Resident Macrophages. *Front Immunol* **6**, 486. 10.3389/fimmu.2015.00486.
59. Gomez Perdiguero, E., and Geissmann, F. (2013). Myb-independent macrophages: a family of cells that develops with their tissue of residence and is involved in its homeostasis. *Cold Spring Harb Symp Quant Biol* **78**, 91-100. 10.1101/sqb.2013.78.020032.
60. Mass, E., Nimmerjahn, F., Kierdorf, K., and Schlitzer, A. (2023). Tissue-specific macrophages: how they develop and choreograph tissue biology. *Nature Reviews Immunology*. 10.1038/s41577-023-00848-y.
61. Ginhoux, F., and Williams, M. (2016). Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity* **44**, 439-449. 10.1016/j.immuni.2016.02.024.
62. Hirayama, D., Iida, T., and Nakase, H. (2017). The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. *Int J Mol Sci* **19**. 10.3390/ijms19010092.
63. Mosser, D.M., Hamidzadeh, K., and Goncalves, R. (2021). Macrophages and the maintenance of homeostasis. *Cell Mol Immunol* **18**, 579-587. 10.1038/s41423-020-00541-3.
64. Mass, E., Nimmerjahn, F., Kierdorf, K., and Schlitzer, A. (2023). Tissue-specific macrophages: how they develop and choreograph tissue biology. *Nat Rev Immunol* **23**, 563-579. 10.1038/s41577-023-00848-y.
65. Nobs, S.P., and Kopf, M. (2021). Tissue-resident macrophages: guardians of organ homeostasis. *Trends Immunol* **42**, 495-507. 10.1016/j.it.2021.04.007.
66. Lipsick, J.S. (1996). One billion years of Myb. *Oncogene* **13**, 223-235.

67. Schulz, C., Gomez Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S.E., Pollard, J.W., et al. (2012). A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* 336, 86-90. 10.1126/science.1219179.
68. Eskenasy, A. (1972). [Morphogenesis and significance of alveolar macrophage reactions]. *Rev Roum Med Intern* 9, 3-13.
69. Smith, S.J., and Mohun, T.J. (2011). Early cardiac morphogenesis defects caused by loss of embryonic macrophage function in *Xenopus*. *Mech Dev* 128, 303-315. 10.1016/j.mod.2011.04.002.
70. Hulsmans, M., Clauss, S., Xiao, L., Aguirre, A.D., King, K.R., Hanley, A., Hucker, W.J., Wulfers, E.M., Seemann, G., Courties, G., et al. (2017). Macrophages Facilitate Electrical Conduction in the Heart. *Cell* 169, 510-522 e520. 10.1016/j.cell.2017.03.050.
71. Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W.M., 3rd, Hao, Y., Stoeckius, M., Smibert, P., and Satija, R. (2019). Comprehensive Integration of Single-Cell Data. *Cell* 177, 1888-1902 e1821. 10.1016/j.cell.2019.05.031.
72. Korsunsky, I., Millard, N., Fan, J., Slowikowski, K., Zhang, F., Wei, K., Baglaenko, Y., Brenner, M., Loh, P.R., and Raychaudhuri, S. (2019). Fast, sensitive and accurate integration of single-cell data with Harmony. *Nat Methods* 16, 1289-1296. 10.1038/s41592-019-0619-0.
73. Akama-Garren, E.H., van den Broek, T., Simoni, L., Castrillon, C., van der Poel, C.E., and Carroll, M.C. (2021). Follicular T cells are clonally and transcriptionally distinct in B cell-driven mouse autoimmune disease. *Nat Commun* 12, 6687. 10.1038/s41467-021-27035-8.
74. van Dijk, D., Sharma, R., Nainys, J., Yim, K., Kathail, P., Carr, A.J., Burdziak, C., Moon, K.R., Chaffer, C.L., Pattabiraman, D., et al. (2018). Recovering Gene Interactions from Single-Cell Data Using Data Diffusion. *Cell* 174, 716-729 e727. 10.1016/j.cell.2018.05.061.
75. Becht, E., McInnes, L., Healy, J., Dutertre, C.-A., Kwok, I.W.H., Ng, L.G., Ginhoux, F., and Newell, E.W. (2019). Dimensionality reduction for visualizing single-cell data using UMAP. *Nature Biotechnology* 37, 38-44. 10.1038/nbt.4314.
76. Melville, L.M.a.J.H.a.J. (2020). UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction. *arXiv*.
77. Browaeys, R., Saelens, W., and Saeys, Y. (2020). NicheNet: modeling intercellular communication by linking ligands to target genes. *Nat Methods* 17, 159-162. 10.1038/s41592-019-0667-5.
