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Understanding immune-aging in myeloma and its precursor states

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Doctor of Philosophy

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Understanding immune-aging in myeloma and its precursor states

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M.S., Northeastern University, 2017
B.Pharm, University of Pune, 2015

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in the Graduate Division of Biological and Biomedical Science
in Cancer Biology
2025

Abstract

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By Sayalee V. Potdar

Myeloma and its precursor monoclonal gammopathy (MGUS) are more common in individuals with Black ancestry and associated with immune dysfunction. Mechanisms linking immune dysfunction to racial ancestry or malignant transformation remain unclear. Here we show that blood/marrow T cells from myeloma patients exhibit enhanced capacity for inflammatory cytokine production and proliferation including in cells with phenotypes previously linked to T-cell exhaustion. In chronologically age-matched cohorts, myeloma patients have greater aging-associated immune changes compared to MGUS patients. MGUS patients with Black ancestry exhibit greater immune- aging compared to White counterparts. Myeloma T cells exhibit altered inflammatory phospho-proteomic signaling and was associated with distinct transcriptional profiles of immune and tumor cells in the bone marrow. Immune-aging correlated with responses to SARS CoV-2 vaccination, providing a correlation between immune aging and immune function in vivo. Maladaptive immune-aging may underlie racial predisposition and impact malignant transformation and immune responses in MM.

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Acknowledgements

I have learned so much during this Ph.D. and this would not be possible without the support, encouragement, and patience of all those stewarding me through this journey.

First, I would like to thank my doctoral advisors, Dr Kavita Dhodapkar, and Dr Madhav Dhodapkar, whose expertise, mentorship and support have shaped this degree. Thank you for challenging me to be a better scientist, and for the opportunities you have provided. I am especially grateful to my primary advisor, Kavita, for her open-door mentorship. I would also like to thank my doctoral committee Dr. Haydn Kissick, Dr. Larry Boise, and Dr. Greg Lesinski for their rigorous and essential feedback and support. I learned so much during and because of our meetings.

I feel fortunate and grateful to every member of the Dhodapkar lab for their support, collaboration, and friendship; this work would not have been possible without them. A special mention to Alyssa and Sam, thank you for being the best fellow-grad buddies I could have asked for and for teaching me the ropes - all the scientific discussions and non-scientific banter got us through. I would like to thank all the clinical collaborators and the cores at Emory, especially the Immune Monitoring Shared Resource, Flow Cytometry Core and the EPC Genomics Core.

I would like to thank all my friends in Atlanta and at Emory. During my time in the city, I made friends who I have come to see as family. I would like to thank all my friends at Emory, especially Maggie, for her unwavering support and much needed laughter during our countless hangouts. This journey would be also impossible without the support and comradery of Ewelina and Rebecca. Outside of Emory, I would like to thank Niru, Harsh, Rishi, Kanchan, Tarun, Molly and all my comedy friends who have made my life outside of lab so rich. I would like to thank all my Boston friends, who were my first home away from home. To my friends at Dana-Farber, especially Julia Frede, Val Dimitrova and Tushara, who have been instrumental in the scientist that I am today. To Deepa and Siddharth, for always cheering me on.

To all my Pune friends, who have encouraged and supported me all through my life, so it is no surprise that they did the same during this journey. To Soham, Sanjana, Aneesha, Krishna, Kirit and Raunak, I am forever grateful for your endless support and friendship. I cannot express enough gratitude to my closest friends- Pallavi, Niharika and Sneha. Thank you for always being my biggest cheerleaders through moments of self-doubt, for comic-relief and for all the love and patience.

Finally, to my wonderful family- your love, support and endless encouragement has meant the world, especially on the toughest days. I would like to thank my parents, Sabina and Vikram, for always believing in me and for always providing a safe space for all that I needed, even if it was through terrible humor. I would not be where I am today without all that you have done for me and continue to do. I would like to thank my brother Shaunak, my best friend for life, for always having my back. I would also like to thank my cousin Ritika and uncle Salil for their support. Lastly, I would like to thank my grandfather, Pappa, I hope I have made you proud.

And to all the patients, whose strength and resilience make research possible and rewarding.

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List of Abbreviations

AAIS	Age-associated immune score
APC	antigen presenting cell
ASCT	autologous haemopoietic stem cell transplantation
BCMA	B-cell maturation antigen
BM	bone marrow
BMMCs	bone marrow mononuclear cells
CAR	Chimeric antigen receptor
CCR7	C-C chemokine receptor type 7
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CD16	Cluster of Differentiation 16
CD28	Cluster of Differentiation 28
CD226	Cluster of Differentiation 226
CD45RA	Cluster of Differentiation RA
CD45RO	Cluster of Differentiation RO
CD127	Cluster of Differentiation 127
CD138	Cluster of Differentiation 138
CITE-seq	Cellular indexing of transcriptomes and epitopes sequencing
CFSE	Carboxyfluorescein succinimidyl ester
CTLA4	cytotoxic T-lymphocyte associated protein 4
CyTOF	cytometry by time of flight

DC	dendritic cell
DEG	differentially expressed gene
DSB	Double stranded breaks
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
flowSOM	flow self-organizing map
GAM	generalized additive models
GM-CSF	granulocyte-macrophage stimulating factor
GSEA	gene set enrichment analysis
hCD45	human Cluster of Differentiation 45
HC	healthy controls
HD	healthy donor
HSPCs	hematopoietic stem/progenitor cells
IFN-a	interferon alpha
IFNg	interferon gamma
Ig	Immunoglobulin
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
IL-17	interleukin-17
KLRG1	killer cell lectin-like receptor G1
Lag-3	lymphocyte-activation gene 3

LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
MC	metacluster
MGUS	Monoclonal Gammopathy of Unknown Significance
MHC	major histocompatibility complex
MISTRG6	M-CSF$^{h/h}$ IL-3/GM-CSF$^{h/h}$ SIRPa$^{h/h}$ TPO$^{h/h}$ RAG2$^{-/-}$ IL2Rg$^{-/-}$ IL6$^{h/h}$
MM	Multiple Myeloma
MSigDB	Molecular Signature Database
NDMM	newly-diagnosed multiple myeloma
NK	natural killer
PCA	Principal component analysis
PB	Peripheral blood
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PMA	phorbol-12-myristate-13-acetate
RBD	receptor-binding domain
sFLC	Serum free light chain
SMM	Smoldering Myeloma
Tbet	T-box expressed in T cell
Tcm	central memory T cell
TCE	T-cell engager

TCR	T cell receptor
Tem	effector memory T cell
Th1	type 1 T helper cell
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TME	tumor microenvironment
TNF-α	tumor necrosis factor 1
t-SNE	t-distributed stochastic neighbor embedding
UMAP	uniform manifold approximation and projection
UMI	Unique Molecular Identifiers

Chapter 1: Introduction

1.1 Myeloma and evolution from precursor states

Cancer has a major impact on human health and is a leading cause of death worldwide. In 2024, there were nearly 2 million new cases diagnosed in the United States alone, with about 0.6 million dying from the disease. Multiple myeloma (MM), which is the focus of this dissertation, had about 36,000 new cases in 2024, and contributed to 1.8% of all new cancer cases and 2% of all cancer deaths, with a 60% 5-year survival rate (1).

Multiple myeloma (MM) is a hematological lymphoid malignancy, which remains incurable, is the second most common hematological malignancy after leukemia and its development is a multistep process that begins in bone marrow (BM) (2). Hematopoietic stem cells undergo differentiation in the BM and secondary lymphoid organs and become B cells, and eventually differentiate into plasma cells. Normal plasma cells in the BM can undergo genetic alterations such as chromosomal translocations and progress to a premalignancy clonal asymptomatic disorder called Monoclonal gammopathy of undetermined significance (MGUS) (2-4). MGUS is heterogenous, much like MM, and is classified based on the involved immunoglobulin (M-protein): non–immunoglobulin M (IgM) such as IgG and IgA, IgM, and light chain, and can progress to MM at a rate of 1% per annum (5). MGUS consistently precedes the development of MM, with or without an identified intervening stage, referred to as smoldering multiple myeloma (SMM) (6, 7)

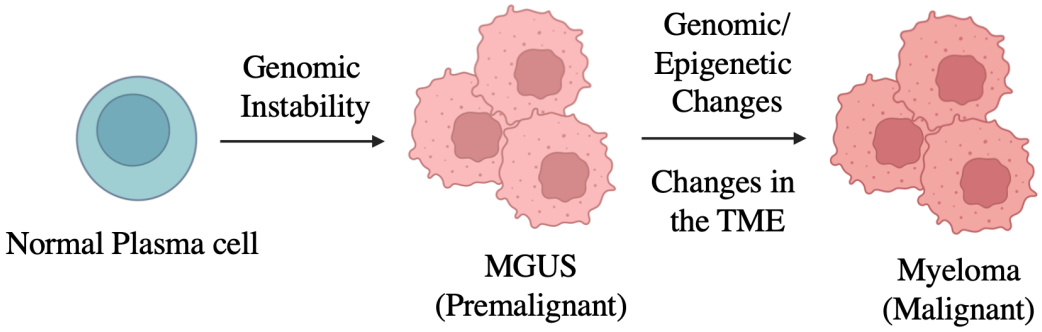

Figure 1.1: Evolution of a plasma cell to a myeloma cell. A schematic showing the multistep progression of a normal plasma cell in a human bone marrow undergoing genomic instability events and progressing to a premalignant stage known as MGUS. These MGUS cells further undergo genetic and epigenetic changes as well as external changes in the tumor microenvironment (TME) to ultimately progress to the malignant myeloma stage.

The International Myeloma Working Group (IMWG) has defined that the clinical diagnosis of MM requires the presence of one or more myeloma-defining events (MDEs) in addition to evidence of 10% or more clonal plasma cells. MDE consists of CRAB features (Hyper**C**alcemia, **R**enal insufficiency, **A**nemia, or lytic **B**one lesions) and three specific biomarkers: clonal bone marrow plasma cells of 60% or higher, serum free light chain (FLC) ratio of 100 or higher (provided involved FLC level is ≥100 mg/L), and more than one focal lesion on magnetic resonance imaging (8). The clinical diagnosis for MGUS and SMM differ from MM (Table 1)

Table 1: IMWG classification for MGUS, SMM and MM.

Criteria	MGUS	SMM	MM
CRAB Features	Absent	Absent	Present
% clonal plasma cells in the BM	<10%	10-60%	>10%
Serum monoclonal plasma cells	<30 g/L	>30 g/L	-

For the clinical diagnosis of MM, the following CRAB features need to be present (9).

- Hyper**C**alcemia: Serum calcium >1 mg/dL higher than upper limit of normal or >11 mg/dL
- **R**enal insufficiency: Creatine clearance <40 mL/min or serum creatinine > 2 mg/dL
- **A**nemia: Hemoglobin value of >2 g/dL below the lower limit of normal, or a hemoglobin value <10 g/dL
- **B**one lesions: one or more osteolytic lesions on skeletal radiography, CT, or PET-CT

The evolution of a normal plasma cell to a malignant myeloma cell with identifiable precursor states serve as a useful tool to understand human cancer progression. The stage of "precancer"

has been a challenge for the cancer research community because while this early neoplastic stage is distinguishable from normal tissues owing to molecular and phenotypic alterations, resulting in abnormal cells that are at least partially self-sustaining and function outside of normal cellular cues that constrain cell proliferation and survival, defining it very early hinges on pathology and does not take into consideration the genetic and molecular alterations it harbors (10). However, it is vital to understand the biology of these precancerous states to clinically intervene at the earliest opportunities and to prevent cancer progression. In the case of MGUS, about 3% of the general population have it and there is a 1% per year progression rate from MGUS to MM. A nationwide cancer screening trial in 2009 demonstrated that MGUS could be consistently documented, and samples could be collected before the diagnosis of MM (3). MGUS is not resectable but both tumor and non-tumor compartments from the patient bone marrow can be readily isolated for research. Over the last decades, with the advent of sequencing, more light has been shed on the genetic make-up and additional tissue-specific biomarkers of MM and its precursor stages (11). Cytogenetic and gene-profiling studies have revealed that most of the alterations seen in MM are already present at the MGUS stage (12-14). Gene-expression profiling of MGUS cells revealed that purified plasma cells from MGUS are much closer to MM cells than normal plasma cells, with harbor overexpression of DNA metabolism and cell cycle control genes (15). Like MGUS, SMM cells also harbor aberrations similar to MM, and the presence of specific chromosome abnormalities such as del(17p), t(4:14), 1q gains, and hyperdiploidy seem to correlate with increased risk of disease progression (16, 17).

Studies comparing plasma cells from MGUS and MM cohorts have revealed increasing proportion of clonal plasma cells with genetic abnormalities, consistent with the expansion of preexisting clones at the transition of MGUS to MM since only a small fraction of clonal plasma

cells present in MGUS carried cytogenetic abnormalities (18). Studies tracking patients with MGUS who progressed to clinical MM show that genomic alterations and intraclonal heterogeneity is established at the MGUS stage and that this progression, in most cases, did not involve new somatic mutations (19, 20).

1.2 Tumor microenvironment

More than 120 years ago, Stephen Paget postulated the hypothesis of the "seed-and-soil", emphasizing the role of a permissive microenvironment for cancer progression (21, 22). The BM consists of cellular (immune, endothelial, adipocytes, mesenchymal stem cells, reticular, and osteolineage cells) and noncellular components, extracellular matrix (ECM), and soluble factors, all of which maintain homeostatic hematopoiesis. In MM, this homeostasis is disrupted, and the BM can aid MM tumor cells in tumor growth, survival, migration, drug-resistance, loss of immune function etc (23). Plasma cells require extrinsic factors for their migration to and survival in the BM, to play a central role in the humoral immune protection of humans (24). In the case of MM, these tumor cells proliferate and colonize in the TME and recirculate, and finally egress from the BM during the extramedullary stages of the disease (25).

Despite harboring complex genomic alterations and clonal evolution, MGUS remains clinically asymptomatic. This suggests the possibility that changes in growth rate and malignant transformation may depend in part on extrinsic factors such as interactions of tumor cells with the tumor microenvironment (TME). To understand this, a study from the Dhodapkar lab looked at the growth of primary human pre-neoplastic and malignant plasma cells together with non-malignant cells *in vivo* (26). A genetically humanized mice model known as MIS$^{(KI)}$TRG was utilized for this study. These are immune-deficient mice with 5 knock-in human alleles (M-CSF,

IL-3, GM-CSF, Thrombopoietin and SIRPα) and exhibit superior multi-lineage engraftment of human hematopoietic stem cells, including innate immune cells. Isolated MGUS tumor cells showed progressive growth in the BM upon xenotransplantation in these mice, supporting the concept that the observed clinical stability of MGUS lesions may indeed depend predominantly on tumor-extrinsic growth controls, emphasizing the role of the immune system and the TME in myeloma disease progression (26).

1.3 Role of the immune system in MGUS and myeloma

1.3.1 Introduction to cancer immune surveillance

Over the last century, it has been well established that the immune system plays a fundamental role in the most, if not all cancers. In 1909, Paul Ehrlich proposed that the immune system usually suppresses tumor formation, a concept known as the "immune surveillance" hypothesis, which is relevant to this day. This concept was then enriched in the 1950s by Burnett and Thomas who showed that the body's immune system is vital in identifying and eliminating diseases cells, including tumor cells (27). Cancer immune surveillance is an important host protection mechanism to inhibit carcinogenesis and to maintain cellular homeostasis. In the interaction of host and tumor cells, three essential phases known as the "Three E's" have been proposed: elimination, equilibrium and escape (28, 29).

Elimination is the first phase where both the innate and adaptive arm of the immune system work in tandem to detect and destroy cancer cells, before they become malignant. These tumor cells express stress-induced molecules such as surface calreticulin, tumor antigens in context of MHC class I molecules, that are recognized by CD8 T cell and, and/or NKG2D ligands recognized by

NK cells. DCs take up and cross-present tumor antigens to T cells and these activated effector cells release IFN-γ that can mediate anti-tumor effects by inhibiting tumor cell proliferation and angiogenesis. Tumor-specific CD4 T cells produce IL2 and home to the tumor site, and aid the effector activity of these CD8 T cells. (30). If this phase successfully eradicates the cancer cells, it completes immunoediting process without progression to the subsequent phases.

Equilibrium is the second stage, and the longest phase where the host immune system and any tumor cell variant that has survived the elimination phase enter a dynamic equilibrium, wherein lymphocytes and cytokines such as IL2 and IFN-γ exert potent selection pressure on the tumor cells that is enough to contain, but not fully extinguish, a tumor bed. The immune system holds this tumor bed containing many genetically unstable and mutating tumor cells in a stage of functional dormancy which also has tumor promoting cytokines such as IL-10 and IL-23 (29, 31). This results in a heterogenous tumor population that has reduced immunogenicity.

Escape is the third phase of cancer immunoediting where the host immune system fails to restrict tumor outgrowth, leading to a clinically malignant stage. Tumor cells evade immune recognition using mechanisms like the loss of tumor antigens, MHC class I or co-stimulatory molecules, express molecules of increased resistance (STAT-3), survival (anti-apoptotic molecule bcl2), immunosuppression and secrete cytokines such as VEGF, TGF-β, IL-6, M-CSF that enhance angiogenesis (29, 32).

1.3.2 Immune surveillance and recognition in MGUS and MM

Evidence for immune surveillance and recognition in MM and its precursor stages has been established in both from murine models as well clinical patient samples. The application of the 3 Es in MM progression is an area of active research, with T cells and NK cells playing important

roles in elimination, the MGUS and SMM disease state representing the equilibrium phase, and clinical MM representing the escape phase (33).

Studies utilizing mass cytometry and single cell transcriptomics have shown that MGUS BM carries phenotypic alterations in immune cells including T, B, natural killer (NK), and myeloid cells (34, 35). Early studies of the bone marrow TME in MGUS provided one of the earliest examples of the presence of preneoplasia-specific T cells in humans (36). MGUS-specific CD4 and CD8 T cell response was detected in T cells from the BM whereas T cells from MM marrow lack this tumor-specific rapid effector function (37). Another study from the Dhodapkar lab looking at antigen-specific response in patients showed that MGUS but not MM patients or healthy donors were able to mount a humoral and cellular immune response against SOX2, which plays a critical role in self-renewal in embryonal stem cells. In addition, the absence of anti-SOX2 T cells is significantly associated with future progression to active MM, highlighting the importance of tumor-specific T cells for preventing myeloma progression (38). In addition to changes in T cells, the TME in MGUS also consists of alterations in innate immune cells, with enrichment of distinct subsets of innate lymphoid cells, which are widely known to impact T-cell function (39).

In murine models, engraftment of MGUS and MM patient bone marrow transplanted into MIS$^{(KI)}$TRG mice, MGUS tumor cells exhibited progressive growth, suggesting that the dormant nature of the MGUS cells in patients is regulated in part by tumor-extrinsic controls, including the immune system (26). In another study using a V-kappa*MYC-CD226 murine model for myeloma, it was found that the CD226-dependent response was mediated by both NK and CD8+ T cells in a perforin and interferon gamma-dependent manner, thus providing an anti-tumor response (40).

All clinically diagnosed cancers have an established immunosuppressive milieu that allows tumor cells to evade antitumor immunity, and the MM TME is no different. However, there is data supporting the presence of immune surveillance in the MM TME, in both, the innate and the adaptive arm (41). In a preclinical model of syngeneic stem cell transplant for MM, it was observed that TIGIT immune checkpoint blockade led to the prevention of CD8+ T cell exhaustion and successful immune control of MM, supporting the role of immune system in tumor control (42).

1.3.3 T cells in Myeloma

T cells have been known to be altered in MM and to consequently have a role in the immunodeficiency associated with the disease, since T cells play a vital role in exerting anti-tumor effects. Several studies have shown that in both MM and MGUS, there is a decrease in the PB CD4/CD8 T cell ratio, which is due to both the decrease in absolute and relative numbers of CD4 T cells and an increase in relative numbers of CD8 T cells (41, 43, 44). Overall, there is an increase in the proportions of memory T cells and depletion of naïve counterparts relative to age matched healthy controls and memory T cells from both MGUS and myeloma patients exhibit greater terminal-effector differentiation. However, memory T cells in MGUS show greater enrichment of stem-like TCF1/7hi cells (45). Both MGUS and MM have shown to have higher Tregs as compared to age matched healthy controls, and that there is an elevated level of CD38hi T regs in MM patients (46, 47). There is an increase in T helper type 1 (Th1) cells in the BM in both MGUS and MM patients and is consistent with findings of an increased Th1/Th2 ratio observed in the PB of MM patients (48, 49). There have also been several studies demonstrating the increase of Th17, a pro-inflammatory T cells in both PB and BM, but specially in the BM

where they are implicated in the development of MM lytic bone lesions via their production of IL-17 (50-52). These Th17 cells significantly inhibit the production of pro-inflammatory Th1 cytokines such as IFN-γ, suggesting a role in maintaining immune suppression in the tumor microenvironment (50, 52).

As MGUS progresses to MM, there is an attrition of TCF1hi stem-like memory T cells (53, 54). In addition, there is an accumulation of senescent T cells expressing high levels of lytic genes such as granzyme A, senescence-associated genes such as KLRG, and proinflammatory cytokines, which may indicate towards the loss of immune surveillance in myeloma (45).

T cell clonality has important clinical and research value and is of interest in understanding myeloma immunology. CD8 T cells in myeloma patients, at various stages of their disease, were found to be specific for cancer germline gene antigens and their frequency correlated with tumor burden (55). Other studies using clonotypic assays have also demonstrated that T cell clonality has prognostic implications in MM by showing that tumor-specific CD8 T cells have strong immune responses and are correlated with both disease burden and clinical outcomes in MM patients (56). In both PB and BM, studies have shown that clonal CD8 T cell expansions are more frequent in precursor disease patients compared with MM patients, suggesting that cytotoxic T cell dysfunction correlates with the progression of disease (37, 57)

When looking at long-term survivors of MM, studies have shown that these survivors have significantly higher frequencies of clonal cytotoxic T cell expansion, higher Th17 cells and lower Tregs in their circulation as compared to patients with a shorter follow-up (58, 59).

All this data indicates that there are drastic alterations in the T cell compartment as the disease progression, and these changes need to be delineated further to improve treatment strategies.

1.4 Aging and cancer

1.4.1 Hallmarks of Aging

Much like the hallmarks of cancer, aging too is driven by several factors, all of which are active areas of research. In 2023, after decades of aging research, the field established 12 hallmarks of aging that fall under 3 major categories: Primary, antagonistic and integrative (60). The primary hallmarks of aging progressively accumulate with time, and consist of genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis and disabled macroautophagy (61). The antagonist hallmarks are responses to damage and comprise of deregulated nutrient-sensing, mitochondrial dysfunction and cellular senescence. Finally, integrative hallmarks are those that when the accumulated damage inflicted by the primary and antagonistic hallmarks cannot be compensated anymore and they are stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis. All the 12 hallmarks of aging interact with one another to speed up the process of aging. However, age-related diseases in humans have higher chances to co-occur and share genomic characteristics when they are causally linked to the same hallmark rather than to different hallmarks (62).

Another important aspect of aging is to understand the difference between biological age and chronological age, with biological age being regarded as being more accurate than chronological age in determining chronic health outcomes. Chronological age refers to the amount of time a person has lived for, whereas the biological age of a person is more nuanced, and is a combination of several factors and biomarkers such epigenetic alteration and DNA methylation (63-65).

1.4.2 Links between Aging and Cancer

Aging is considered one of the most significant risk factors for various malignant diseases and the prevalence of these diseases increases as adults age, reaching a peak around 85 or 90 years, when the incidence of new cancer diagnoses starts to decline (1). People over the age of 65 make up 60% of all new cancer diagnoses and 70% of cancer deaths and with advances in healthcare maximizing human life expectancy and the elderly population continues to grow, cancer rates are predicted to increase in the coming years (66). Adults over the age of 65 years with a cancer diagnosis exhibit an increased incidence of comorbidities and aging-related conditions compared with those without cancer and their younger counterparts (67). This may be due to two reasons: A certain individual may have a higher disposition to cancer if they have a higher biological aging or the development of malignancy may result the deterioration of general health due to long-distance effects of the cancer on other organs, causing rapid aging (60). Aging and cancer research done in tandem alludes to the fact that they either share or diverge in several disease mechanisms and biological processes (Table 2) (68-70)

Table 1.2: Shared Hallmarks between Aging and Cancer

Hallmark	Aging	Cancer
Genomic Instability	Increased	Increased
Epigenetic alterations and reprogramming such as DNA methylation, chromatin remodeling and histone modifications	Present	Present
Cellular senescence	Increased	Increased
Inflammation	Chronic inflammation	Tumor-promoting inflammation
T cell alterations such as TCR diversity and effector T cells	Present	Present
Dysbiosis	Present	Present

1.4.3 Effect of aging on T cells

T cells are crucial for immune functions and prevent disease progression in most living organisms and so an aged T cell system is characterized by progressive dysfunction, higher vulnerabilities to infection and cancer as well as increased autoimmunity (71). The thymus plays a vital role in the maturation and selection of a diverse T cell repertoire and as we age, we undergo thymic involution due to reduction in thymic mass (72). This results in a diminished generation of naive T cells that reach circulation, a compensatory clonal expansion of high-differentiated memory, and an exhaustion of stem cell-like T cells, all of which compromises the adaptive immune response (73). Along with an increase in memory T cells as we age, there is also an alteration in the phenotype of cells and the cytokines they produce. Studies indicate that there are increased levels of memory cells producing type I (TNF and IFNg – particularly in CD28- CD8+ T cells) and type II (IL4 and IL10) cytokines, leading to a pro-inflammatory state (74).

Like with other cell types, T cells also undergo genetic and epigenetic alterations with age, and this can lead to several clinical manifestations. Chromosomal alterations in aging T cells and mutations in hematopoietic stem cells are linked with an increased incidence of T cell leukemia (73). Mitochondrial dysfunction, which is another major feature of aging T cells, can cause genomic instability, resulting in telomere attrition and the reduced activity of DNA repair enzymes (75, 76). Telomere length is a well-established indicator of cell proliferation and thus its "age" and attrition is seen in both CD4 and CD8 T cells as we age, and this affects primarily naive, early differentiated and the lymphoid progenitor cell subsets (77). Another important aspect of T cell aging is the reduction in TCR repertoire due to the reduction of the number of

naïve T cells and clonal expansion of terminally differentiated effectors, which hampers the body's immune response (78).

For a long time, the field believed that the accumulation of memory T cells is a result of aging and remain quiescent till they received the right antigenic stimulation. However, this idea has been revisited since cumulative evidence supports the notion that cytokine-activated T cells, known as 'virtual memory T cells' present similar phenotypes to conventional antigen-experienced memory cells and accumulate with aging in mice and tend to show features of senescence (79, 80). However, this is still an active area of research, especially in human samples.

1.4.4 Inflammatory signatures seen in aging and cancer

Increased chronic inflammation or "Inflammaging" is a state of systemic chronic, low-grade inflammation that develops in older individuals without any overt infection (81). Chronic inflammation has systemic manifestations, as well as with pathological local phenotypes such as arteriosclerosis, neuroinflammation, osteoarthritis etc. There are several mechanisms of chronic inflammation such as Reactive Oxygen Species (ROS), ER stress, Insulin resistance, NF-kB signaling, TLR signaling, misfolded proteins, NLRP3 inflammasomes, and pro-inflammatory miRNAs. All these mechanisms interact with other hallmarks of aging via a complex network of signaling pathways, potentially accelerating aging (82).

Sequencing studies have shown that immune function in patient blood declines with enhanced inflammation, identifying an age associated T cell population that is composed of exhausted memory cells and mediates pro-inflammatory effects via granzyme K (83). Pro-inflammatory cytokines in T cells such as IFN, TNF-α, IL1 and IL6 are commonly elevated with age, due to a dysregulation in the mTOR pathway (84-86). In the context of viral infections, pro-inflammatory

type 1 IFNs inhibit telomerase activity and act as drivers of T cell senescence (87). With aging, there are many inflammation-enabling systemic alterations in T cell populations like the hyperfunction of pro-inflammatory Th1/17 cells, defective immunosurveillance which has a negative impact on the elimination of virus-infected, malignant or senescent cells, loss of self-tolerance, and the reduced maintenance and repair of biological barriers (88-90).

In the context of age-related diseases such as cardiovascular diseases, genomic instability, which is a hallmark of aging, favors clonal hematopoiesis of indeterminate potential (CHIP), with the expansion of myeloid cells that often bear a proinflammatory phenotype (91). A 3-year longitudinal study in older individuals to characterize pro and anti-inflammatory cytokines showed that there are systemic defects in JAK-STAT signaling pathways, due to elevated levels of basal phospho-STAT proteins (92).

It has been well established that tumor-promoting inflammation in the tumor microenvironment is one of the enabling characteristics of cancer development. Genomic instability and other factor contribute to oncogene activation, which results in the expression of proinflammatory transcription factors (such as NF-κB, STAT3 or HIF1α) within tumor cells. These activated transcription factors mediate the expression of key cytokines and chemokines such as TNFα and IL-6 as well as inflammatory enzymes like COX-2, forming a complex network of inflammatory responses within the TME. In response these chemokines, the innate and adaptive immune arm are recruited to mediate an immune response. Inflammatory enzymes catalyze key steps in prostaglandin synthesis, which further regulate several physiological processes involved in cancer-related immunity and inflammation, thus driving tumorigenesis (93, 94). Thus, in cancer-related inflammation, multiple pathways are involved in trying to suppress effective anti-tumor immunity.

1.4.5 Immune aging and vaccine response

Vaccines contain antigens that are either derived from the pathogen or produced synthetically to represent components of the pathogen to effectively mount an immune response in the event of any exposure to a specific pathogen (95). For a vaccine to be effective, the immune system must retain its ability to generate immune memory so that the host can produce a rapid and specific response to the pathogen when encountered again. However, as discussed before, aging compromises the ability of the immune system, including humoral immunity, to respond sufficiently to pathogens and cancers and thus, dampens vaccine response. There have been many studies done in humans showing that younger people had higher protective titers against influenza (96), there was a strong decline in TBE and tetanus titers with age and that people over the age of 60 were unable to mount a response (97) and in a study assessing vaccination-induced changes in the human B-cell repertoire and pneumococcal IgM and IgA antibody at different ages, it was found that IgA and IgM responses were significantly impaired in the old subjects (98). In the context of the COVID-19 infection, age is associated with reduced efficacy of vaccines and linked to higher risk of severe infection (99, 100). Reduced immunogenicity was observed following the vaccination with virus geometric mean titer (GMT) values being higher in younger individuals than their older counterparts (101). A study looking at the efficacy of the SARS-CoV-2 vaccine in two geriatric animal models showed that there was reduced protection in the geriatric animal population (102).

1.4.6 Age calculators

Biological aging is not linear, but rather a result of complex systems working together and understanding it is vital in predicting disease risk and evaluating aging interventions. Since the

1980's, there has been a lot of interest in identifying reliable biomarkers which are age dependent to accurately evaluate an individual's biological age (103). Currently there are several "age calculators" or "molecular aging clocks" that utilize biomarkers (genomic, epigenomic, transcriptomic, proteomic etc), mathematical models and machine learning algorithms to try to accurately predict the biological/physiological age of an individual.

- DNAmAge: One of the most common "age calculators" that researchers have explored are epigenetic age calculators, since DNA methylation is one of the most established aging biomarkers (104, 105). These "epigenetic clocks" (Horvat clock and Hannum clock) were the first openly available datasets for DNA methylation patterns from multiple human tissues ranging from prenatal tissues to those from centenarians and is referred to as DNAmAge (64, 106). Studies examining if an epigenetic clock can predict cancer incidence or mortality have shown that these are stronger predictors for cancer mortality than cardiovascular disease and that there is a dose-responsive relationship between increased DNAmAge and cancer incidence and mortality; for each one-year increase in the difference between chronological and epigenetic age, there was a 6% increased risk of developing cancer within three years and a 17% increased risk of cancer-associated mortality in the next five years (107, 108). While promising, these epigenetic clocks have several drawbacks like a correlation with certain blood cell types that also show age-related change and not accounting for tissue variability as epigenetic aging can differ between tissues, which has led to a gap in knowledge about mortality prediction in any other tissues barring blood (109). Hannum's blood-based age estimator was also designed for adult blood samples, leading to biased estimates in children.

To improve the accuracy of these epigenetic clocks by accounting for phenotypic changes due to physiological dyregulation, a phenotypic age estimator called DNAm PhenoAge was designed. This phenotypic age estimator was constructed by generating a "phenotypic age" which was a weighted average of 10 clinical characteristics: chronological age, albumin, creatinine, glucose and C-reactive protein levels, lymphocyte percentage, mean cell volume, red blood cell distribution width, alkaline phosphatase and white blood cell count, instead of chronological age and then a penalized regression model like that of DNAmAge was used. The DNAm PhenoAge is a greater predictor of mortality, health span or cardiovascular disease (110). Another DNA methylation age clock known as the "DNAm GrimAge" came about in 2013, which is a mortality risk estimate and is a linear combination of chronological age, sex, and DNAm-based surrogate biomarkers for seven plasma proteins and disease promoting exposures such as smoking pack-years (94). While the DNAm GrimAge clock outperformed previous epigenetic clocks while predicting mortality risk, it is not a very informative predictor of chronological age and like the previous models, is not adjusted for cell-type heterogeneity (111).