78. Hanzelmann, S., Castelo, R., and Guinney, J. (2013). GSVA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics* 14, 7. 10.1186/1471-2105-14-7.
79. Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43, e47. 10.1093/nar/gkv007.
80. Baccin, C., Al-Sabah, J., Velten, L., Helbling, P.M., Grünschläger, F., Hernández-Malmierca, P., Nombela-Arrieta, C., Steinmetz, L.M., Trumpp, A., and Haas, S. (2020). Combined single-cell and spatial transcriptomics reveal the molecular, cellular and spatial bone marrow niche organization. *Nat Cell Biol* 22, 38-48. 10.1038/s41556-019-0439-6.
81. Kanehisa, M., and Goto, S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Research* 28, 27-30. 10.1093/nar/28.1.27.

82. Kanehisa, M., Furumichi, M., Sato, Y., Kawashima, M., and Ishiguro-Watanabe, M. (2023). KEGG for taxonomy-based analysis of pathways and genomes. *Nucleic Acids Res* 51, D587-D592. 10.1093/nar/gkac963.
83. Supek, F., Bosnjak, M., Skunca, N., and Smuc, T. (2011). REVIGO summarizes and visualizes long lists of gene ontology terms. *PLoS One* 6, e21800. 10.1371/journal.pone.0021800.
84. Van Gassen, S., Callebaut, B., Van Helden, M.J., Lambrecht, B.N., Demeester, P., Dhaene, T., and Saeys, Y. (2015). FlowSOM: Using self-organizing maps for visualization and interpretation of cytometry data. *Cytometry A* 87, 636-645. 10.1002/cyto.a.22625.
85. Kang, J.A., and Jeon, Y.J. (2020). Emerging Roles of USP18: From Biology to Pathophysiology. *Int J Mol Sci* 21. 10.3390/ijms21186825.
86. Zhang, H., Wang, D., Zhong, H., Luo, R., Shang, M., Liu, D., Chen, H., Fang, L., and Xiao, S. (2015). Ubiquitin-specific Protease 15 Negatively Regulates Virus-induced Type I Interferon Signaling via Catalytically-dependent and -independent Mechanisms. *Scientific Reports* 5, 11220. 10.1038/srep11220.
87. Sarasin-Filipowicz, M., Wang, X., Yan, M., Duong, F.H., Poli, V., Hilton, D.J., Zhang, D.E., and Heim, M.H. (2009). Alpha interferon induces long-lasting refractoriness of JAK-STAT signaling in the mouse liver through induction of USP18/UBP43. *Mol Cell Biol* 29, 4841-4851. 10.1128/MCB.00224-09.
88. Yoshimura, A., Naka, T., and Kubo, M. (2007). SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol* 7, 454-465. 10.1038/nri2093.
89. Obajemu, A.A., Rao, N., Dilley, K.A., Vargas, J.M., Sheikh, F., Donnelly, R.P., Shabman, R.S., Meissner, E.G., Prokunina-Olsson, L., and Onabajo, O.O. (2017). IFN-lambda4 Attenuates Antiviral Responses by Enhancing Negative Regulation of IFN Signaling. *J Immunol* 199, 3808-3820. 10.4049/jimmunol.1700807.
90. Sharma, S., Campbell, A.M., Chan, J., Schattgen, S.A., Orlowski, G.M., Nayar, R., Huyler, A.H., Nündel, K., Mohan, C., Berg, L.J., et al. (2015). Suppression of systemic autoimmunity by the innate immune adaptor STING. *Proc Natl Acad Sci U S A* 112, E710-717. 10.1073/pnas.1420217112.
91. Puranik, A.S., Leaf, I.A., Jensen, M.A., Hedayat, A.F., Saad, A., Kim, K.W., Saadalla, A.M., Woollard, J.R., Kashyap, S., Textor, S.C., et al. (2018). Kidney-resident macrophages promote a proangiogenic environment in the normal and chronically ischemic mouse kidney. *Sci Rep* 8, 13948. 10.1038/s41598-018-31887-4.
92. Gomez Perdiguero, E., Klapproth, K., Schulz, C., Busch, K., Azzoni, E., Crozet, L., Garner, H., Trouillet, C., de Bruijn, M.F., Geissmann, F., and Rodewald, H.R. (2015). Tissue-resident macrophages originate from yolk-sac-derived erythromyeloid progenitors. *Nature* 518, 547-551. 10.1038/nature13989.