- GlycanAge: Over the last two decades, several large-scale studies have shown reliable effects of aging on protein glycosylation as measured from human serum or plasma (112, 113). Glycosylation is a key post-translational mechanism that regulates function of immunoglobulins, with multiple systemic repercussions to the immune system, making this assessment valuable. The GlycanAge index was associated with health variables such as fibrinogen, HbA1c, BMI, triglycerides and uric acid after correction for age and sex and had less variance than other age predictors such as telomere length. However, these were not longitudinal studies and so it was hard to predict whether aging caused the

observed changes in IgG glycosylation or if these glycosylation changes in IgG contribute to aging by promoting inflammation (112).

- IMM-AGE: This aging calculator was developed to assess immune aging, by considering immunosenescence and different compartment of the immune system, that age at different rates (114). Alpert et al conducted a longitudinal study of blood samples from 135 healthy adults over a period of 9 years and by mass cytometry, changes in the circulating immune-cell subpopulations were assessed. They showed that while immune-cell frequencies changed at different rates, there were 33 immune cell subsets that consistently correlated with age across their 9 years of data collection and bucketed them as those that went up with age (CD57+ CD8 T cells, CD8 and CD4 effector T cells, Tregs, PD1+ CD8 T cells etc) and those that decreased with age (Naïve CD8 and CD4 T cells, CXCR5+ CD4 T cells, CD27+ CD8 T cells, B cells etc). They also identified a gene signature that correlated with IMM-AGE. They also cross verified their algorithm to test the association between IMM-AGE and cardiovascular disease and utilized the Framingham Heart Study, which has over 2000 participants between the ages of 40 and 90 years (115). They calculated the DNA methylation age of these participants and conducted correlation studies with IMM-Age and found that IMM-Age was >500-fold more significant than DNA methylation age in predicting overall survival, highlighting the pivotal role of immune aging in survival.

1.5 The epidemiology of Myeloma

1.5.1 Myeloma and aging

Myeloma is predominantly a disease of older adults with the median age at diagnosis being 70 years and only less than 2% of patients are less than 40 years old (116, 117). With advances in medicine and the world's aging population increasing, myeloma is becoming more prevalent. MGUS, the precursor state, has been detected in about 5% of individuals ≥70 years of age (2). Age is also an important determinant of treatment approaches in myeloma, including whether the patient is eligible for autologous haemopoietic stem cell transplantation (ASCT) and most randomized trials limit this to patients ≤65 years of age. However, several studies have demonstrated similar outcomes with ASCT in older patients, alluding to the fact that the biological age of a patient is more relevant than the chronological age while determining the eligibility (2, 118).

1.5.2 Myeloma and race

The incidence of multiple myeloma is 2–3-times higher in black individuals than in white individuals, with the median age at diagnosis being 66 years in blacks and 70 years in whites (119). This racial difference is seen only the incidence rate and no racial survival disparity among blacks and whites was seen. A study of 365 participants showed that MGUS is significantly more common in blacks, and more often has features associated with higher risk of progression to MM (120). Since these racial differences are seen in the precursor state, there have been studies showing that the excess risk of multiple myeloma in black patients is due to an increase in the risk of MGUS rather than an increase in the risk of progression from MGUS to multiple myeloma (121). This racial disparity is even more evident amongst patients younger

than 50 years where rates of myeloma are 2.6 times higher in Black men and 3.3 times higher in Black women than the rates for White men and women, respectively (122).

These differences can be divided into two broad groups: biological or social determinants. Biological determinants include factors such as hereditary (First-degree relatives of patients with myeloma have a 2–3 times higher risk of developing the disease), genetic variance, obesity (this is one of the known risk factors for myeloma and nearly 48% black adults are clinically obese, as compared to 34% whites) and other myeloma-specific biological differences such as black MGUS patients having lower levels of monoclonal protein, an earlier age of onset, lower prevalence of IgM MGUS, and a higher frequency of abnormal serum free light chain (sFLC) ratios compared to their white counterparts (123).

Understanding and addressing these racial disparities are vital for researchers and physicians deliver effective treatments, because currently, these health disparities greatly affect the clinical care that black patients get compared to their white counterparts (123).

1.6 Summary, scope, and goals for this project

Despite significant advances in immunotherapy, multiple myeloma (MM) remains to be an incurable malignancy. The immune system's critical role in eliminating malignant cells underscores the importance of understanding immune dysfunction in myeloma progression and treatment resistance. While recent single-cell transcriptomic studies of MM and its precursor state, monoclonal gammopathy of undetermined significance (MGUS), have suggested T-cell exhaustion based on murine chronic viral infection models, functional validation of these exhaustion signatures remains incomplete.

The first aim of this dissertation is to comprehensively characterize blood and bone marrow T cells in MGUS and MM through functional and single-cell transcriptomic analyses, providing deeper insights into immune dysfunction beyond traditional exhaustion paradigms, and the implications of the signatures that we see. The second aim addresses a critical gap in myeloma research: despite higher incidence and earlier onset of myeloma in Black individuals, this population remains understudied. We seek to elucidate the immunological basis for racial disparities in myeloma incidence by examining distinct immune signatures between racial groups in both MGUS and MM patients.

Through these investigations, we aim to advance the understanding of T cell biology in MM while addressing crucial questions about racial disparities in myeloma pathogenesis and progression. These insights may inform the development of more effective therapeutic strategies in the clinic.

Chapter 2: T cells from Myeloma patients lack global exhaustion

The following chapter has been published in part as an original research paper published in Blood Advances

Immune-aging is linked to clinical malignancy, racial ancestry and vaccine responses in myeloma

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2.1 Key Findings

- T cells from myeloma and MGUS patients retain their capacity to be polyfunctional after TCR stimulation.
- T cells in the MM marrow are enriched for phenotypes of advanced differentiation and exhibit distinct functional phenotypes such as GM-CSF and IL17-producers, previously implicated in tissue inflammation or bone disease.
- T cells from myeloma and MGUS patients retain their proliferative capacity after TCR stimulation

2.2 Introduction

T cell exhaustion is a state of dysfunction seen during chronic infections when T cells exhibit a reduced capacity to secrete cytokines, proliferate and have an increased expression of inhibitory immune checkpoints such as PD1, TIM3, LAG3, CTLA4 and TIGIT (124). CD8 T cell exhaustion was first described with chronic LCMV infection using murine models during which virus-specific CD8 T cells persist but lack effector function (125). Another murine chronic LCMV model showed that virus-specific CD8 T cells initially develop the ability to perform effector functions but with persistent chronic infection, these functions are lost in a hierarchical manner during chronic infection with some functions that are exhausted early (e.g., IL-2, cytotoxicity, and proliferation), whereas others (e.g., IFN-γ) persist longer (126). While there are many similarities in exhausted T cells in chronic viral infections and tumors such as inhibitory immune checkpoints and transcriptional programs such as TOX, there are several differences like the tumor microenvironment. Exhausted T cells from chronic viral infection have a stronger type I interferon signal as compared to those in tumors (127) Within, TCF1+ self-renewing

population that terminally differentiated exhausted T cells are derived from, in tumors, they lack expression of CXCR5 (128).

T cells in MM patients express inhibitory immune checkpoints such as PD-1, TIGIT etc, which is a well-established hallmark of T cell exhaustion (129, 130). However, this data has been mostly extrapolated from models of chronic viral infection, and whether the research established in these viral models can be translated to tumor settings is still an active area of research due to established differences between the two. In addition, functional validation in single-cell transcriptomic/immunophenotypic studies is limited.

The clinical success of endogenous T cell therapies in myeloma patients argues against global T cell exhaustion and demonstrates the active anti-tumor effects of immune system in MM (131) Patient T cells have been redirected in the clinic in the form of chimeric antigen receptor (CAR) T cells against MM-associated targets such as B-cell maturation antigen (BCMA) and GPRC5D and have yielded positive results (132) Similarly, the FDA has approved several bispecific antibody therapies for relapsed/refractory MM, including teclistamab (targeting BCMA), talquetamab (targeting GPRC5D), and elranatamab (targeting BCMA), which have demonstrated significant clinical efficacy (133). These findings underscore the importance of further evaluating MM T cell functionality.

2.3 Results

To understand the functional properties of immune cells in patients with plasma cell disorders, we combined functional assays with immune profiling in biospecimens from MGUS, newly diagnosed MM (NDMM) and age-matched healthy controls. We employed single-cell mass cytometry to study the capacity of T cells from blood/marrow to produce cytokines (IFN-γ, TNF-

α, IL2, GM-CSF and IL17) following stimulation by anti-CD3/CD2/CD28 antibodies. First, we looked at cytokine production in CD4 T cells, where CD4 T cells from NDMM/MGUS patients in both blood and bone marrow retained capacity for production of all cytokines (Figure 2.1)

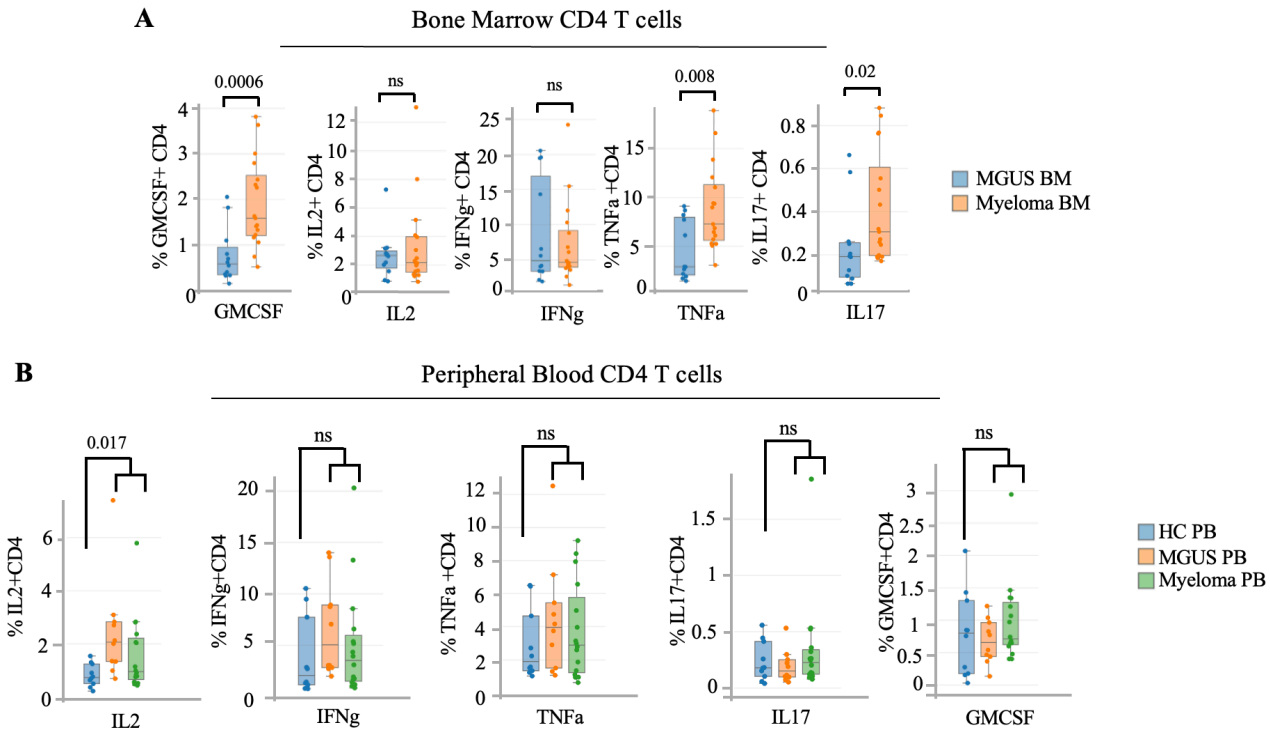

Fig 2.1: Cytokine production in CD4 T cells post CD2/3/28 antibodies. Cytokine secretion (GM-CSF, IL2, IFN-γ, TNF-α, and IL17) by BM (A) and PB (B) CD4 T cells following TCR stimulation in BMMNCs and PBMCs from HC (n=10), MGUS (n=12) and MM (n=17). Each dot represents unique patient sample. Box plots represent median with Q1-Q3 and error bars as min-max. P-values were calculated using a two tailed Mann Whitney. ns, not significant.

Next, we performed FlowSOM, which is a clustering algorithm for visualization and analysis of cytometry data, used to distinguish cell populations in an unsupervised way (134). FlowSOM analysis of CD4 T cells identified 3 metaclusters (MC) enriched in the myeloma bone marrow, MC2 with CD57+ memory T cells and two cytokine-producing MCs, MC10 expressing GM-CSF, and MC8 with polyfunctional phenotype secreting IL2, IFN-γ, IL17 and TNF-α, which also expressed markers such as PD-1, LAG-3 and TIGIT previously linked to T cell exhaustion (Figure 2.2)

Similarly, CD8 T cells from NDMM/MGUS patients in both blood and bone marrow retained capacity for production of all tested cytokines (Figure 2.3). FLOWSOM done on CD8 T cells identified 3 MCs as enriched in MM marrow, of which MC4 and MC6 had a TEMRA phenotype, while MC5 exhibited polyfunctional phenotype including IL17 (Figure 2.4). Most of the IFN-γ and TNF-α producing CD8 T cells in MM marrow had a terminal effector phenotype and KLRG1+ differentiated T cells-maintained capacity for cytokine production (Figure 2.5)

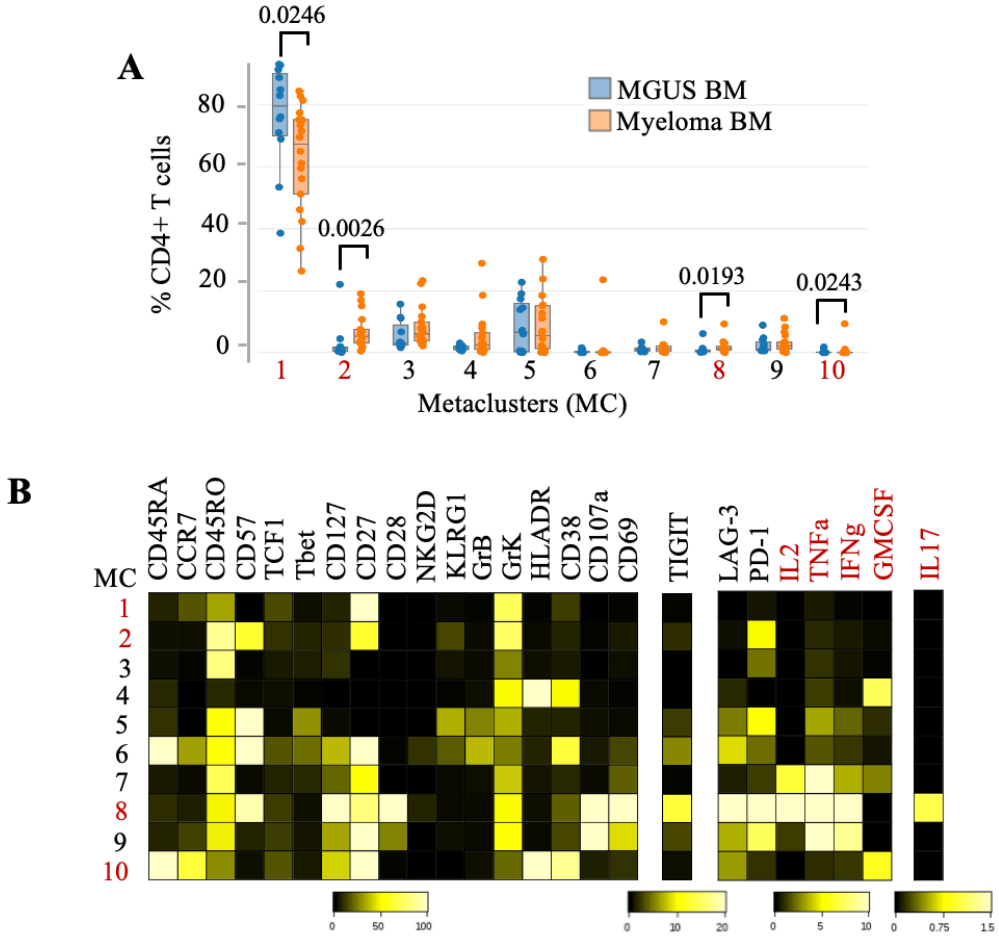

Fig 2.2: FLOWSOM in CD4 T cells post CD2/3/28 antibodies. FlowSOM clustering of mass cytometry data on BM CD4+T cells from MGUS (n=12) and MM (n=17), A: Showing proportions of metaclusters in MGUS/MM patients. B: Cluster heatmaps showing phenotypes. Each dot represents unique patient sample. Box plots represent median with Q1-Q3 and error bars as min-max. P-values were calculated using a two tailed Mann Whitney. ns, not significant.

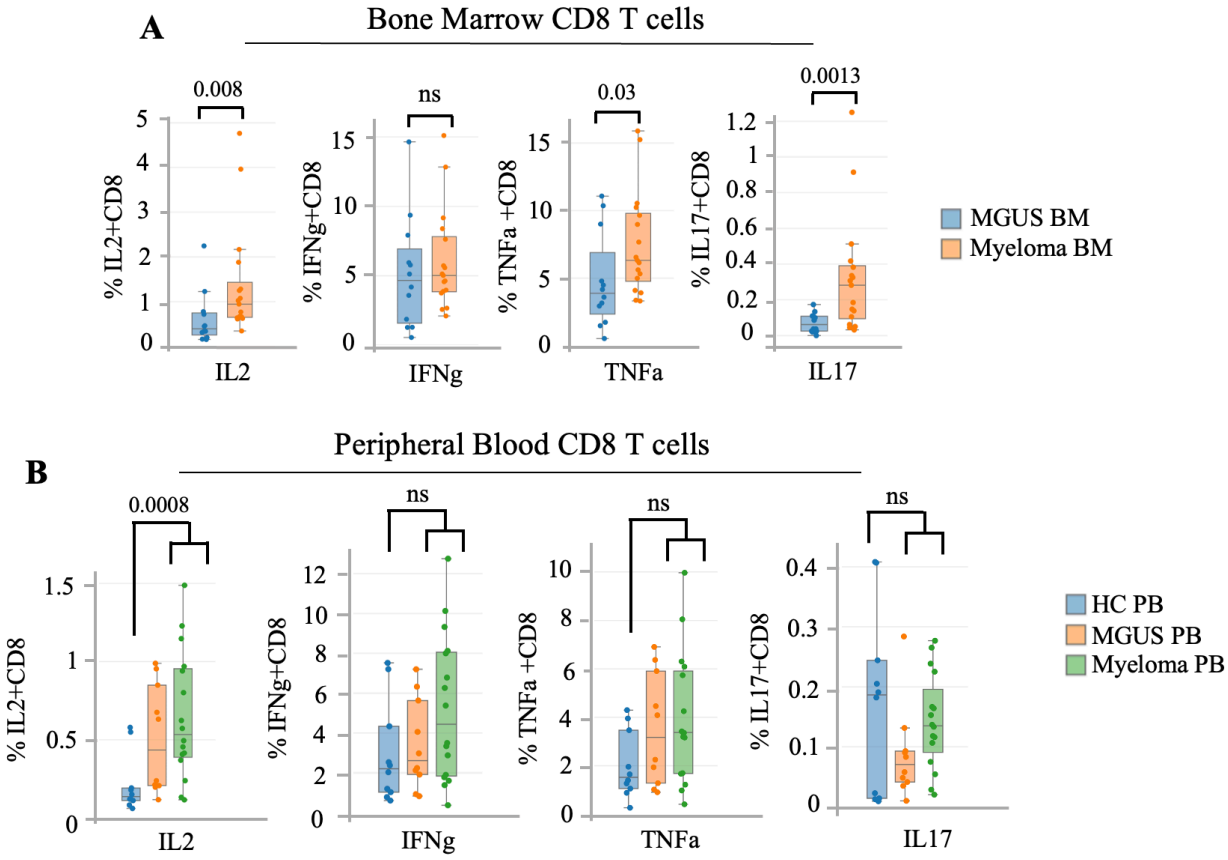

Fig 2.3: Cytokine production in CD8 T cells post CD2/3/28 antibodies. Cytokine secretion (IL2, IFN-γ, TNF-α, and IL17) by BM (A) and PB (B) CD8 T cells following TCR stimulation in BMMNCs and PBMCs from HD (n=10), MGUS (n=12) and MM (n=17). Each dot represents unique patient sample. Box plots represent median with Q1-Q3 and error bars as min-max. P-values were calculated using a two tailed Mann Whitney. ns, not significant.

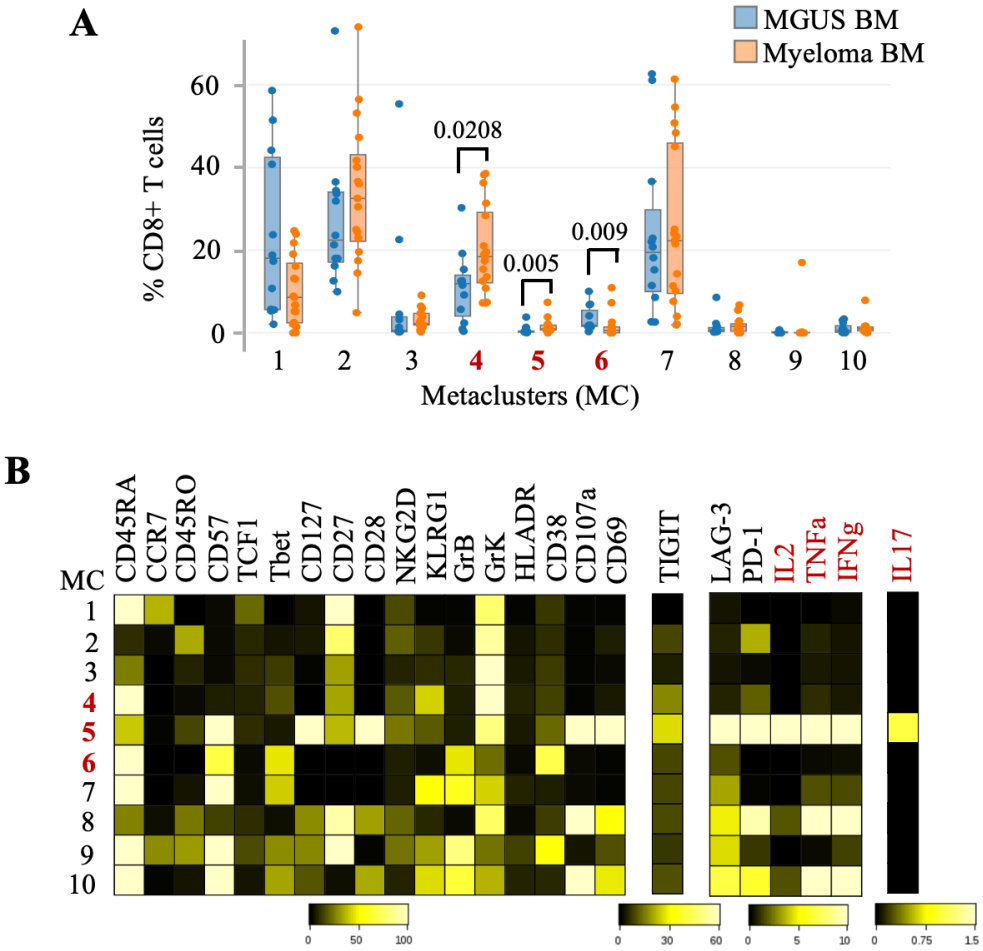

Fig 2.4: FLOWSOM in CD8 T cells post CD2/3/28 antibodies. FlowSOM clustering of mass cytometry data on BM CD8+T cells from MGUS (n=12) and MM (n=17), A: Showing proportions of metaclusters in MGUS/MM patients. B: Cluster heatmaps showing phenotypes. Each dot represents unique patient sample. Box plots represent median with Q1-Q3 and error bars as min-max. P-values were calculated using a two tailed Mann Whitney. ns, not significant.

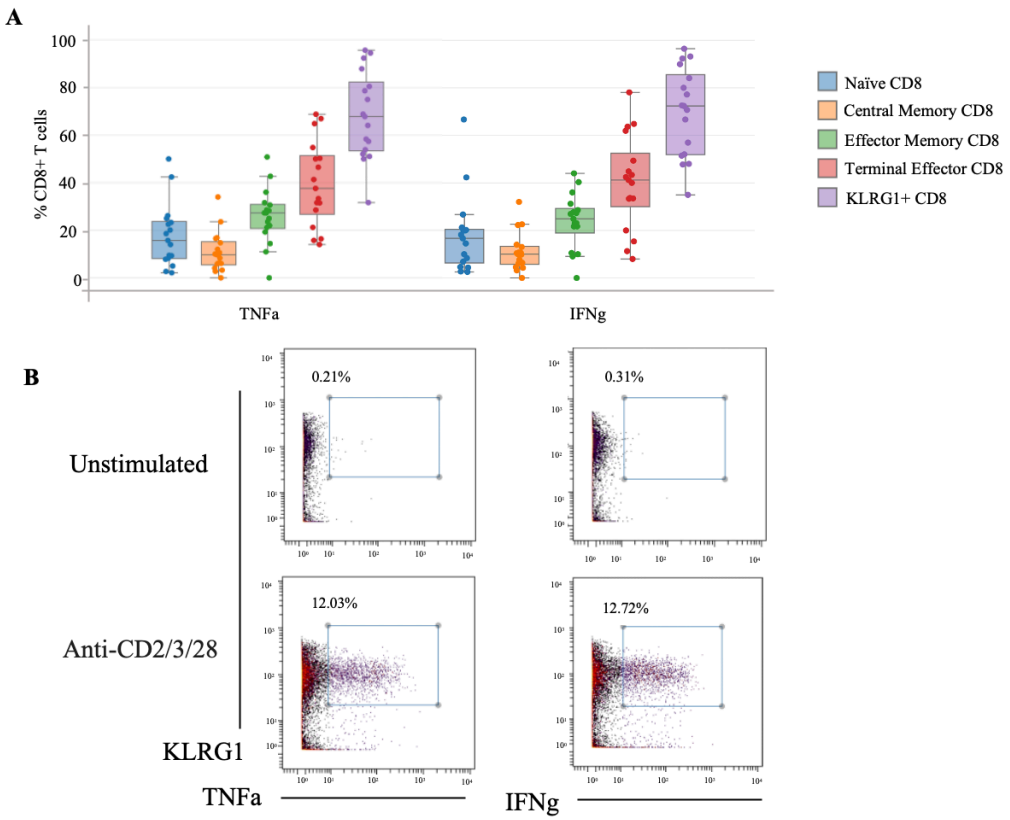

Fig 2.5 Cytokine producing CD8 T cells in MM marrow have a terminal effector phenotype. Mononuclear cells from MM bone marrow (n=17) were stimulated with CD2/3/28 beads and examined for cytokine secretion using mass cytometry. (A) Cytokine secretion in CD8 bone marrow from different T cell subsets. (B) Representative dot plots for unstimulated and stimulated CD8+ T cells from MM bone marrow.

Next, we examined the capacity of T cells from MGUS and MM patients to proliferate after TCR stimulation. CFSE was used as a cell tracer and flow-cytometry was performed 7 days after anti-CD3/CD2/CD28 stimulation, along with age-matched healthy controls (HC). Both circulating as well as bone-marrow T cells from patients with MGUS/MM also exhibited capacity for T cell proliferation in culture, again supporting lack of global T cell exhaustion.

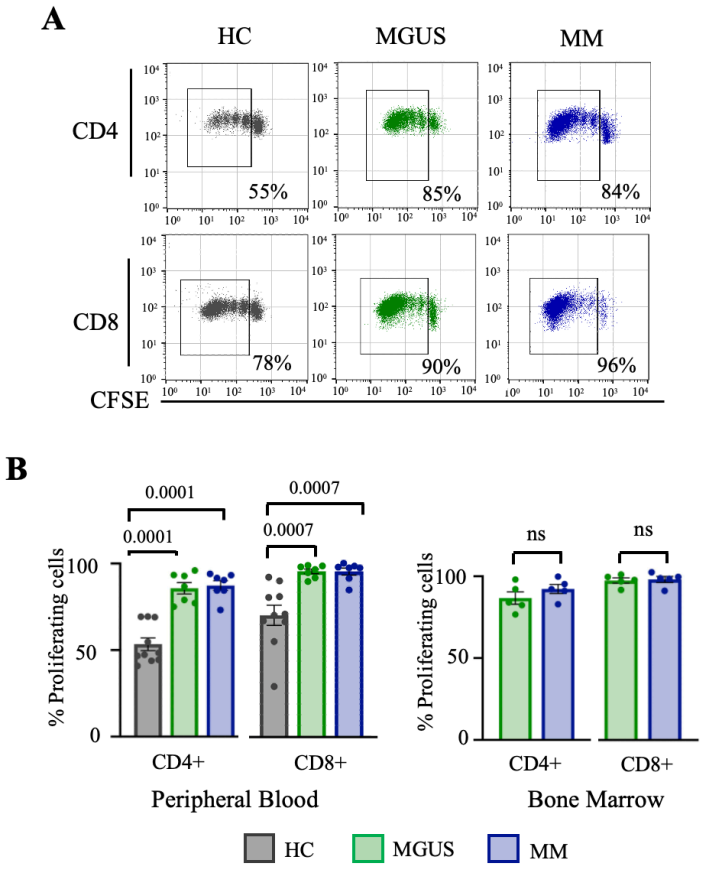

Fig 2.6 CFSE proliferation in CD4 and CD8 T cells post CD2/3/28 stimulation. T cell proliferation following TCR stimulation. g. Representative plot showing proliferation of CFSE-labeled PB T cells. Bar graphs show proliferating CD4 and CD8 T cells in blood (A: HC n=10, MGUS n=7 and MM n=7) and BM (B: MGUS n=5 and MM n=5). Bar graphs represent mean+/-SEM. Each dot represents unique patient sample. P-values were calculated using a two tailed Mann Whitney.

2.4 Discussion

Together these data demonstrate that T cells from MM and its precursor MGUS respond well to TCR stimulation, and these T cells from both, blood and bone marrow are polyfunctional and can proliferate. This suggests lack of global exhaustion, even though it has been previously shown that T cells in MM patients express inhibitory immune checkpoints such as PD-1 (130).

The cytokine data demonstrates that T cells in the MM marrow are enriched for phenotypes of advanced differentiation and exhibit distinct functional phenotypes such as GM-CSF and IL-17 producers. MM-enriched cell types such as Th-$_{GM}$ cells may promote tissue inflammation, as in autoimmunity and recruit myeloid cells during the MGUS-MM transition (135, 136). Similarly, Th-17 cells have been linked to lytic bone disease, which is a hallmark of malignancy in MM (50, 51) producers. All this data implicates that T cells from MM, especially in the bone marrow lack global T cell exhaustion, and rather show markers of tissue inflammation linked to aging, which is discussed in detail in Chapter 3.

2.5 Materials & Methods

Patients and specimen collection:

Peripheral blood and bone marrow specimens were obtained from patients with myeloma and MGUS following informed consent approved by Emory Institutional Review Board. De-identified blood specimens from healthy donors were also purchased from New York Blood Center, All-Cells and LifeSouth Community Blood Center. Mononuclear cells (MNCs) were isolated using Ficoll density gradient centrifugation.

Intracellular cytokine analysis:

MNCs from patients with MGUS, myeloma or healthy controls were thawed and rested for 1 hour in RPMI+5%PHS and then stimulated using ImmunoCult Human CD3/CD28/CD2 T Cell Activator (StemCell Technologies) in RPMI media containing Golgistop (BD Biosciences) for 4 hours. Immunophenotyping and cytokine analysis was performed utilizing a 38-marker mass cytometry panel (Table 2.2) as described above.

T cell proliferation:

MNCs were thawed and rested for 1 hour in RPMI+5%PHS and labeled with CellTrace CFSE (Invitrogen) to track proliferation. Cells were cultured in RPMI+5%PHS for 6 days either alone or with anti-CD2/3/28 beads (Miltenyi T Cell Activation/Expansion Kit, human). Cells were analyzed for CFSE dilution as a marker for proliferating T cells using flow cytometry. Markers used are indicated in Table 2.3

Statistical analysis

Statistical analysis of CyTOF and flow cytometry was performed using Cytobank and GraphPad Prism. Two-tailed Mann–Whitney was used to compare data between groups.