93. Epelman, S., Lavine, K.J., and Randolph, G.J. (2014). Origin and functions of tissue macrophages. *Immunity* 41, 21-35. 10.1016/j.immuni.2014.06.013.
94. Stamatziades, E.G., Tremblay, M.E., Bohm, M., Crozet, L., Bisht, K., Kao, D., Coelho, C., Fan, X., Yewdell, W.T., Davidson, A., et al. (2016). Immune Monitoring of Trans-endothelial Transport by Kidney-Resident Macrophages. *Cell* 166, 991-1003. 10.1016/j.cell.2016.06.058.
95. Arnold, L.W., and Lannigan, J. (2010). Practical issues in high-speed cell sorting. *Curr Protoc Cytom Chapter 1, Unit 1* 24 21-30. 10.1002/0471142956.cy0124s51.

96. Shokolenko, I.N., Wilson, G.L., and Alexeyev, M.F. (2014). Aging: A mitochondrial DNA perspective, critical analysis and an update. *World J Exp Med* 4, 46-57. 10.5493/wjem.v4.i4.46.
97. Lima-Junior, D.S., Krishnamurthy, S.R., Bouladoux, N., Collins, N., Han, S.J., Chen, E.Y., Constantinides, M.G., Link, V.M., Lim, A.I., Enamorado, M., et al. (2021). Endogenous retroviruses promote homeostatic and inflammatory responses to the microbiota. *Cell* 184, 3794-3811.e3719. 10.1016/j.cell.2021.05.020.
98. Mu, X., Ahmad, S., and Hur, S. (2016). Endogenous Retroelements and the Host Innate Immune Sensors. *Adv Immunol* 132, 47-69. 10.1016/bs.ai.2016.07.001.
99. Volkman, H.E., Cambier, S., Gray, E.E., and Stetson, D.B. (2019). Tight nuclear tethering of cGAS is essential for preventing autoreactivity. *eLife* 8, e47491. 10.7554/eLife.47491.
100. Zhao, B., Xu, P., Rowlett, C.M., Jing, T., Shinde, O., Lei, Y., West, A.P., Liu, W.R., and Li, P. (2020). The molecular basis of tight nuclear tethering and inactivation of cGAS. *Nature* 587, 673-677. 10.1038/s41586-020-2749-z.
101. Jenal, U., Reinders, A., and Lori, C. (2017). Cyclic di-GMP: second messenger extraordinaire. *Nature Reviews Microbiology* 15, 271-284. 10.1038/nrmicro.2016.190.
102. Cheng, X., Ning, J., Xu, X., and Zhou, X. (2022). The role of bacterial cyclic di-adenosine monophosphate in the host immune response. *Front Microbiol* 13, 958133. 10.3389/fmicb.2022.958133.
103. Aliakbar Tehrani, Z., Rulíšek, L., and Černý, J. (2022). Molecular dynamics simulations provide structural insight into binding of cyclic dinucleotides to human STING protein. *Journal of Biomolecular Structure and Dynamics* 40, 10250-10264. 10.1080/07391102.2021.1942213.
104. Parvatiyar, K., Zhang, Z., Teles, R.M., Ouyang, S., Jiang, Y., Iyer, S.S., Zaver, S.A., Schenk, M., Zeng, S., Zhong, W., et al. (2012). The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response. *Nat Immunol* 13, 1155-1161. 10.1038/ni.2460.
105. Pesavento, C., and Hengge, R. (2009). Bacterial nucleotide-based second messengers. *Current Opinion in Microbiology* 12, 170-176. <https://doi.org/10.1016/j.mib.2009.01.007>.
106. Li, Z., Zhang, X., Cheng, L., Xu, X., Zhou, X., Wu, H., and Peng, X. (2019). Signal Transduction of Streptococci by Cyclic Dinucleotide Second Messengers. *Curr Issues Mol Biol* 32, 87-122. 10.21775/cimb.032.087.
107. Newton, A.C., Bootman, M.D., and Scott, J.D. (2016). Second Messengers. *Cold Spring Harb Perspect Biol* 8. 10.1101/cshperspect.a005926.
108. Fitzgerald-Bocarsly, P., Dai, J., and Singh, S. (2008). Plasmacytoid dendritic cells and type I IFN: 50 years of convergent history. *Cytokine Growth Factor Rev* 19, 3-19. 10.1016/j.cytogfr.2007.10.006.
109. Bencze, D., Fekete, T., and Pázmándi, K. (2021). Type I Interferon Production of Plasmacytoid Dendritic Cells under Control. *Int J Mol Sci* 22. 10.3390/ijms22084190.