Table 2.1: Antibody panel for Intracellular cytokine analysis

	Antibody	Clone	Vendor		Antibody	Clone	Vendor
1	CD45	HI30	Standard Biotools	27	CCR7	G043H7	Standard Biotools
2	CD107a	H4A3	Standard Biotools	28	#IL-17A	BL168	Standard Biotools
3	#IL-2	MQ1-17H12	Standard Biotools	29	CD3	UCHT1	Standard Biotools
4	CD69	FN50	Standard Biotools	30	*#Granzyme K	GM26E7	Biolegend
5	#TNFa	Mab11	Standard Biotools	31	CD38	HIT2	Standard Biotools
6	#IFNg	B27	Standard Biotools	32	CXCR4	12G5	Standard Biotools
7	CD57	HCD57	Standard Biotools	33	CD25	3C7	Standard Biotools
8	HLADR	HI30	Standard Biotools	34	KLRG1	SA231A2	Biolegend
9	CD4	RPA-T4	Standard Biotools	35	CD56	NCAM16.2	Standard Biotools
10	CD8	RPA-T8	Standard Biotools	36	#Perforin	B-D48	Standard Biotools
11	*#TCF1	7F11A10	Biolegend	37	#Granzyme B	GB11	Standard Biotools
12	CD14	RMO52	Standard Biotools	38	CD16	3G8	Standard Biotools
13	CD127	A019D5	Standard Biotools				
14	LAG3	11C3C65	Standard Biotools				
15	CD45RA	HI100	Standard Biotools				
16	TIGIT	MBSA43	Standard Biotools				
17	CD27	L128	Standard Biotools				
18	CD11c	Bu15	Standard Biotools				
19	#GM-CSF	BVD2-21C11	Standard Biotools				
20	CD28	CD28.2	Standard Biotools				
21	#Tbet	D6N8B	Standard Biotools				
22	#CTLA-4	14D3	Standard Biotools				
23	*#EOMES	WD1928	ThermoFisher Scientific				
24	CD45RO	UCHL1	Standard Biotools				
25	PD-1	EH12.2H7	Standard Biotools				
26	NKG2D	ON72	Standard Biotools				

All metal conjugated antibodies purchased from Standard Biotools
*Purified antibodies were purchased from Biolegend/ThermoFisher Scientific, and metal tagged using Standard Biotools metal conjugation kit following manufacturers methods and titrated prior to use.
#Intracellular markers
After antibody staining, cells were incubated with intercalation solution and mixed with EQ Four Element Calibration Beads (Cat. #201708)

Table 2.2: Antibody panel for Proliferation Assays

Antibody	Species Reactivity	Vendor	Clone	Dilution
CD3	Human	Biolegend	HIT3a	1:50
CD4	Human	Biolegend	RPA-T4	1:50
CD8	Human	Biolegend	SK1	1:50

Other dyes used:

- LIVE/DEAD Fixable Violet Dead Cell Stain (ThermoFisher Scientific)
- CellTrace CFSE (Invitrogen)

2.6 Acknowledgements

MVD is supported in part by funds from NIH R35CA197603, Paula and Rodger Riney Foundation and SCOR award from LLS. KMD is supported in part by funds from NIH CA238471 and AR077926. The authors acknowledge Winship immune monitoring resource supported by P30CA138292.

Chapter 3: Immune cells from Myeloma patients are immune aged

The following chapter has been published in part as an original research paper published in Blood Advances

Immune-aging is linked to clinical malignancy, racial ancestry and vaccine responses in myeloma

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3.1 Key Findings

- T cells from MM patients exhibit greater immune-aging compared to age-matched MGUS cohorts and exhibit an inflammatory phenotype.
- Black MGUS patients have a higher age-associated immune score (AAIS) as compared to their age-matched white counterparts.
- Vaccine response in MM patients correlated with immune-aging: Patients with a higher AAIS has a dampened response to the SARS-Cov2 vaccine.

3.2 Introduction

As we age, the human body undergoes several changes, one of which is dramatic alterations in the immune system. The aging immune system has several known features such as a dampened response to infections, cancers and an increase in an inflammatory signature (137). Features observed in MM T cells post TCR stimulation in Chapter 2 did not support global T cell exhaustion that is often implicated in cancer, but rather tissue inflammation linked to aging. Immune-aging has been an area of active research in the field myeloma since myeloma affects the elderly, and charactering the immune system is vital in leveraging immune-based therapies such as monoclonal antibodies, bispecific antibodies and CAR-T cells. Furthermore, the clinical success of these T cell therapies in myeloma patients argues against global T cell exhaustion (131).

As discussed in the introduction to this dissertation, there are several age calculators that consider the alterations that accumulate as we age, such as immune responses decline with age, it is important to characterize age-related alterations in the innate and adaptive immune cell populations, antigen receptor repertoires and tumor microenvironments (114, 138). Since MM

disproportionally affects individuals of black ancestry, it is vital that any aging calculator that is developed represents healthy donors of black ancestry as well as those of white ancestry (123). To overcome this, we designed an immune-age calculator that reflected this racial diversity using modalities such as linear regression modeling and mass cytometry analysis and studied a cohort of 100+ healthy donors across 7 decades of life to quantify age-associated immune changes. To comprehensively characterize immune system alterations during malignant transformation, we employed multi-modal analyses combining CITE-seq transcriptomic and proteomic profiling of MM and MGUS patients. We validated our findings through phosphoproteomic analysis and the assessment of vaccine responses in MM patients, revealing novel insights into disease progression and racial disparities in MM pathogenesis.

3.3 Results

Aging of the immune system is associated with altered immune composition, which has been utilized to quantify immune aging. As prior studies quantifying immune aging did not include racially diverse cohorts to reflect our myeloma and MGUS cohort, we first analyzed a cohort of healthy individuals (n=107) (Table 3.1) across 7 decades of life utilizing a custom mass cytometry panel to identify immune phenotypes that correlated with age (Table 3.5). 37 immune cell subtypes were manually gated and then using $p < 0.001$ as a cut-off, we narrowed down 19 cell subtypes (Figure 3.1). Specimens from MGUS/MM patients were analyzed with the same panel and data from aging-associated variables were interpolated to compute aging-associated immune score (AAIS) in MM/MGUS patients like methods described earlier. Patient characteristics are described in Table 3.2.

Table 3.1: Healthy donor characteristics used for Immune-age calculator

Healthy Controls		
Total samples	n=107	
Median Age in yrs (Range)	28 (0.1-71)	
	Variables	n (%)
Sex	Female	56 of 106 (53%)
	Male	50 of 106 (47%)
Race	White	48 of 97 (48%)
	Black	39 of 97 (40%)
	Other	10 of 97 (12%)

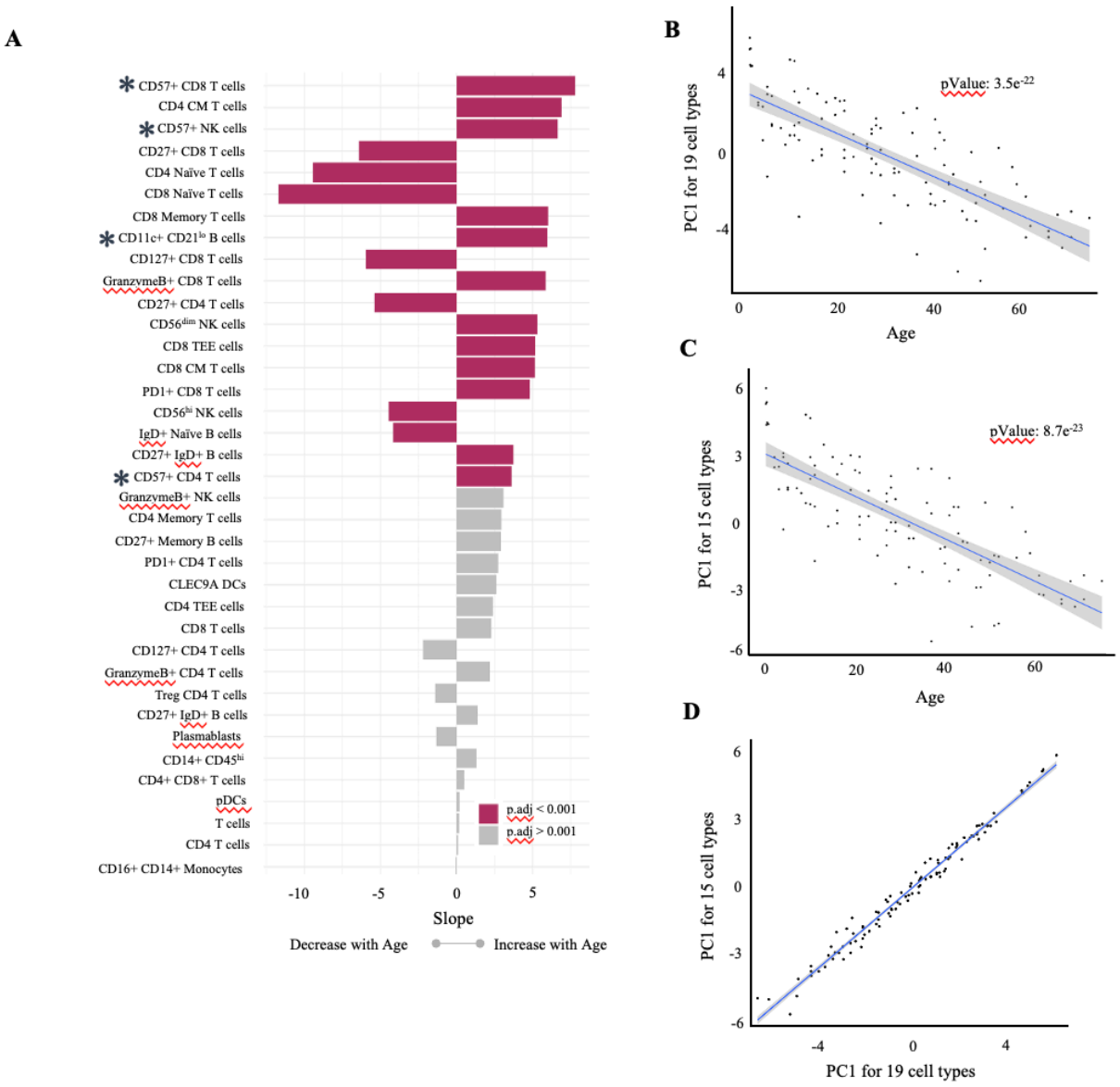

Fig 3.1 Calculation for aging-associated immune score. Blood specimens from 107 healthy donors across 7 decades of age were analyzed using mass cytometry.

A. Manually gated 37 cell subsets were analyzed for correlation with chronologic age. Bars in pink show cell types with p<0.001. Cell types marked with asterisks are not included in the 15 cell subsets.

B. Linear regression plot showing correlation between PC1 value and chronologic age for 19 cell subsets
C. Linear regression plot showing correlation between PC1 value and chronologic age for 15 cell subsets
D. Linear regression plot showing correlation between PC1 value for 15 cell subsets and 19 cell subsets

Table 3.2: Patient characteristics

	Healthy Controls n=27	MGUS n=42	NDMM (MM_1) n=50	MM following SARS CoV-2 vaccination (MM_2) n=83	P value
Median Age in yrs (Range)	60 (33-75)	64 (41-89)	64 (40-85)	62 (45-80)	0.95
	n (%)	n (%)	n (%)	n (%)	
Age in yrs					
Under 55	11 (41)	8 (19)	8 (19)	16 (19)	0.63
55-75	15 (56)	23 (56)	27 (64)	56 (67)	
Above 75	1 (3)	10 (25)	7 (17)	11 (14)	
Sex					
Female	9 (33)	26 (67)	18 (43)	40 (48)	0.1
Male	18 (67)	14 (33)	24 (57)	43 (52)	
Race					
White	15 (55)	20 (48)	17 (34)	43 (52)	0.13
Black	3 (11)	19 (45)	22 (44)	37 (45)	
Other	1 (4)	1 (2)	3 (6)	1 (1)	
Undisclosed	8 (30)	2 (5)	8 (16)	2 (2)	
Sex and Race					
White Female	6 (33)	10 (29)	4 (11)	21 (24)	0.27
White Male	9 (50)	8 (24)	13 (34)	27 (30)	
Black Female	1 (6)	12 (35)	11 (29)	20 (23)	
Black Male	2 (11)	4 (12)	10 (26)	20 (23)	
IgH					
IgA	-	9 (23)	7 (21)	16 (34)	0.35
IgG	-	23 (59)	16 (47)	22 (47)	
Other	-	7 (18)	11 (32)	9 (19)	
IgL					
Kappa	-	24 (69)	17 (52)	35 (74)	0.09
Lambda	-	11 (39)	16 (48)	12 (26)	
Cytogenetic Risk					
Standard	-	-	18 (67)	30 (67)	>0.99
High	-	-	9 (33)	15 (33)	

P value for age was calculated using Kruskal-Wallis ANOVA test between MGUS, NDMM (MM_1) and MM following SARS CoV-2 vaccination (MM_2). P values for proportions were calculated using Chi-Squared test and comparisons were made between MGUS, MM_1 and MM_2. For cytogenetic risk, only the MM cohorts were compared. For race, White vs all others were combined to perform Chi-Squared test.

In a cohort of MGUS (n=36) and newly-diagnosed MM (NDMM) patients (MM_1, n=41) matched for chronologic age (Chr Age), NDMM patients had significantly higher aging-associated immune score (AAIS) compared to MGUS. Together, these data suggest that malignant transformation in MM may be associated with progressive immune aging. Both MGUS and MM are more common in individuals with Black ancestry. Black MGUS patients also had higher AAIS compared to age-matched white counterparts, however no race-dependent differences were observed for the NDMM cohort. Together these data suggest an impact of racial ancestry on immune-aging trajectories may begin early in MGUS and provide insights into prior studies showing MM development at an earlier age in Black patients.

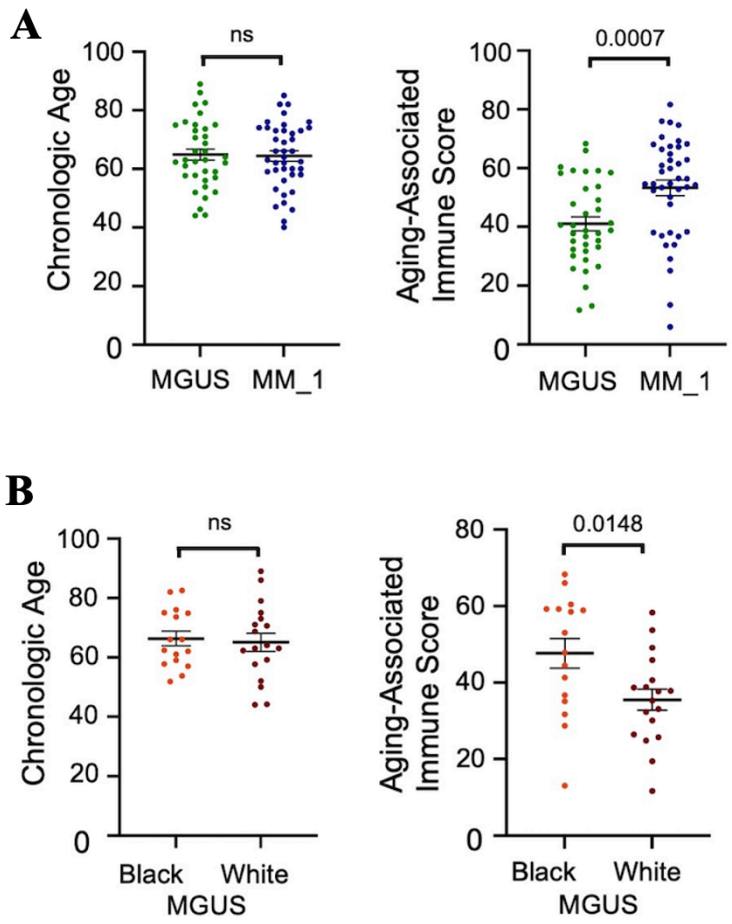

Fig 3.2 Chronological ages and Aging-associated immune score (AAIS) in patients.

A. MGUS (n=36) and newly-diagnosed MM patients (MM_1) (n=41).

B. Black (n=16) and white (n=18) MGUS patients.

Each dot represents unique patient sample. P-values were calculated using a two tailed Mann Whitney.

To further understand transcriptional changes associated with immune aging phenotypes, we analyzed BM cells from MM/MGUS patients with cellular indexing of transcriptomes and epitopes (CITE-seq) sequencing (Figure 3.3). Patients were then divided into immunologically younger/older cohorts based on median AAIS. These groups were comparable in terms of chronological age. Immune composition of different cell types did not differ between younger/older MGUS/MM cohorts, except for a higher proportion of CD16+ monocytes in older MGUS patients (Figure 3.4). We compared transcriptomes of immunologically younger/older cohorts to identify differentially expressed pathways in specific cell types (Figure 3.5). Pathway analysis of differentially-expressed genes in CD4/CD8 T cells between immunologically younger/older MM patients revealed greater PD1 signaling in older MM patients, while interferon-response signatures were enriched in older MGUS and MM patients. Enriched pathways in myeloid and tumors cells from immunologically-older MM patients were also consistent with greater immune activation. All pathways for MM and MGUS have been listed in Tables 3.3 and 3.4.

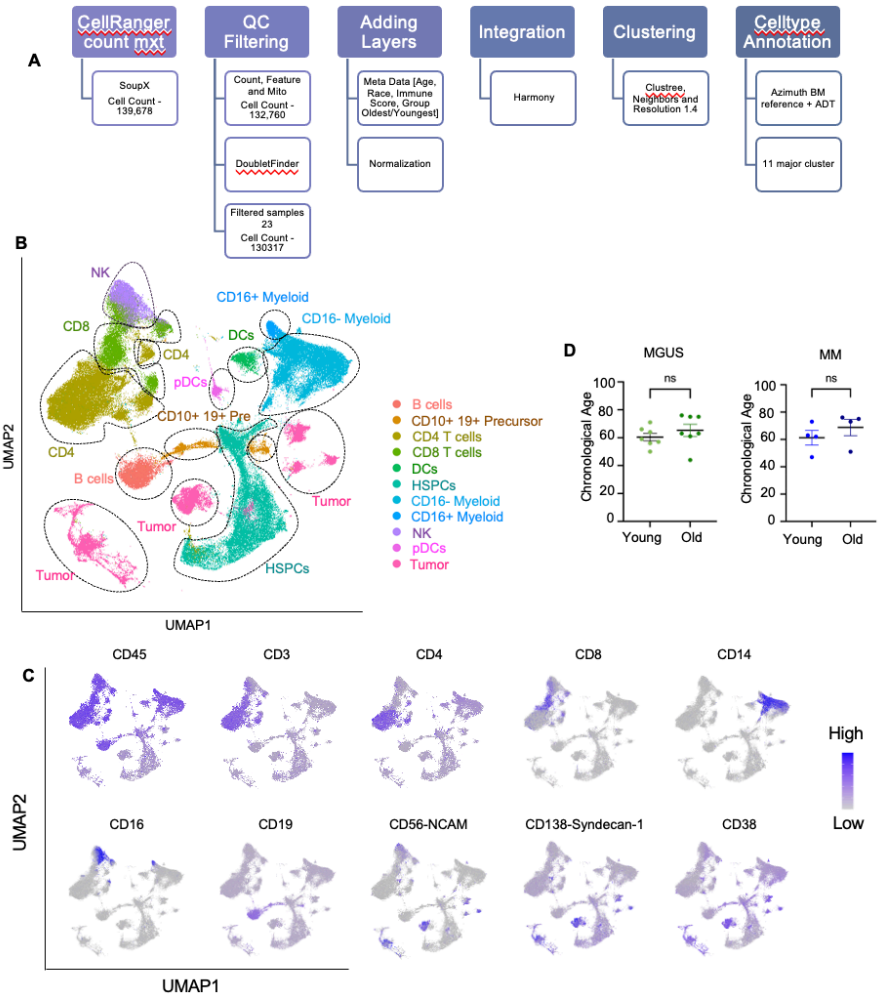

Fig. 3.3. CITE-Seq analysis for BMMCs from MGUS (n=14) and MM (n=9) patients

a. CITE-seq QC and analysis pipeline

b. Uniform manifold approximation and projection (UMAP) graph for all cells sequenced based on the transcriptome. 60 distinct clusters could be identified and were then classified into cell types using ADT staining overlayed with Azimuth BM

c. UMAP showing antibody staining for cell type markers

d. Chronological ages for MGUS patients: Immune young (n=7) and immune old (n=7) and MM: Immune young (n=4) and immune old (n=4), based on AAIS above/below median

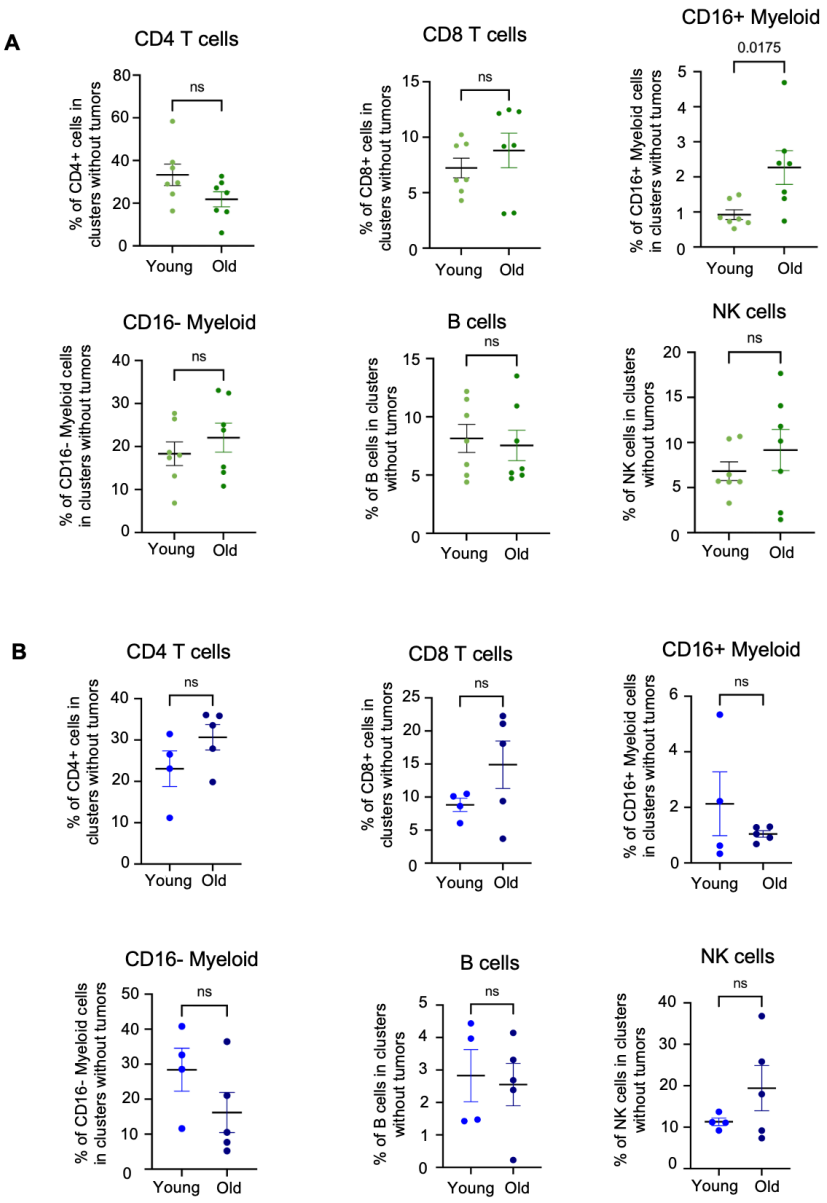

Fig. 3.4. Immune composition based on CITE-Seq in BM between immune young/old MGUS/MM patients. Patients were classified as immune young/old based on AAIS above/below the median for the group. Data shown as percent in total cells excluding the tumor compartment

a. Cell distribution in MGUS patients: Immune young (n=7) and immune old (n=7)

b. Cell distribution in MM patients: Immune young (n=4) and immune old (n=5)

Each dot represents unique patient sample. *P*-values were calculated using a two tailed Mann Whitney.

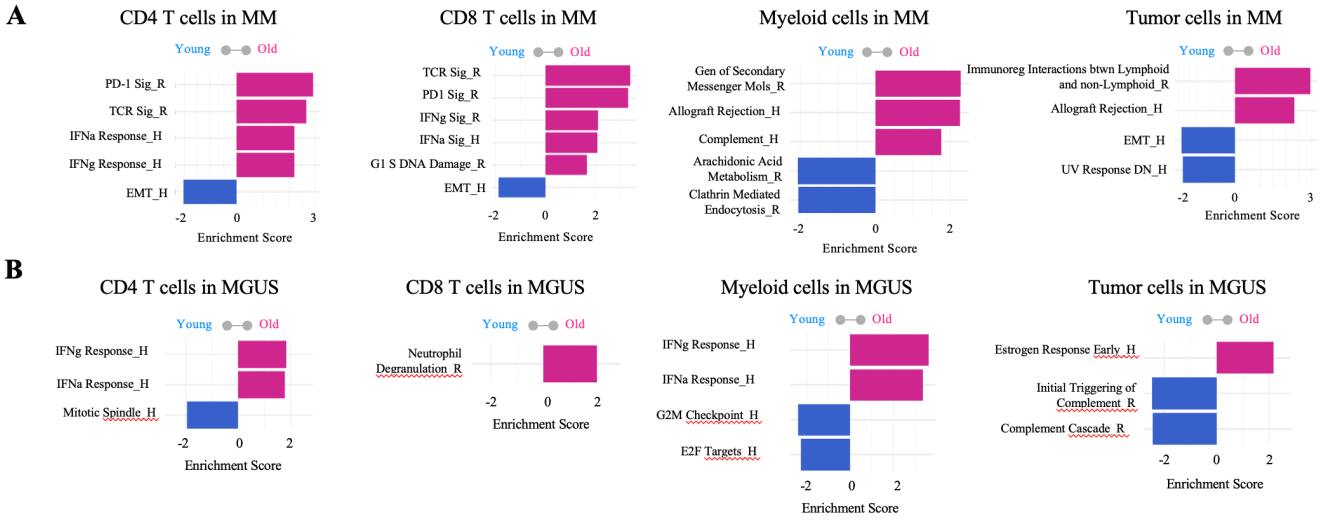

Fig. 3.5. Age-related changes in transcriptional profiles in MM and MGUS BM: Top enriched pathways based on pathway analysis (hallmark:_H, Reactome:_R) of differentially expressed genes analyzed by single cell transcriptomics of bone marrow cells from immune-old (n=5) (AAIS 60-82 yrs) and immune-young (n=4) (AAIS 37-54 yrs) MM patients and immune-old (n=7) (AAIS 33-60 yrs) and immune-young (n=7) (AAIS 13-32 yrs) MGUS patients in CD4 T cells, CD8 T cells, Myeloid cells and Tumor cells. Pathways in pink are enriched in immune-aged and those in blue are enriched in immune-young.

Table 3.3 Pathways upregulated in Immune-Old vs Immune-Young in MM patients

MM Patients	Pathway	FDR q-value	Enriched in ImmuneOld vs Immune Young
CD4 T cells	HALLMARK_INTERFERON_ALPHA_RESPONSE	0.00613503	Immune Old
	HALLMARK_INTERFERON_GAMMA_RESPONSE	0.00306752	Immune Old
	REACTOME_PD_1_SIGNALING	0	Immune Old
	REACTOME_TCR_SIGNALING	0.00045758	Immune Old
	HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	0.00292799	Immune Young
CD8 T cells	REACTOME_TCR_SIGNALING	0	Immune Old
	REACTOME_PD_1_SIGNALING	0	Immune Old
	REACTOME_INTERFERON_GAMMA_SIGNALING	0.0357775	Immune Old
	HALLMARK_INTERFERON_ALPHA_RESPONSE	0.01585563	Immune Old
	REACTOME_G1_S_DNA_DAMAGE_CHECKPOINTS	0.094865119	Immune Old
	HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	0.051705554	Immune Young
Myeloid cells	HALLMARK_ALLOGRAFT_REJECTION	0.00433228	Immune Old
	HALLMARK_COMPLEMENT	0.08844044	Immune Old
	REACTOME_GENERATION_OF_SECOND_MESSENGER_MOLECULES	0.00695171	Immune Old
	REACTOME_ARACHIDONIC_ACID_METABOLISM	0.09362445	Immune Young
	REACTOME_CLATHRIN_MEDIATED_ENDOCYTOSIS	0.08603592	Immune Young
Tumor	HALLMARK_ALLOGRAFT_REJECTION	0	Immune Old
	REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_AND_A_NON_LYMPHOID_CELL	0	Immune Old
	HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	0.00398362	Immune Young
	HALLMARK_UV_RESPONSE_DN	0.00324853	Immune Young

Table 3.4 Pathways upregulated in Immune-Old vs Immune-Young in MGUS patients

MGUS Patients	Pathway	FDR q-value	Enriched in ImmuneOld vs Immune Young
CD4 T cells	HALLMARK_INTERFERON_GAMMA_RESPONSE	0.02336653	Immune Old
	HALLMARK_INTERFERON_ALPHA_RESPONSE	0.017578891	Immune Old
	HALLMARK_MITOTIC_SPINDLE	0.014533921	Immune Young
CD8 T cells	REACTOME_NEUTROPHIL_DEGRANULATION	0.06828997	Immune Old
Myeloid cells	HALLMARK_INTERFERON_GAMMA_RESPONSE	0	Immune Old
	HALLMARK_INTERFERON_ALPHA_RESPONSE	0	Immune Old
	HALLMARK_G2M_CHECKPOINT	0	Immune Young
	HALLMARK_E2F_TARGETS	0.00322403	Immune Young
Tumor	HALLMARK_ESTROGEN_RESPONSE_EARLY	0.0032069	Immune Old
	REACTOME_INITIAL_TRIGGERING_OF_COMPLEMENT	0.01080602	Immune Young
	REACTOME_COMPLEMENT_CASCADE	0.00669137	Immune Young

To further validate transcriptional evidence of enhanced inflammation in MM at a proteomic level, we performed single-cell phosphoproteomic analysis at baseline and following in-vitro stimulation in a cohort of MM and age-matched healthy donors. T cells and CD16+/CD16- monocytes from MM expressed higher baseline levels of several phosphoproteins (pSTAT1, pSTAT3, pSTAT5, pERK and p38) (Figure 3.6)

Following stimulation with LPS and interferon-a, MM T cells exhibited reduced fold increase in phosphoproteins compared to healthy controls (Figure 3.7). In contrast, myeloid cells (particularly CD16+ monocytes) expressed higher levels of phosphoproteins relative to age-matched healthy donors (Figure 3.7). These data further support enhanced basal inflammatory sigaling in MM immune cells and are remarkably similar to phosphoproteomic profiles described in studies of immune aging (92).

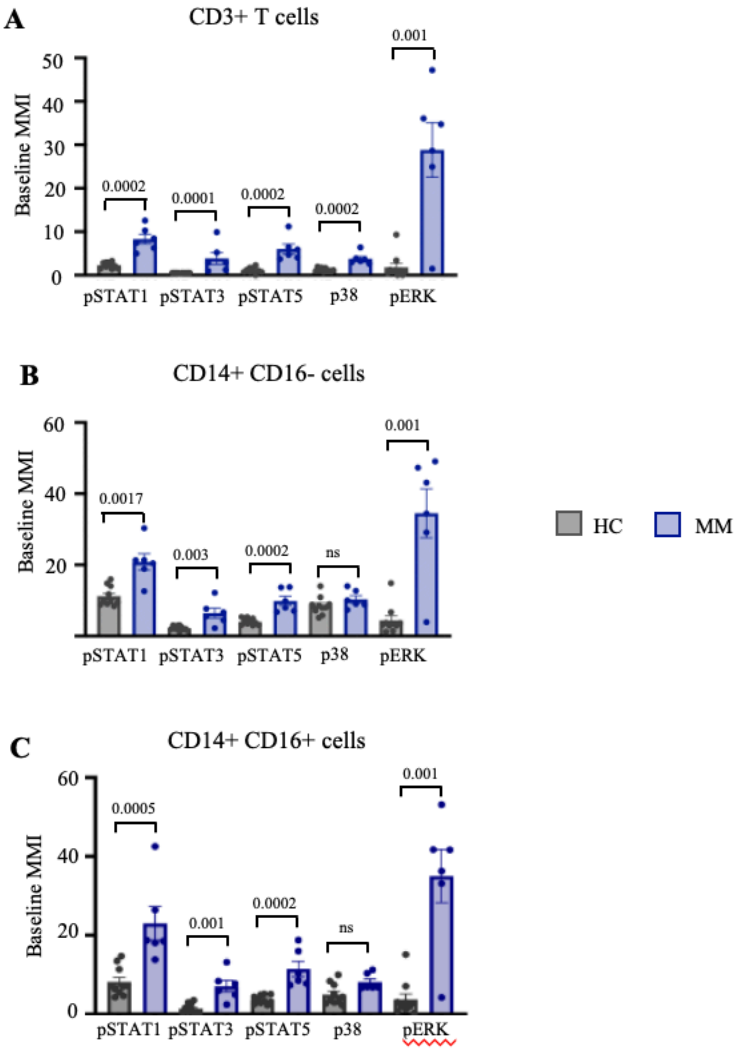

Fig. 3.6. Detection of phosphoproteins at baseline (pSTAT1, pSTAT3, pSTAT5, p38 and pERK) by mass cytometry in circulating CD3+T cells (A), CD14+16- monocytes (B) and CD14+16+ monocytes (C) in HC (n=10) and MM (n=6) at baseline. Bar graphs represent mean+/-SEM. Each dot represents unique patient sample. P-values were calculated using a two tailed Mann Whitney

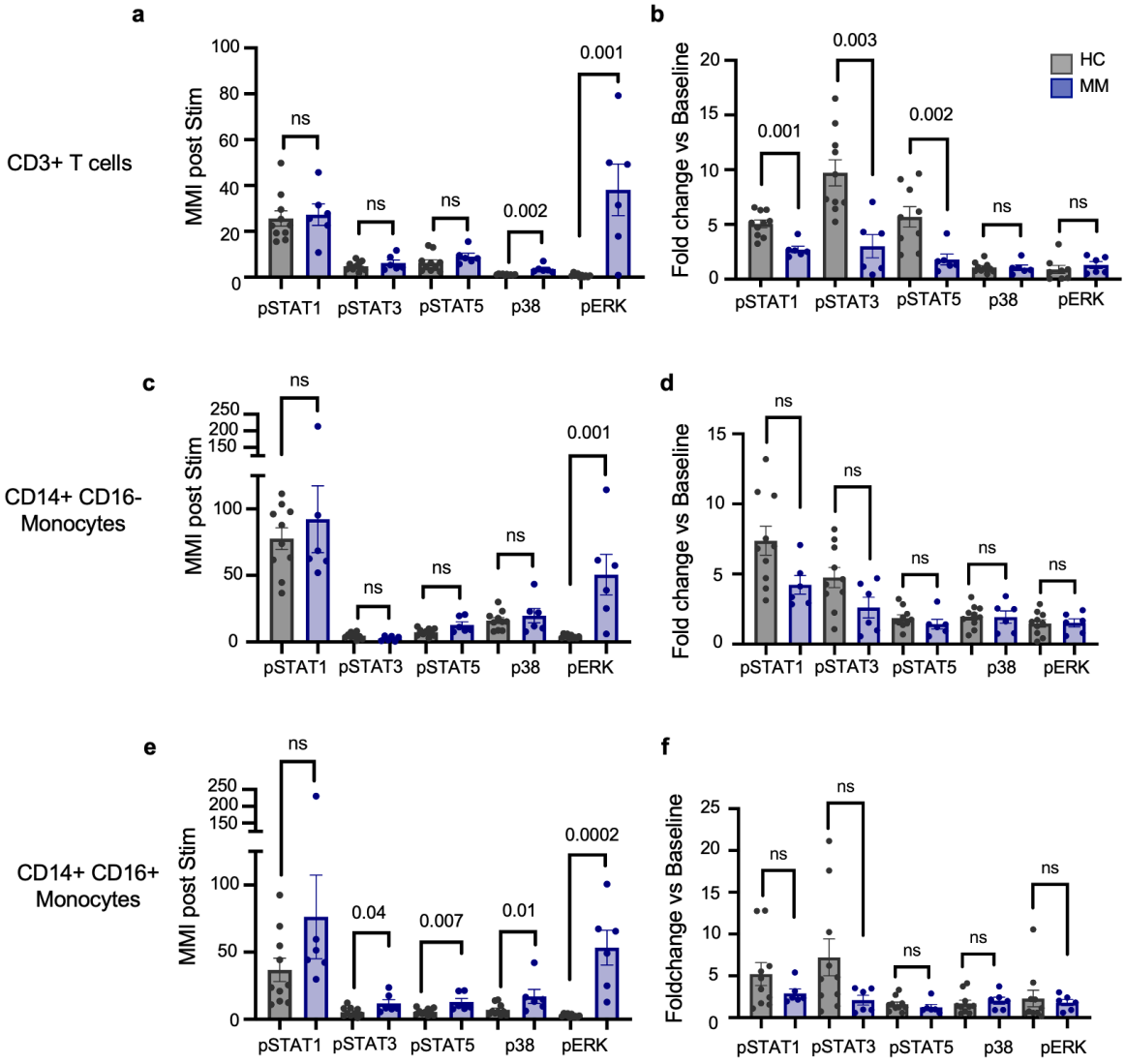

Fig 3.7. Detection of phosphoproteins (pSTAT1, pSTAT3, pSTAT5, p38 and pERK) following stimulation with LPS and interferon-a by mass cytometry in circulating CD3+T cells, CD14+16- monocytes and CD14+16+ monocytes in HC (n=10) and MM (n=6)

Left panels: median metal intensity (MMI) following stimulation.

Right panels: Fold change compared to baseline (shown in Fig. 2d-f).

Bar graphs represent mean+/- SEM. Each dot represents unique patient sample. P-values were calculated using a two tailed Mann Whitney.

Reduced immune response to vaccines has been recognized as a major consequence of immune aging in humans (139). To evaluate the impact of immune aging phenotypes in MM on immune function in vivo, we analyzed a cohort of MM patients (MM_2, n=83) who had received SARS CoV-2 vaccination (140). This cohort comprised of an equal split of white and black and male and female patients. Biospecimens from this cohort were analyzed to assess immune aging. Evaluation of immune aging in this cohort was based on 15 aging-associated subsets analyzed in these patients (Fig 3.1). As with the initial MM cohort, patients in this cohort had significantly higher AAIS compared to MGUS patients of similar chronologic age (Fig 3.8). Patients who developed vaccine-induced immunity, defined as positive titers for receptor-binding domain (RBD) antibodies, had lower AAIS compared to patients lacking these responses (Fig 3.8).

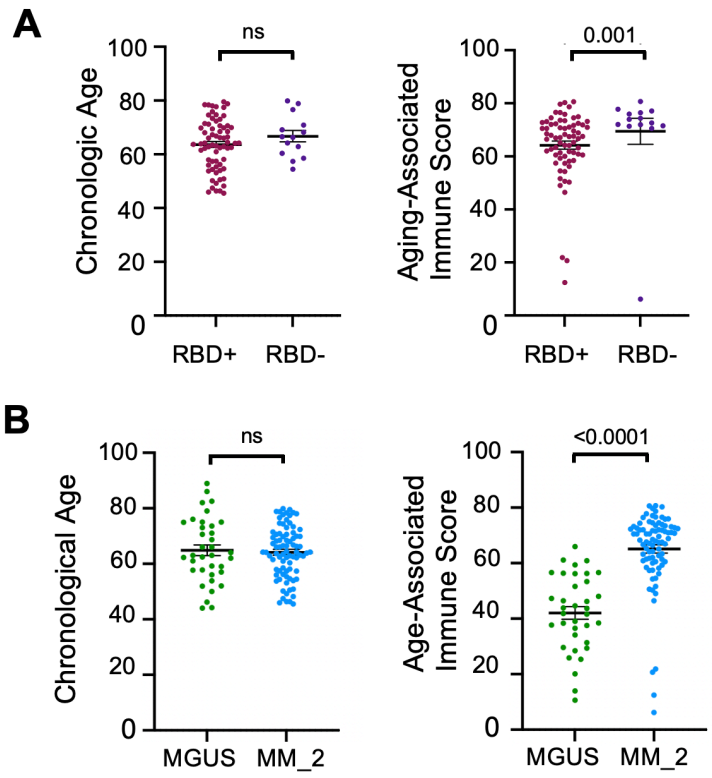

Fig 3.8. Impact of immune aging on vaccine response in myeloma patients

A: Chronologic age (ChrAge) and Aging-associated immune score (AAIS) in MM patients who developed receptor-binding domain (RBD)-binding antibodies following SARS CoV-2 vaccination (RBD+; n=69) versus those who did not (RBD-; n=14).

B: ChrAge and AAIS in MGUS (n=36) and MM patients who had received SARS CoV-2 vaccination (MM_2) (n=83).

Each dot is a unique patient. P values were calculated using 2 tailed Mann Whitney. ns, not significant.

3.4 Discussion

These data demonstrate that in spite of transcriptional similarities with exhausted T cells described in prior studies (131), T cells in MM marrow lack global T-cell exhaustion as was discussed in Chapter 2, where we demonstrated functional validation to show this phenotype. This implicates that transcriptome-based studies showed be followed with functional validation for the phenotypes observed. For example, PD1 signaling in MM T cells that was upregulated in the immune-old cohort may reflect aging, as opposed to canonical T-cell exhaustion (141). MM also is associated with greater immune aging compared to MGUS. The inflammatory state observed in the cytokine profiling and transcriptomic studies was further validated using phospho-proteomic analysis, where MM immune cells showed increased baseline inflammation-associated phosphoproteins and altered responses following activation.

MM and MGUS are more common in individuals with black ancestry, and this is an area of active exploration in the field (123). We see that MGUS patients with black ancestry exhibited enhanced immune aging. These data therefore support potential contribution of immune aging in malignant transformation, as well as racial predisposition with earlier onset of MM in black individuals. Finally, to evaluate the impact of immune aging on immune function in vivo, we analyzed another cohort of MM patients undergoing SARS-Cov2 vaccination. Immune but not chronologic aging was associated with responses to vaccination.

These findings provide novel insights into immune system alterations during malignant transformation and establish crucial links to racial predisposition in MM pathogenesis. Our work demonstrates significant correlations between immune aging signatures and in vivo immune function, as shown by vaccine response in these patients. These discoveries enhance our

understanding of immune dysfunction in multiple myeloma while highlighting the clinical relevance of racial disparities in disease progression.

Future investigations should focus on elucidating the molecular mechanisms driving accelerated immune aging in multiple myeloma and determining how these alterations impact the efficacy of emerging immunotherapeutic approaches. Understanding these mechanisms may prove critical for optimizing treatment strategies and improving outcomes across diverse patient populations. Additionally, longitudinal studies examining the evolution of immune dysfunction throughout disease progression could provide valuable insights for therapeutic intervention timing and selection.

Materials & Methods

Patients and specimen collection:

Peripheral blood and bone marrow specimens were obtained from patients with myeloma and MGUS following informed consent approved by Emory Institutional Review Board. De-identified blood specimens from healthy donors were also purchased from New York Blood Center, All-Cells and LifeSouth Community Blood Center. Mononuclear cells (MNCs) were isolated using Ficoll density gradient centrifugation.

Immunophenotyping with single-cell mass cytometry (CyTOF):

Immunophenotyping was performed utilizing a 37-marker mass cytometry panel (Table 2.1). PBMCs were stained with cell surface antibodies, fixed, permeabilized, and washed in accordance with the manufacturer's cell-surface and nuclear staining protocol as previously described (140). After antibody staining, cells were incubated with intercalation solution, mixed with EQ Four Element Calibration Beads and acquired using a Helios mass cytometer (all from Standard Biotools). Gating and data analysis were performed using Cytobank (https://www.cytobank.org). Dead cells and doublets were excluded using cisplatin intercalator and DNA content with iridium intercalator.

Development of immune-age calculator

PBMCs from a cohort of 107 healthy donors spanning seven decades of life (age range 0–75 years, Sex: 50 males and 56 females, Race: 48 White, 39 Black, 10 Others and 10 Undisclosed) (Table 3.3) were analyzed by mass cytometry using a 37-marker panel (Table 3.5). 37 distinct cell populations were identified using manual gating and analyzed for association with chronologic age. Of these, 19 immune cell markers (Fig 3.1) exhibited significant age-associated

changes identified through three analytical approaches: Pearson correlation coefficients, generalized additive models (GAM), and simple linear regression. Pearson correlations quantified the strength of association between age and each marker, while GAMs captured non-linear trends using the mgcv package in R (version 4.3.0). Simple linear regression models (lm(Age ~ cell)) were used to assess linear relationships between age and each marker. Selection of the 19 markers was based on the strength of the coefficients and the significance of p-values (< 0.001) from these models. Immune cell markers percentages from each donor were used as input for principal component analysis (PCA), performed using the `prcomp` function from the stats package in R (version 4.3.0). The first principal component (PC1) was selected to calculate the Immune-Age of the samples. A linear regression model (lm(Age ~ PC1)) was developed using PC1 as the independent variable and age as the dependent variable. Model training included repeated cross-validation (CV) to ensure robustness.

CITE-seq library prep:

BMMCs (n=23) were thawed, incubated at 37°C for 1 hour to rest. Cells were stained using a TotalSeq-C antibody cocktail (Table 3.6) following the 10X Genomics protocol for Chromium Single-Cell Immune Profiling with Feature Barcoding Technology (ver. 1.0). Single cells were isolated using the Chromium Controller (10X Genomics). Gene expression and CITE-seq libraries were prepared using the following kits from 10× Genomics: Chromium Next GEM Single Cell 5' Kit v2 (PN-1000263), Chromium 5' Feature Barcode Kit (PN-1000541), Chromium Single-Cell 5′ Library Construction Kit (PN-1000190), Chromium Next GEM Chip K Single Cell Kit (PN- 1000287), Dual Index Kit TT Set A (PN-1000215) and Dual Index Kit TN Set A (PN- 1000250). Sequencing was conducted on an Illumina NovaSeq 6000 at a targeted depth of 50,000 reads/cell for GEX libraries and 5000 reads/cell for CITE-seq libraries.

CITE-seq Data Analysis:

Initial demultiplexing of raw base calls by sample index was performed by Cell Ranger MKFASTQ Software suite (10x Genomics Cell Ranger v7.0.1) (142). The FASTQ files were aligned to reference genome GRCh38 (GENCODE v32/Ensembl 98), barcoded for unique UMI, filtered, deduplicated, and converted into count matrix of gene expression and surface proteins using Cell Ranger Multi pipeline v7.0.1 (available at 10x website). Removal ambient RNA contamination and batch effect from the count matrix data we used SoupX (143). The raw and filtered feature matrices were used as an input for SoupX. The contamination rate was calculated using autoEstCont() and the counts were corrected by adjustCounts() command. The resultant matrix was used for downstream analysis. Quality control and dimensionality reduction were performed in R v4.3.0 (https://www.r-project.org) using the Seurat package v5.1.0 (144). As initial QC of 23 samples (MGUS n=14 and MM n=9) filtered out the cells that expressed < 200 genes, > 65000 UMI count or > 10% mitochondrial genes; respectively for each sample. Additional doublet were removed from these bone marrow samples using DoubletFinder (145). These adjusted cell counts for each sample were merged (cell count n= 130317) for downstream analysis. Merged object was normalized and scaled with SCTransform using method glmGamPoi, regressing mitochondrial percentage with 'vst'. Surface protein assays were normalized across cells using centered log ratio transformation. Next, Principal component analysis was calculated using RunPCA and 1:45 PCs were used for clustering on manual inspection by elbow plot. The merged dataset is integrated using the harmony (146). The integrated object was refined based on the Jaccard similarity using the FindNeighbors function on harmony corrected PCA embeddings.

Resolution parameter for graph-based clustering was chosen based on Clustree inspection. Finally for visualization, UMAP dimensional reduction were calculated using 45 principal components with resolution of 1.4 to identify 60 cell clusters. These clusters were further classified into major categories, B, T cells (CD4 and CD8), NK, Myeloid (CD16+ and CD16-), pDCs, hematopoietic stem/progenitor cells (HSPCs), B cell precursors and tumor cell types, based on the protein expression of lineage-associated markers and Azimuth BM reference datases (144) Figure 3.3 shows steps used for QC and data processing.

For comparing transcriptomes from immunologically old/young patients, AAIS was computed using mass cytometry data from paired blood samples. The dataset was grouped as MM-old (n = 5) vs MM-young (n = 4) and MGUS-old (n = 7) vs MGUS-young (n = 7) based on AAIS being above/below the median for the group. For each cell subpopulation, differentially expressed genes between old vs. young were identified using FindMarker function, method MAST from Seurat package. We used the average log2 fold change of +/- 0.585 and p-values < 0.05 for all signature genes to estimate the enrichment of each cell subpopulation group. Pathway analysis of significantly differentially expressed genes between clusters of interest was performed using the preranked workflow with gene set enrichment analysis (GSEA) software and the Molecular Signature Database (MSigDB) from the Broad Institute.

Phospho-CyTOF:

MNCs from myeloma patients and age-matched healthy controls were thawed and rested in RPMI+5%PHS for 1 hour. 3 million cells from each sample were either left unstimulated or stimulated with a cocktail of IFNα (50ng/mL) (Sigma-Aldrich) and LPS (5ug/mL) (MD Bioproducts) and incubated at 37 °C for 15 minutes. Cells were then fixed using 1.6%

formaldehyde and stained for surface markers followed by methanol fixation , permeabilization and staining for phosphoproteins per manufacturers protocol (Table 3.7). Cells were incubated with intercalation solution, mixed with EQ Four Element Calibration Beads and acquired using a Helios mass cytometer (all from Standard Biotools). Gating and data analysis were performed using Cytobank (https://www.cytobank.org). Dead cells and doublets were excluded using cisplatin intercalator and DNA content with iridium intercalator.

Detection of SARS-CoV-2 spike RBD binding antibodies (147)

Recombinant SARS-CoV-2 RDB was coated on Nunc MaxiSorp plates at a concentration of 1 μg/mL in 100 uL phosphate-buffered saline (PBS) at 4°C overnight. Plates were blocked for two hours at room temperature in PBS/0.05%Tween/1% BSA (ELISA buffer). Serum or plasma samples were heated to 56°C for 30 min, aliquoted, and stored at −20°C before use. Samples were serially diluted 1:200 in dilution buffer (PBS-1% BSA-0.05% Tween-20). 100 μL of each dilution was added and incubated for 30 minutes at room temperature. 100 uL of horseradish peroxidase-conjugated isotype and subclass specific secondary antibodies, diluted 1 to 2,000 in ELISA buffer, were added and incubated for 30 minutes at room temperature. Development was performed using 0.4 mg/mL o-phenylenediamine substrate (Sigma) in 0.05 M phosphate-citrate buffer pH 5.0, supplemented with 0.012% hydrogen peroxide before use. Reactions were stopped with 1 M HCl and absorbance was measured at 490 nm. Between each step, samples were washed four times with 300 uL of PBS-0.05% Tween. Prior to development, plates were additionally washed once with 300 uL of PBS. Secondary antibodies used for development were as follows: anti-hu-IgM-HRP, anti-hu-IgG-HRP, and anti-hu-IgA-HRP (Jackson Immuno

Research, and Mouse anti-hu-IgG1 Fc-HRP, Mouse anti-hu-IgG2 Fc-HRP, Mouse anti-hu-IgG3 Fc-HRP, or Mouse anti-hu-IgG4 Fc-HRP (Southern Biotech).

Table 3.5: Antibody panel for Immunophenotyping

	Antibody	Clone	Vendor		Antibody	Clone	Vendor
1	CD45	HI30	Standard Biotools	27	CD38	HIT2	Standard Biotools
2	*CD28	CD28.2	Biolegend	28	CD127	A019D5	Standard Biotools
3	*CXCR5	J252D4	Biolegend	29	CD19	HIB19	Standard Biotools
4	CD45RA	HI100	Standard Biotools	30	CD3	UCHT1	Standard Biotools
5	CD31	WM59	Standard Biotools	31	*#GZM K	GM26E7	Biolegend
6	CD4	RPA-T4	Standard Biotools	32	IgM	MHM-88	Standard Biotools
7	CD8	RPA-T8	Standard Biotools	33	#GZN B	GB11	Standard Biotools
8	CD20	2H7	Standard Biotools	34	HLADR	L243	Standard Biotools
9	*#TOX	REA473	Miltenyi Biotec	35	PD-1	EH12.2H7	Standard Biotools
10	CD25	2A3	Standard Biotools	36	CD56	NCAM16.2	Standard Biotools
11	IgD	IA6-2	Standard Biotools	37	CD16	3G8	Standard Biotools
12	*#EOMES	WD1928	ThermoFisher Scientific				
13	CD21	BL13	Standard Biotools				
14	BDCA2	201A	Standard Biotools				
15	#TCF1	7F11A10	Biolegend				
16	CD27	L128	Standard Biotools				
17	CD14	HCD14	Standard Biotools				
18	CD33	WM53	Standard Biotools				
19	CCR7	G043H7	Standard Biotools				
20	#TBET	D6N8B	Standard Biotools				
21	CLEC9a	8F9	Standard Biotools				
22	CD11c	Bu15	Standard Biotools				
23	CD57	HCD57	Standard Biotools				
24	CD95	DX2	Standard Biotools				
25	CD45RO	UCHL1	Standard Biotools				
26	NKG2D	ON72	Standard Biotools				

All metal conjugated antibodies purchased from Standard Biotools
*Purified antibodies were purchased from Biolegend/ThermoFisher Scientific/Miltenyi Biotec, and metal tagged using Standard Biotools metal conjugation kit following manufacturers methods and titrated prior to use.
#Intracellular markers
After antibody staining, cells were incubated with intercalation solution and mixed with EQ Four Element Calibration Beads (Cat. #201708)

Table 3.6: TotalSeq-C antibody panel used for CITE-Seq

	Antibody	Clone		Antibody	Clone
1	CD117 (c-kit)	104D2	29	CD10	HI10a
2	CD226 (DNAM-1)	11A8	30	CD45	HI30
3	CD223 (LAG-3)	11C3C65	31	CD19	HIB19
4	CD314 (NKG2D)	1D11	32	CD38	HIT2
5	CD303 (BDCA-2)	201A	33	CD161	HP-3G10
6	CD274 (B7-H1, PD-L1)	29E.2A3	34	IgD	IA6-2
7	KLRG1 (MAFA)	2F1/KLRG1	35	CD11b	ICRF44
8	CD20	2H7	36	CD185 (CXCR5)	J252D4
9	CD16	3G8	37	CD1c	L161
10	CD66b	6/40c	38	HLA-DR	L243
11	CD14	63D3	39	CD194 (CCR4)	L291H4
12	CD123	6H6	40	CD141 (Thrombomodulin)	M80
13	CD370 (CLEC9A/DNGR1)	8F9	41	Ig light chain κ	MHK-49
14	CD209 (DC-SIGN)	9E9A8	42	Ig light chain λ	MHL-38
15	CD127 (IL-7Ra)	A019D5	43	CD138 (Syndecan-1)	MI15
16	TIGIT (VSTM3)	A15153G	44	CD27	O323
17	CD25	BC96	45	CD33	P67.6
18	CD294 (CRTH2)	BM16	46	CD57	QA17A04
19	CD21	Bu32	47	CD56 (NCAM)	QA17A16
20	CD244 (2B4)	C1.7	48	CD4	RPA-T4
21	CD79b (Igβ)	CB3-1	49	CD8a	RPA-T8
22	CD28	CD28.2	50	CD11c	S-HCL-3
23	CD279 (PD-1)	EH12.2H7	51	CD155 (PVR)	SKII.4
24	CD69	FN50	52	CD45RO	UCHL1
25	CD183 (CXCR3)	G025H7	53	CD3	UCHT1
26	CD196 (CCR6)	G034E3	54	CXCR4	12G5
27	CD197 (CCR7)	G043H7	55	4-1BB	4B4-1
28	CD45RA	HI100			

TotalSeq-C antibodies were purchased from BioLegend and used following manufacturers methods.

Table 3.7: Antibody panel for Phospho-CyTOF analysis

	Antibody	Clone	Vendor
1	CD45	H130	Standard Biotools
2	CD19	H1B19	Standard Biotools
3	HLA-DR	L243	Standard Biotools
4	CD20	H1	Standard Biotools
5	CD56	NCAM16.2	Standard Biotools
6	#p-STAT5	47	Standard Biotools
7	CD123	6H6	Standard Biotools
8	#p-STAT1	4a	Standard Biotools
9	#p-38	D3F9	Standard Biotools
10	#p-STAT3	4/p-STAT3	Standard Biotools
11	CD11c	Bu15	Standard Biotools
12	CD14	M5E2	Standard Biotools
13	CLEC9a	8F9	Standard Biotools
14	CD66b	80H3	Standard Biotools
15	CD33	WM53	Standard Biotools
16	CD16	3G8	Standard Biotools
17	BDCA3 (CD141)	M80	Standard Biotools
18	CD11b	ICRF44	Standard Biotools
19	CD8	SK1	Standard Biotools
20	CD3	UCHT1	Standard Biotools
21	#pERK	D13.14.4E	Standard Biotools
22	CD4	SK3	Standard Biotools

All metal conjugated antibodies purchased from Standard Biotools
#Intracellular markers
After antibody staining, cells were incubated with intercalation solution and mixed with EQ Four Element Calibration Beads (Cat. #201708)

2.6 Acknowledgements

MVD is supported in part by funds from NIH R35CA197603, Paula and Rodger Riney Foundation and SCOR award from LLS. KMD is supported in part by funds from NIH CA238471 and AR077926. The authors acknowledge Winship immune monitoring resource supported by P30CA138292.

Chapter 4: General Discussion and Closing Remarks

4.1 Introduction

The overall goal of this dissertation was to better understand immune alterations in MM and its precursor state MGUS, and to illustrate correlations with age-associated immune dysfunction seen in patients. While there have been significant advances in the field's understanding of the role that the immune system plays in myeloma progression, leading to significant treatment improvements, myeloma remains incurable (148, 149). The therapeutic potential of the immune system in myeloma progression has been demonstrated by the evidence of anti-tumor responses in both MGUS and MM TME (37, 150), the clinical success of immunomodulatory drugs (lenalidomide, pomalidomide) and monoclonal antibodies (Daratumumab, Isatuximab etc) (151) and the recent success of CAR-T cells and T- cell targeting bispecific antibodies (132, 152) in the treatment of myeloma patients. This clinical success also argues against T-cell exhaustion, a phenotype based large on comparisons with transcriptomic signatures from murine chronic viral infection models (131).

MM and MGUS are more common in individuals with black ancestry, owing to several complex disparities in incidence, diagnosis, access to treatment and clinical trials, and underrepresentation in research, which ultimately impact outcomes (123). However, mechanisms linking immune dysfunction to racial ancestry or malignant transformation remain unclear.

This dissertation elucidates the functional state of T cells in MM, revealing enhanced inflammatory cytokine production by bone marrow T cells, indicative of accelerated immune aging. These findings were corroborated through integrated analyses of bone marrow immune and tumor cell transcriptional signatures, phosphoproteomic profiling, and functional vaccine response studies.

4.2 Investigation of immune-aging in myeloma

In efforts to functionally phenotype T cells from MGUS and myeloma bone marrow, we observed that after TCR stimulation, these T cells were polyfunctional and retained their ability to proliferate. This data indicated the lack global exhaustion in myeloma. Further characterization of T cells from myeloma BM revealed that they in fact have enhanced inflammatory cytokine production such as GMCSF and IL17, even in phenotypes previously thought to be "exhausted" such as PD-1 and LAG-3+ cells (Chapter 2).

We hypothesized that these immunophenotypic changes such as increase in terminal effectors and inhibitory checkpoints previously described in MM may relate instead to altered trajectories of immune aging. Current knowledge of immune-aging in myeloma and how it affects disease progression is scarce, with some evidence pointing towards senescence in a clonal population of terminally differentiated T cells with low expression of the classic exhaustion markers PD-1 and CTLA4 (153) and an increase of senescent T cells and drop in the naïve T cell pool with subsequent lines of therapy (154). Using a cohort of nearly 300 racially diverse MM/MGUS patients/healthy controls, we found that MM is associated with greater immune aging as compared to chronologically age matched MGUS patients (Figure 3.2). These findings were also supported by transcriptomic analysis, where immunologically older MM and MGUS patients were enriched for PD1 and interferon-response signatures (Figure 3.5). Phospho-proteomic analysis further validated these findings where MM T cells and monocytes had higher baseline inflammatory signaling and a dampened response to stimulation as compared to age-matched healthy controls (Figure 3.6, Figure 3.7).

Since the reduced responsiveness of vaccines is a hallmark of aging, we validated that with our MM cohort that received the SARS CoV-2 vaccination, where the group of patients who

developed vaccine-induced immunity were immunologically younger than those that did not (Figure 3.8).

4.3 Racial differences in myeloma and its precursor states

Myeloma and its precursor states affects black individuals more than white individuals and so to capture these racial differences, we ensured that the healthy donor cohort we used to validate functional differences as well as those used to create the immune-age calculator represented the MGUS and MM patient cohort with an equal split of white and black donors (Table 3.1, Table 3.2). This ensured that the immune-age calculator captured the impact of racial differences on any age-associated immune changes. Black MGUS patients were immunologically older compared to age-matched white counterparts, but no race-dependent differences were observed in the newly diagnosed myeloma cohort (Figure 3.2). Together these data suggest an impact of racial ancestry on immune-aging trajectories may begin early in MGUS and provide insights into prior studies showing MM development at an earlier age in Black patients (2, 123).

4.4 Future directions

T cell exhaustion and its impact on anti-tumor immunity remains to be a critical area of investigation in MM research, as effective tumor recognition by T cells is fundamental for mounting robust anti-tumor responses. Although exhausted and senescent T cells share a similar dysfunctional role in antitumor immunity owing to the decrease in fitness of T cells, they are distinctly different in terms of phenotypic and functional characteristics during tumor progression (155, 156). Our data suggests a lack of global exhaustion, owing to the T cells abilities to respond to TCR stimulation, further validated by the clinical success of CAR-T cells and bispecific

antibodies (132). This suggests a need to systematically characterize the functional state of T cells in MM. T cells within the MM marrow are likely to be heterogeneous and consist of both tumor-specific T cells (including neoantigen-specific T cells which as more likely to respond to checkpoint blockade therapies) and bystander T cells (150, 157). The re-activation and clonal expansion of tumor-reactive T cells are critical to the success of immune checkpoint blockade (ICB), adoptive transfer of TILs and immunomodulatory drugs (IMIDs) (158). Unfortunately, PD-1 blockade as a single agent has been clinically underwhelming in the treatment of MM (159). MGUS has been characterized with an enrichment of stem-like memory T cells (TCF1+, TCF7+) which is lost as the disease progressed to myeloma (45) and higher levels of naïve T cells (160), showing that the MM bone marrow microenvironment is more immunosuppressive than MGUS. The spatial location of these T cells in the BM is key for their interactions with tumor cells and APCs, and high-dimensional spatial analyses have shown that T cell entry into MM clusters is regulated by CD2/58 co-stimulation and antigen-presenting CLEC9A+ dendritic cells (136). All these findings reinforce the need to better characterize the heterogeneity of T cells in MM progression.

Similarly, there is a need to characterize the immune-aging phenotype that we observed in greater depth. While with the FlowSOM analysis, we were able to discern specific phenotypes such as within the CD4 T cells, there were CD57+ memory CD4 T and two cytokine-producing subsets and in CD8 T cells, a subset which showed a TEMRA phenotype and a polyfunctional MC which produced IL17, further functional validation of these specific populations is important to understand the inflammatory state of T cells.

In addition to this, further functional validation of other T cell aging markers would provide deeper insight into myeloma T cell biology. Defects in telomere length leading to critically short

telomeres have been implicated in several age-related diseases, premature ageing syndromes, as well as in cancer (161). Aging T cells also exhibit an increase in DNA damage markers, such as γH2AX foci and β-galactosidase activity, both of which are strongly associated with cellular aging (73). γH2AX, a well-established marker of double-strand breaks (DSBs), contributes to genomic instability and has been implicated in chronic inflammatory conditions. Inflammation disrupts the balance between pro-inflammatory cytokines (TNFα, IL-6 etc) and anti-inflammatory cytokines (IL-12, IFNγ etc), leading to the production of DNA-damaging reactive oxygen and nitrogen species. This exacerbates γH2AX accumulation and further drives immune dysfunction. (162). These insights open promising therapeutic avenues, including utilizing γH2AX as a biomarker to monitor immune aging and disease progression. Additionally, targeted interventions such as telomerase reactivation, DNA repair-enhancing therapies, and clinical strategies aimed at mitigating DSBs could be integrated with existing immunotherapies to enhance their clinical efficacy.

Another important characteristic of T cell aging is the diminished generation of naive T cells, leading to a compensatory clonal expansion of memory T cells, and a reduction in the diversity of the peripheral T cell repertoire. This decline in T cell receptor (TCR) diversity has significant clinical implications, as it weakens immune responses to pathogens and malignancies. Our findings highlight a potential clinical opportunity: classifying patients into “immune-young” and “immune-old” populations. Patients with a more diverse TCR repertoire (characteristic of the “immune-young” group) are likely to exhibit stronger immune responses, including improved tumor recognition and clearance. This classification could serve as a predictive tool for immunotherapy outcomes, enabling more personalized therapeutic strategies. It has been shown that harvesting less mature autologous T cells prior to CAR-T cell manufacturing has been

associated with improved outcomes in MM (163). This is applicable to MGUS as well, where there is evidence that T cells from MGUS are able to mount immune responses and our data shows that MGUS patients have a lower immune-age score than MM patients (Figure 3.2) (37). Recent clinical trials and studies have begun to explore some earlier interventions in the context of SMM, with some observed benefits and delayed progression to MM in certain patient subsets treated with IMiDs such as lenalidomide (164, 165). Lenalidomide has been shown to increase the proportion of CD28 T cells in MM (166) and the accumulation of CD28- T cells is an established change associated with aging (167). Studies looking at the potential reversal of T cell aging have shown that p38 signaling, which is increased in terminally differentiated CD27-CD45RA+ compared with the CD27-CD45RA- (EM) T cell populations, is involved in the induction of increased apoptosis and impaired telomerase activity in these populations after TCR activation (168). In our data, we see an increased expression of baseline p38 in MM T cells (Figure 3.6), and inhibition of p38 could have clinical potential, as an adjunct to allogenic T cell immunotherapies.

MM is characterized by significant heterogeneity in clinical characteristics and cytogenetic abnormalities, which are found in most MM patients and their prognostic value has been well studied (169). Translocation t(4;14), t(14;16) and t(14;20) have been associated with poor prognosis, and their presence identifies high-risk (HR) disease, while patients with t(11;14), t(6;14) and/or trisomies are considered to have standard-risk (SR) disease (169). Mass cytometry done to characterize T cell heterogeneity revealed that TME of patients with trisomies is populated by more senescent/terminally differentiated and exhausted T cell subsets (160). The presence of trisomies is a negative prognostic factor in MGUS/SMM but a positive one in NDMM, after therapy has been administered, making them especially sensitive to lenalidomide (170).

Understanding the relationship between immune-aging and cytogenetic abnormalities may provide valuable insights into clinical outcomes.

As previously discussed, the incidence of myeloma and its precursor states is 2–3-times higher in black individuals than in white individuals (119). In this dissertation we see that MGUS patients with black ancestry exhibited enhanced aging, which indicates racial predisposition with earlier onset of myeloma in black individuals. This has great clinical implications since several studies have shown that black patients face a delay in diagnosis, where the average length of time between MM diagnosis and start of treatment with a novel therapy is 5.2 months for black patients compared to 2.7 months for white patients (171).

In summary, this dissertation advances our understanding of immune system function in myeloma and its precursor states. We identified distinct functional profiles of myeloma bone marrow T cells, characterized by enhanced inflammatory cytokines, proliferation, and phospho-signaling, with transcriptomic validation. Our work revealed connections between immune aging in myeloma and its malignant transformation, racial predisposition, and vaccine response. These findings may prove instrumental in improving immune therapies for myeloma patients.

Chapter 5: References

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